Clonal heterogeneity of FLT3-ITD detected by high-throughput amplicon sequencing correlates with adverse prognosis in acute myeloid leukemia

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ABSTRACT

In acute myeloid leukemia (AML), internal tandem duplications (ITDs) of FLT3 are frequent mutations associated with unfavorable prognosis. At diagnosis, the FLT3-ITD status is routinely assessed by fragment analysis, providing information about the length but not the position and sequence of the ITD. To overcome this limitation, we performed cDNA-based high-throughput amplicon sequencing (HTAS) in 250 FLT3-ITD positive AML patients, treated on German AML Cooperative Group (AMLCG) trials. FLT3-ITD status determined by routine diagnostics was confirmed by HTAS in 242 out of 250 patients (97%). The total number of ITDs detected by HTAS was higher than in routine diagnostics (\(n = 312\) vs. \(n = 274\)). In particular, HTAS detected a higher number of ITDs per patient compared to fragment analysis, indicating higher sensitivity for subclonal ITDs. Patients with more than one ITD according to HTAS had a significantly shorter overall and relapse free survival. There
was a close correlation between FLT3-ITD mRNA levels in fragment analysis and variant allele frequency in HTAS. However, the abundance of long ITDs (≥75nt) was underestimated by HTAS, as the size of the ITD affected the mappability of the corresponding sequence reads. In summary, this study demonstrates that HTAS is a feasible approach for FLT3-ITD detection in AML patients, delivering length, position, sequence and mutational burden of this alteration in a single assay with high sensitivity. Our findings provide insights into the clonal architecture of FLT3-ITD positive AML and have clinical implications.

INTRODUCTION

Mutations in the fms-related tyrosine kinase 3 (FLT3) gene are prevalent in newly diagnosed acute myeloid leukemia (AML) cases, affecting up to 39% patients. Internal tandem duplications (ITDs) represent the most common type of FLT3 mutation, being most frequent in patients with normal karyotype (cytogenetically normal AML, CN-AML) and in patients positive for the translocation t(6;9)(p23;q34) or t(15;17)(q22;q21) [1–4]. FLT3-ITD is associated with an unfavorable prognosis due to reduced duration of complete remission (CR), shorter event free survival (EFS) and shorter overall survival (OS) [1, 5–7]. The European Leukemia Net included the FLT3-ITD mutation as prognostic risk factor into a clinical risk-stratification that may guide physicians in therapy decisions [8, 9]. Patients carrying FLT3-ITD mutations with high allelic ratio benefit from a more intensive consolidation treatment such as allogeneic stem cell transplantation [7, 10–12]. Since the recent approval of the tyrosine kinase inhibitor (TKI) Midostaurin in combination with induction chemotherapy, FLT3-mutated patients may profit from this targeted treatment. [13–15] FLT3-ITDs are predominately located in exon 14 and 15, affecting the juxtamembrane domain (JM) and tyrosine kinase domain 1 (TKD1) of the FLT3 receptor [16]. Depending on the FLT3-ITD insertion site and respective functional domain, differential outcome and response to treatment with conventional chemotherapy as well as TKIs were observed; especially non-JM ITDs displayed a resistance to TKIs [10, 16–21]. Although always leading to an in-frame transcript, FLT3-ITDs vary in length (between three to over 400 nucleotides (nt)) and sequence [1, 2, 4, 6, 17, 22, 23]. Whether the ITD length has an impact on outcome remains controversial [1, 22, 24, 25]. However, a prognostic relevance was observed for the mutant to wild-type (WT) allelic ratio which corresponds to the size of the clone(s) with the FLT3-ITD. A high allelic burden is associated with poor prognosis (shorter OS, EFS), and outcome is even worse for patients with a loss of WT FLT3 [1, 4, 6, 16, 17, 26–29]. Additionally, up to five distinct clones with different FLT3-ITDs were observed per patient [16]. In light of these findings, the assessment of FLT3-ITD characteristics is of important prognostic value. In routine diagnostics, the FLT3-ITD status is assessed by capillary electrophoresis of PCR-amplified cDNA (hereafter referred to as ‘fragment analysis’) [26]. However, this assay only provides the length but not the position and the sequence of the insertion. Therefore, it is attractive to overcome these methodological limitations by the use of high-throughput amplicon sequencing (HTAS) as an alternative strategy for FLT3-ITD detection. Techniques based on next generation sequencing (NGS) have the potential to assess multiple parameters simultaneously with scalable sensitivity [27, 30–32]. Previous studies already highlighted the potential of FLT3-ITD detection by NGS, especially with regards to diagnosis and disease monitoring, e.g. minimal residual disease (MRD) detection, as evaluation of treatment response and early detection of relapse [16, 17, 27, 30–34]. Establishment of NGS assays in diagnostic routine requires high sensitivity at low costs as well as fast turn around time and reliable results. To evaluate the applicability and accuracy of NGS-based FLT3-ITD detection for routine diagnostics, we compared FLT3-ITD detection by HTAS and fragment analysis in 250 adult FLT3-ITD positive AML patients.

