Point Mutations in the FLT3-ITD Region Are Rare but Recurrent Alterations in Adult AML and Associated With Concomitant KMT2A-PTD

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FLT3-ITD mutations are common druggable alterations in patients with acute myeloid leukemia (AML) and associated with poor prognosis. Besides typical ITD mutations, point mutations and deletions in the juxtamembrane domain (JMD) have been observed. However, due to the low frequency of these alterations, there is only limited information on molecular and clinical associations. To evaluate the prognostic impact of non-ITD mutations in the FLT3 JMD region, we analyzed a large cohort of 1,539 adult AML patients treated in different protocols of the Study Alliance Leukemia, using next-generation sequencing. Non-ITD point mutations and deletions within the FLT3 JMD were identified with a prevalence of ~1.23% (n = 19). Both FLT3-ITD and non-ITD mutations were associated with a higher rate of NPM1 (42%–61%; p < 0.001) and DNMT3A mutations (37%–43%; p < 0.001), as well as an increased percentage of peripheral blood (54%–65%) and bone marrow blast cells (74%; p < 0.001), compared to FLT3-wild-type patients. Most significantly, AML patients with FLT3 non-ITD mutations had a higher rate of concomitant KMT2A-PTD mutations (37.5%; p < 0.001) as compared to FLT3-ITD (7%).
INTRODUCTION

The FMS-like tyrosine kinase 3 (FLT3) is a transmembrane receptor tyrosine kinase, which is expressed by hematopoietic stem cells and stimulates the development of myeloid and lymphoid progenitor cells (1). Mutations of the FLT3 gene are identified in ~30% of patients with acute myeloid leukemia (AML). About 20%–25% of AML patients show internal tandem duplications (FLT3-ITD), affecting the juxtamembrane domain (JMD) and/or the tyrosine kinase domain-1 (TKD1) of FLT3 (2). In addition, mutations in exon 20, coding for the TKD2 region (most frequently affecting codons D835 and 1836), can be detected in 7%–10% of AML patients (FLT3-TKD) (1, 2).

Both FLT3-ITD and FLT3-TKD mutations constitutively activate the FLT3 kinase, inducing proliferation of leukemic populations, although differences in the signaling induced have been reported between these two mutations (1). While the clinical significance of FLT3-TKD mutations is uncertain, the presence of FLT3-ITD mutations in AML patients confers a poor prognosis with an increased risk of relapse and shorter overall survival (2, 3). Consequently, the FLT3-ITD mutational status is included in the current risk classification of the ELN-2017 recommendations and is recognized as target for specific TK inhibitors (2, 4, 5). However, several factors modify the prognostic impact of FLT3-ITD mutations, such as the allelic ratio and the presence of a concomitant NPM1 mutation (4).

In addition to typical ITD mutations, previous reports indicated the presence of rare non-ITD mutations (small deletions and point mutations) in the FLT3 JMD of AML patients (6–16). However, due to the low frequency of these alterations, there are so far only casuistic reports available, with limited information on molecular and clinical associations. To investigate the prevalence and prognostic impact of non-ITD mutations in the FLT3 JMD of adult patients with AML, we analyzed a large cohort of 1,539 patients with newly diagnosed AML to correlate these mutations with clinical characteristics, co-mutations, and outcome.

METHODS

Patients

All patients (n = 1,539) investigated had newly diagnosed AML, were registered in clinical protocols of the Study Alliance Leukemia (SAL) (AML96, AML2003 or AML60+, SORAML), and had available biomaterial at diagnosis. Detailed descriptions of the treatment protocols have been published previously (17–20); all protocols included intensive induction chemotherapy and consolidation treatment according to cytogenetic risk groups. The study was in agreement with the Helsinki declaration and approved by the ethical board of the Technical University Dresden (EK98032010).