RESULTS

HTAS reliably identifies FLT3-ITD subclones of prognostic relevance

For our comparative analysis we selected 250 AML patient samples obtained at initial diagnosis, all FLT3-ITD positive according to routine diagnostics (Figure 1), as well as 17 FLT3-ITD negative AML samples. All patients were treated on AMLCG trials (AMLCG 1999 [35], AMLCG 2004 [36] or AMLCG 2008 (ClinicalTrials.gov identifier: NCT01382147)) and received an intensive, high-dose cytarabine based induction therapy. The study cohort included patients with all cytogenetic aberrations and was not restricted to patients under the age of 60 years (Table 1). Performing HTAS, we sequenced the same FLT3-ITD mutational hot-spot region as covered by cDNA fragment analysis using identical primer sites in both assays (Figure 2 and Table 2). The output of both methods is shown by an exemplary patient in Figure 3. Overall, HTAS detected a total of 312 FLT3-ITDs in 242 of 250 FLT3-ITD positive patients (97%), compared to 274 ITDs detected by fragment analysis (Supplementary Table 1). The median length of ITD measured by HTAS was 51 nt (range:12–175 nt) at a median allele frequency of 12.2%
(range: 0.5–91.1%). By fragment analysis, the median ITD length was 54 nt (range: 15–153 nt) at a median FLT3-ITD mRNA level of 0.40 (range: 0.01–0.96). In 11 patients, we observed differences in length between HTAS and routine assays (fragment analysis or Sanger sequencing using cDNA template) with a median variation in length of 6 nt (range: 1–45 nt; median ITD size: 66 nt, range: 12–90 nt; n = 11/242; 5%). Validation of the insertion length on the genomic level by targeted sequencing and/or fragment analysis of amplified gDNA confirmed either the results from HTAS or diagnostic routine, each in about half of the cases (Supplementary Table 2). In paired samples (HTAS and Sanger sequencing, n = 182), we found identical insertion sites of the dominant ITD in all patients. The highest number of FLT3-ITDs was detected in the zipper motif of the JM, followed by the β1-sheet of the TKD1 and then the hinge region of the JM (Figure 4). Neither the insertion site nor the length of ITDs showed any significant correlations with clinical outcome (OS, RFS, and CR rate; Supplementary Tables 3 and 4). In contrast to other studies [16, 37], patients with ITDs in the TKD1 did not show worse clinical outcome compared to patients with ITDs in the JM in the dominant clone (Supplementary Figure 1).

Using HTAS, one ITD was detected in 190 (78.5%) patients, two ITDs were detected in 40 patients (16.5%), three ITDs were detected in nine patients (3.75%) and four ITDs were detected in three patients (1.2%; Figure 5A). In eight patients, who were tested FLT3-ITD positive in diagnostic routine, no ITD was detected by HTAS at a variant allele frequency (VAF) above the detection limit (0.5%). However, in four of these eight patients, HTAS detected an ITD consistent with routine results regarding length and position at a VAF below the cut-off. According to routine, another two of these eight patients had each a deletion (three and ten nucleotides) neighbouring or within the ITD. For the remaining two patients, no information about the FLT3-ITD mutational burden (FLT3-ITD mRNA level) was available from routine diagnostics.

Furthermore, HTAS missed eight subclonal ITDs each in one patient reported by routine diagnostics, including three with a length > 75 nt (median FLT3-ITD mRNA level: 0.09, range: 0.06–0.25), while 46 additional subclonal ITDs were detected in 34 patients by HTAS only (median VAF: 1.79%, range: 0.50–19.21%; median ITD-supporting reads: 1759, range: 460–23878). The distribution of ITDs over FLT3 domains was the same in HTAS and Sanger sequencing with regards to the dominant clone (Supplementary Figure 2). Out of the 46 additional FLT3-ITD clones by HTAS 38 (83%) were validated, ten of which displayed differences in length compared to the genomic level (targeted sequencing and/or fragment analysis with gDNA template; Supplementary Table 5).

Overall, HTAS detected more ITDs per patient (mean: 1.27, range: 1–4) compared to fragment analysis (mean: 1.14, range: 1–3; Figure 5B). In contrast to fragment analysis, HTAS revealed a significantly shorter OS and RFS for patients with more than one ITD (HTAS: p-value (OS): 0.038, p-value (RFS): 0.042; fragment analysis: p-value (OS): 0.230, p-value (RFS): 0.157; compare Figure 6A and 6B versus 6C and 6D). However, the CR

Figure 1: Study design. Flow chart illustrating the selection of patient samples. *Residual patients were lost during follow-up. HTAS (high-throughput amplicon sequencing), ITD (internal tandem duplication).
rate did not show any obvious correlation with the number of detected ITDs (data not shown). A multivariate analysis, including NPM1 mutation status, karyotype and number of FLT3-ITD mutations per patient (single versus multiple), did not show significant correlations with clinical outcome (Supplementary Table 6). However, there was a trend for longer RFS associated with single FLT3-ITD mutations detected by HTAS (p-value: 0.057). A serial dilution of cDNA derived from the heterozygous FLT3-ITD positive cell line MOLM-13 in cDNA derived from the FLT3-WT cell line HL60 analysed by both methods confirmed higher sensitivity of HTAS (10^-3, ITD-supporting reads: 58–73 with a coverage of 79,376× to 93,019×) in a 96 sample-setting as compared to fragment analysis (10^-1, Figure 7). Out of 17 control patients which were FLT3-ITD negative according to routine diagnostics, HTAS detected a very small subclonal ITD (VAF: 0.58%) in one patient (UPN C-1; Supplementary Table 1). This subclone could not be validated by gDNA-based fragment analysis (Supplementary Table 5).

Table 1: Patient characteristics

| Characteristics               | patients (no.) |
|-------------------------------|----------------|
| cohort size (patients no.)    | 250            |
| median age (years; range)     | 59; 18–80      |
| sex (m/f) (patients no.; [%]) | 121/129; 48/52 |
| median follow-up (months; range)| 50; 0–136     |
| median overall survival (months; range) | 9; 0–136 |

| Morphologic parameters        | patients (no.) |
|-------------------------------|----------------|
| median WBC count (n = 243), (leucocytes/mL; range) | 48,350; 100–391,200 |
| median BM-blasts (n = 229), (%; range) | 83; 10–100 |
| median PLT (n = 243), (PLT/mL; range) | 55,000; 1,220–592,000 |
| median LDH (n = 239), (LDH/mL; range) | 691; 87–6,251 |

| AML type (n = 245)            | patients (no.) |
|-------------------------------|----------------|
| de novo                       | 205            |
| s-AML                         | 27             |
| t-AML                         | 13             |

| FAB-type (n = 236)            | patients (no.) |
|-------------------------------|----------------|
| M0                            | 9              |
| M1                            | 71             |
| M2                            | 60             |
| M3                            | 0              |
| M4                            | 67             |
| M5                            | 24             |
| M6                            | 4              |
| M7                            | 1              |