Molecular Analysis

Molecular studies were performed on genomic DNA isolated from bone marrow (BM) aspirates or peripheral blood (PB) taken at diagnosis. DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) and quantified with the NanoDrop spectrophotometer. In addition to conventional fragment analysis, profiling of FLT3 JMD/TKD1 mutational status and associated co-mutations was performed by targeted resequencing using the TruSight Myeloid assay (Illumina, San Diego, CA, USA) (Supplementary Methods). Briefly, for FLT3 the panel covers the entire ITD region (AA 572–630). Libraries were sequenced paired-end (150 bp) on a NextSeq instrument (Illumina) and analyzed using the Sequence Pilot Software (JSI medical systems) with a 5% variant allele frequency (VAF) mutation calling cutoff. KMT2A-PTD (partial tandem duplication) mutations (formerly MLL-PTD) were analyzed on cDNA (reverse transcribed from 1 μg of total RNA; SuperScript VILO cDNA Synthesis Kit; Invitrogen, Carlsbad, CA, USA), using the Mentreplex AMLplex QS Kit (Biotype, Dresden, Germany) on a 3130xl Genetic Analyzer (Applied Biosystems, Foster, CA, USA).

Statistical Analysis

Categorical variables between groups were compared using the chi-squared test or a 2-sided Fisher’s exact test. For continuous variables the non-parametric Mann–Whitney U test was applied. p values <0.05 were considered significant. To evaluate relapse-free survival (RFS) and overall survival (OS), the Kaplan–Meier method and the log-rank test were used. For multivariable analysis of prognostic factors, Cox-proportional hazard regression models were used for survival endpoints, and logistic regression models were used for CR. All statistical analyses were performed using the R environment for statistical computing version 4.0.3.

RESULTS

Characterization of FLT3 Non-ITD Mutations in the JMD/TKD1 Region

FLT3-ITD mutations were detected in 324 of 1,539 (21.1%) AML patients. Non-ITD mutations within the FLT3 JMD were found in 19 cases (1.23%) (Figure 1A). Patients with mutations in the TKD2 region (FLT3 Exon 20; D835 and 1836; n = 104) were excluded from this analysis. Patients without JMD/TKD1 mutation were classified as FLT3-ITD wild-type (wt) (n = 1,196).
Most non-ITD mutations were single-nucleotide missense variants (SNV; n = 15; Figure 1B). Deletions were found in 4 patients, exclusively comprising small in-frame deletions (1–4 amino acid residues). FLT3 non-ITD mutations were detected with a median VAF of 28% (range 7%–50%) at subclonal levels in the majority of patients (73.7%; Table S1). Recurrent point mutations affected residues V592 (n = 7), Y572 (n = 3), and L576 (n = 2) (Figure 1B). Compared to FLT3-wt patients, both ITD and non-ITD mutations had increased rates of concomitant mutations in NPM1 (60.7% and 42.1% vs. 23.4%; p < 0.001) and DNMT3A (43% and 37% vs. 24%; p < 0.001; Table 1 and Figure 1C). Vice versa, FLT3-wt patients were significantly more often affected by mutations in NPM1, KIT, and JUN/KRAS, RUNX1, SRSF2, TP53. (D) Kaplan–Meier analysis showing the probability of overall survival (OS) for AML patients without molecular alterations in the FLT3-ITD region (wt; n = 1,196; black), ITD mutation (n = 324; red), and non-ITD mutation (n = 19; blue). (E) Results of the multivariable analysis of prognostic factors for overall survival, (OS); relapse-free survival, (RFS) and complete remission, (CR1).

**Associations of FLT3 Non-ITD Mutations With Clinical Features and Outcome**

For patients with FLT3 non-ITD mutations, the median follow-up was 108 months (IQR 80–127 months). The first complete remission (CR1) after initial treatment was achieved in 16/19 patients with FLT3 non-ITD mutation. Genes with <10% mutation rate are not shown: EZH2, ASXL1, IKFZ1, NRAS, RUNX1, SRSF2, TP53. (D) Kaplan–Meier analysis showing the probability of overall survival (OS) for AML patients without molecular alterations in the FLT3-ITD region (wt; n = 1,196; black), ITD mutation (n = 324; red), and non-ITD mutation (n = 19; blue). (E) Results of the multivariable analysis of prognostic factors for overall survival, (OS); relapse-free survival, (RFS) and complete remission, (CR1).
TABLE 1 | Clinical and molecular characteristics of patients.