| Categories according to ELN (n = 246) | patients (no.; [%]) |
|----------------------------------------|---------------------|
| favourable                              | 0; 0                |
| intermediate-I                         | 172; 70             |
| intermediate-II                        | 52; 21              |
| adverse                                | 22; 9               |

| Karyotype (n = 250)                   | patients (no.; [%]) |
|---------------------------------------|---------------------|
| Normal                                 | 176; 70             |
| Complex                                | 74; 30              |

| Molecular genetics                    | patients (no.; [%]) |
|---------------------------------------|---------------------|
| FLT3-ITD+ (n = 250)                   | 250; 100            |
| NPM1+ (n = 189)                       | 118; 47             |
| CEBP4+ (n = 116)                      | 9; 4                |
| KMT2A-PTD+ (n = 241)                  | 20; 8               |

WBC (white blood cell), PLT (platelet counts), BM (bone marrow), LDH (lactate dehydrogenase), AML (acute myeloid leukemia), MDS (myelodysplastic syndrome), ELN (European Leukemia Net), FAB (French American British Classification), ITD (internal tandem duplication), PTD (partial tandem duplication), no. (number).
HTAS reliably detects small and intermediate insertions but underestimates the mutational burden of long FLT3-ITDs

The VAF in HTAS showed a strong correlation with the mutational burden detected by fragment analysis (Pearson: 0.758, p-value: 0.001, n = 220; Figure 8). Consistent with previous reports [26, 38], high FLT3-ITD mRNA levels (>0.5) measured by fragment analysis showed a significant correlation with shorter relapse-free survival (RFS) and overall survival (OS) (Supplementary Figure 3A and 3B). This correlation could also be observed for FLT3-ITD levels by HTAS for RFS and as trend for OS (Supplementary Figure 3C and 3D). Given that the FLT3-ITD/FLT3-WT ratio based on the sum of all ITD clones is recommended by the ELN for standard of care [8], we calculated the total FLT3-ITD mutational burden per patient. Adding the mutational FLT3-ITD burden of ITD subclones increased the significance of the correlations between ITD load and outcome (Supplementary Figure 4A–4C). This was also evident in multivariate analysis (Supplementary Table 6). A total FLT3-ITD mRNA load below 50% was an independent favorable prognostic factor for outcome measured by fragment analysis for both RFS (p-value: 0.005) and OS (p-value: 0.020). For HTAS a low ITD mutation load correlated significantly with longer RFS in multivariate analysis (p-value: 0.025).

The VAF levels of FLT3-ITD measured by HTAS were up to 5-times lower than the FLT3-ITD mRNA level measured by fragment analysis. Interestingly, the difference in mutational burden between HTAS and fragment analysis increased with ITD length irrespective of clonal dominance (Spearman: 0.530, p-value: 0.001, Supplementary Figure 5A and 5B). Using HTAS, long ITDs were detected on average with lower VAF compared to short ITDs (Supplementary Figure 5C, Spearman: −0.249, p-value: 0.001, n = 312), while fragment analysis measured FLT3-ITD mRNA levels more accurately, regardless of ITD length, with only a minor decrease for long ITDs (Supplementary Figure 5D; Spearman: −0.054, p-value: 0.418, n = 228). In HTAS, the number of ITD-supporting reads was negatively correlated with ITD length, while the total number of reads was similar in short and long ITDs (Pearson: 0.309, p-value: 0.001, n = 242). This correlation is likely due to the fact that long ITDs were more difficult to map to the reference sequence. Samples harbouring ITDs with a length < 75 nt showed significantly fewer unmapped reads compared to samples harbouring ITDs with a length > 75 nt (Mann-Whitney-U test, p-value: <0.001; Figure 9). The ITD position was also related to the difference in mutational burden between HTAS and fragment analysis (Supplementary Figure 6), with longer insertions at cDNA nucleotide positions encoding C-terminal domains.