| Parameter                      | FLT3-ITD n=324 | FLT3 non-ITD n=19 | p-value<sup>a</sup> | FLT3 wt n=1196 | p-value<sup>b</sup> |
|--------------------------------|----------------|------------------|---------------------|----------------|---------------------|
| No. of patients (n)            |                |                  |                     |                |                     |
| Age, years, median (IQR)       | 55 (44–64)     | 51 (45–60)       | 0.289               | 56 (45–66)     | 0.405               |
| Sex, n/nval (%)                |                |                  |                     |                |                     |
| Female                         | 169/324 (52.2) | 12/19 (62.3)     | 0.486               | 548/1196 (45.8) | 0.049               |
| Male                           | 155/324 (47.6) | 7/19 (36.3)      |                     | 648/1196 (54.2) |                     |
| Disease status, n/nval (%)     |                |                  |                     |                |                     |
| De novo                        | 290/322 (90.1) | 17/19 (86.2)     | 0.045               | 974/1180 (81.6) | 0.044               |
| sAML                           | 25/322 (7.8)   | 0/19 (0)         |                     | 155/1180 (13.1)|                     |
| tAML                           | 7/322 (2.2)    | 2/19 (10.5)      |                     | 51/1180 (4.3)  |                     |
| Normal karyotype, n/nval (%)   |                |                  |                     |                |                     |
| No                             | 77/303 (25.4)  | 10/16 (62.6)     | 0.003               | 568/1125 (50.5)| 0.001               |
| Yes                            | 226/303 (74.6) | 6/16 (37.5)      |                     | 557/1125 (49.5)|                     |
| Complex karyotype, n/nval (%)  |                |                  |                     |                |                     |
| No                             | 297/305 (97.4) | 17/17 (100)      | 0.001               | 908/1083 (83.8)| 0.001               |
| Yes                            | 8/305 (2.6)    | 0/17 (0)         |                     | 175/1083 (16.2)|                     |
| Follow-up, median (IQR)        |                |                  |                     |                |                     |
| t(11;v), n/nval (%)            |                |                  |                     |                |                     |
| No                             | 289/301 (99.3) | 10/12 (83.3)     | <0.001              | 989/1018 (97.2)| 0.001               |
| Yes                            | 2/301 (0.7)    | 2/12 (16.7)      |                     | 29/1018 (2.8)  |                     |
| ECOG score, n/nval (%)         |                |                  |                     |                |                     |
| 0–1                            | 170/266 (63.9) | 13/18 (72.2)     | 0.646               | 717/987 (72.6)| 0.021               |
| 2–4                            | 96/266 (36.1)  | 5/18 (27.8)      |                     | 270/987 (27.4)|                     |
| Laboratory, median (IQR)       |                |                  |                     |                |                     |
| WBC (Gpt/l)                    | 46.09 (17.1–100.65) | 39.6 (13.4–63.5) | 0.527               | 13.3 (3.59–40.68)| <0.001          |
| Hemoglobin (mmol/l)            | 5.77 (5.01–6.77) | 5.34 (5.25–6.9)  | 0.653               | 5.9 (5.03–7.09) | 0.166               |
| PLT (Gpt/l)                    | 58 (30.5–102.5) | 42 (29.5–89)     | 0.474               | 48 (26–92)     | 0.028               |
| Peripheral blasts (%)          | 63 (25–84)     | 54 (20–86.5)     | 0.960               | 33 (9–66)      | <0.001              |
| LDH (U/l)                      | 602.4 (389–964) | 591 (432–859)    | 0.757               | 411 (256–692.25)| <0.001          |
| BM blasts (%)                  | 74 (57.36–85)  | 74 (64.25–83.5)  | 0.539               | 59 (41.5–74.5) | <0.001              |
| Frequent co-mutations, n/nval (%) |                |                  |                     |                |                     |
| KMT2A-PTD                      | 9/128 (7)      | 6/16 (37.5)      | 0.001               | 19/424 (4.5)  | <0.001              |
| NPM1                           | 196/323 (60.7) | 8/19 (42.1)      | 0.173               | 279/1191 (23.4)| <0.001              |
| DNMT3A                         | 139/324 (43)   | 7/19 (37)        | 0.779               | 282/1196 (24) | <0.001              |
| IDH2                           | 30/324 (9)     | 4/19 (21)        | 0.202               | 186/1196 (16) | 0.011               |
| WT1                            | 40/324 (12)    | 3/19 (16)        | 0.933               | 68/1196 (6)  | <0.001              |
| KIT                            | 5/324 (2)      | 0/19 (0)         | 1.000               | 70/1196 (6)  | 0.004               |
| NRAS                           | 30/324 (9)     | 1/19 (5)         | 0.858               | 211/1196 (18) | <0.001              |
| Clinical outcome<sup>c</sup>   |                |                  |                     |                |                     |
| Follow-up, median (IQR) in months | 100 (70–118) | 108 (80–127) | 0.001               | 86 (40–115) |                     |
| CR rate, n (%)                 | 252 (77.8)     | 16 (85)          | 0.513               | 818 (86.4)    | 0.001               |
| RFS, median (95% CI) in months<sup>d</sup> | 9.69 (7.98–14.2) | 18.5 (13.8–NA) | 0.181               | 22.8 (18.3–27.4) | 0.001           |
| OS, median (95% CI) in months<sup>d</sup> | 13.1 (10.5–17.6) | 31.7 (19.8–NA) | 0.109               | 17.9 (15.9–21.4) | 0.107 |

Significant differences are presented in bold.

<sup>a</sup>Results of the univariate logistic regression model for CR1 and Cox regression model for RFS/OS; NA means infinity.

<sup>b</sup>The median RFS is calculated with the positively selected subgroup of patients with a CR.

<sup>c</sup>p-values for the comparison of all three groups FLT3-ITD, FLT3 non-ITD and FLT3 wt.

<sup>d</sup>tAML, therapy-related acute myeloid leukemia; sAML, secondary acute myeloid leukemia; WBC, white blood cells; BM, bone marrow; PB, peripheral blood; LDH, lactate dehydrogenase; ELN, European LeukemiaNet; OS, overall survival; RFS, relapse-free survival; CR, complete remission; ED30, early death within 30 days; EFS, event-free survival; n number; nval, number of valid non-missing observations; IQR, interquartile range; 95% CI, 95% confidence interval.

aberrant karyotype between both groups. Likewise, general similarities were observed for most laboratory parameters, such as WBC counts (median 39.6–46.09 Gpt l<sup>–1</sup>), PB (median 54–63 Gpt l<sup>–1</sup>), and BM blasts (median 74%). In contrast, FLT3-wt status was associated with a higher rate of ELN-2017 adverse risk (38.2%; <i>p</i> < 0.001) and complex aberrant karyotype (16.2%; <i>p</i> < 0.001), as well as lower WBC counts and lower rates of PB and BM blasts compared to FLT3 mutant patients. With respect to clinical outcome, FLT3 non-ITD mutations were not associated with CR rate (85%), relapse-free survival (median 18.5 months), or overall survival (median 31.7 months) in univariate analyses (Figure 1D and Table 1). Likewise, the multivariable analysis revealed that non-ITD mutations (as well as FLT3-ITD mutations) were not an independent prognostic factor for outcome (Figure 1E and Table S3). Interestingly, there was an insignificant trend toward inferior RFS (HR 6.64; CI 0.691–63.9; <i>p</i> = 0.101; Figure 1E) for patients with the mutual presence of FLT3 non-ITD and KMT2A-PTD mutations (Figure S1 and Table S3). Regarding the effect of
transplantation on outcome, only two (out of 19) patients with FLT3 non-ITD mutations underwent allogeneic hematopoietic stem cell transplantation (alloHSCT) without subsequent event (Figure S2). Thus, compared to FLT3-ITD and -wt patients, no significant differences were observed.

**DISCUSSION**

In the present study, we analyzed 1,539 adult AML patients for the prevalence and prognostic impact of non-ITD mutations in the JMD/TKD1 region of FLT3. We confirm that JMD point mutations and deletions are rare, but recurrent alterations in patients with AML at a frequency in the range of previous estimates (8, 9). Compared to AML, higher rates (~4-fold) of JMD non-ITD mutations were previously detected in patients with acute lymphoblastic leukemia (ALL) (8). Among non-ITD mutations in our cohort, the point mutations Y572C, V592G (9), L576Q (10), G583S (8), Y591H (11), and V592A (12, 13) as well as the deletion EY598_599del (14) were previously recognized as gain-of-function mutations that result in constitutive kinase activation and stimulate AML growth through aberrant STAT5 signaling (9, 12, 14). In addition, in one patient we identified a novel and likely damaging (PolyPhen score = 1) point mutation at Y599N (30% VAF), not previously reported in the literature. However, functional data indicate that any alteration of the JMD sequence interferes with the kinase auto-inhibition, similar to the effect of FLT3-ITD mutations (9, 11, 15). Accordingly, in vitro studies illustrated the response of AML cells with non-ITD JMD mutations to pharmacologic FLT3 inhibition (9, 10, 12, 14–16), which supports the idea to use any of the approved TKIs in patients with this type of mutation (5). In line with this, we demonstrate high similarities between patients with FLT3-ITD and non-ITD mutations, with respect to major laboratory parameters (i.e., an increased percentage of blast cells in PB and BM) and the rate of co-mutated driver variants, which is consistent with the mutational landscape and clinical phenotype typically observed for patients with FLT3-ITD mutations (1–3, 21). For example, a high rate of concomitant NPM1 and DNMT3A mutations but mutual exclusivity with downstream effectors such as NRAS in FLT3 mutant patients has been described in detail (21). Similar to our data, partial tandem duplications (PTD) of the histone methyltransferase KMT2A (MLL) occur in 3%–5% of AML cases and are typically enriched in patients with FLT3-ITD mutations (as compared to FLT3-wt) (21, 22). In addition, we show a not previously reported high association of KMT2A-PTD mutations with FLT3 JMD point mutations, which adds on initial observations on the genomic landscape of KMT2A-PTD-mutated AML (22). More importantly, although the presence of FLT3 non-ITD mutations alone was not an independent prognostic factor, patients with dual non-ITD and KMT2A-PTD mutations showed a trend for inferior outcome. While KMT2A-PTD mutations are typically associated with poor prognosis, isolated KMT2A-PTD mutations are not sufficient to establish leukemic transformation of hematopoietic cells, highlighting the importance of cooperating genetic lesions, such as FLT3 mutations in KMT2A-rearranged leukemias (22–24). Likewise, functional data indicate the transcriptional regulation of FLT3 by KMT2A via aberrant MEIS1 gene expression (25). More recently, the combined inhibition of menin-MLL (MLL1, KMT2A) and FLT3 demonstrated a synergistic therapeutic opportunity in these leukemia subtypes (26). However, mutations of genes associated with RAS signaling (i.e., FLT3) may be frequently lost or emerge during AML progression, with relevance for post-remission strategies (27). In this regard, future investigations are needed to evaluate the impact of FLT3 non-ITD mutations for relapse development. In conclusion, we show that FLT3 non-ITD mutations are rare but recurrent alterations in AML and associated with similar clinical features like FLT3-ITD variants. Interestingly, our data point at a functional interaction of FLT3 non-ITD mutations with KMT2A-PTD and 11q-abnormalities as cooperating genetic events, which might be indicative of a distinct pathway of genesis in this subset of AML.

**DATA AVAILABILITY STATEMENT**

The datasets presented in this article are not readily available because patients did not consent to have data uploaded to a public database. Requests to access the datasets should be directed to the corresponding author on reasonable request: christian.thiede@uniklinikum-dresden.de.

**ETHICS STATEMENT**

The studies involving human participants were reviewed and approved by Technical University Dresden (EK98032010). The patients/participants provided their written informed consent to participate in this study.

**AUTHOR CONTRIBUTIONS**

Conception of the work: CT, SS. Sample/Data Collection: All authors. Acquisition/Analysis of Data: CT, SS. Bioinformatic Analysis: SS. Statistical Analysis: MK, SZ. Interpretation of Data: CT, SS. Drafted the manuscript: SS. Administrative support: GE, MB. All authors have read and approved the manuscript that is being submitted.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.862991/full#supplementary-material
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Conflict of Interest: CT is CEO and co-owner of AgenDix GmbH, a company performing molecular diagnostics. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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