Figure 2: FLT3 mutations. Schematic illustration displaying AML-specific FLT3 mutations according to their receptor domain localization (modified from Opatz et al. [72]). FLT3-ITDs are located within the juxtamembrane (JM) and tyrosine kinase domain (TKD) 1, whereas point mutations are frequently found in TKD1 and TKD2. FLT3 cDNA region covered by HTAS and fragment analysis is indicated by the primer binding marks (R5 and R6). ITD (internal tandem duplication), JM-B (JM binding motif), JM-S (JM switch motif), JM-Z (JM zipper motif), HR (hinge region), β1 (β1-sheet), NBL (nucleotide binding loop), β2 (β2-sheet).
of FLT3 (Spearman: 0.536, \( p \)-value: 0.001, \( n = 312; 
Supplementary Figure 7). Validation of ITDs using gDNA in 43 patient samples furthermore revealed differences in the FLT3-ITD levels from those measured using cDNA in several cases. Besides the underestimation of the mutational burden for long ITDs by HTAS, discrepancies might be attributed to transcriptional imbalance favouring either the WT or the ITD allele. Overall and in line with published data [23, 39], FLT3-ITD levels measured by HTAS with cDNA template showed a strong correlation with the genomic levels measured by fragment analysis (Spearman: 0.846, \( p \)-value: <0.001,

| Table 2: FLT3 primers |
|-----------------------|
| **Primer for fragment analysis of FLT3** |
| forward | reverse |
| cDNA | 5′-FAM-tgt cga gca gta ctc taa aca tg-3′ (R5) | 5′-atc cta gta cct tcc caa act c-3′ (R6) |
| gDNA | 5′-FAM-gca aat tag gta tga aag cca gc-3′ (11F) | 5′-cct tca gca ttt tga cgg caa cc-3′ (12R) |

**Primer for high-throughput amplicon sequencing of FLT3**

| forward | reverse |
| 5′-aat gat acg gec aac aac gag atc act ctt cta cae gac gtct ctg atc t – BARCODE – tgt cga gca gta ctc taa aca tg -3′ | 5′ - cca cga gaa gac ggc ata cga gat cgg tct cgg cat tcc tgc tga acc gtct ccc gat t – BARCODE – atc cta gta cct tcc caa ac c – 3′ |

FAM (fluorescein amidite).

of FLT3 (Spearman: 0.536, \( p \)-value: 0.001, \( n = 312; 
Supplementary Figure 7). Validation of ITDs using gDNA in 43 patient samples furthermore revealed differences in the FLT3-ITD levels from those measured using cDNA in several cases. Besides the underestimation of the mutational burden for long ITDs by HTAS, discrepancies might be attributed to transcriptional imbalance favouring either the WT or the ITD allele. Overall and in line with published data [23, 39], FLT3-ITD levels measured by HTAS with cDNA template showed a strong correlation with the genomic levels measured by fragment analysis (Spearman: 0.846, \( p \)-value: <0.001,

![Figure 3: FLT3-ITD detection output by fragment analysis and by HTAS.](image)

Exemplary results are shown for UPN 42 (A) Electropherogram displays FLT3 amplicon signals as fragment peaks, with the distance of the peak positions corresponding to the size of the ITD and the area under the peak curves used for calculation of the mutational burden. (B) Tabular representation of variant detection results from HTAS in VCF format (Pindel output). WT (wild type), ITD (internal tandem duplication), Chrom (Chromosome), Pos (cDNA position), Ref (reference sequence), Alt (alternative sequence), VAF (variant allele frequency) *computed separately and added manually.
n = 86 ITDs; Supplementary Figure 8A) and targeted haloplex sequencing (Spearman: 0.752, p-value: <0.001, n = 41 ITDs, Supplementary Figure 8B). Comparison of genomic and transcriptional FLT3-ITD levels measured by fragment analysis excluded gross methodological differences. However, the distribution pointed towards a transcriptional imbalance in favour of the ITD allele, consistent with a moderate correlation (Spearman: 0.554, p-value: <0.001, n = 40 ITDs, Supplementary Figure 8C). Internal cell line controls were sequenced in each of the four instrument runs. The FLT3-ITD positive cell line MOLM-13 displayed the expected FLT3-ITD (size: 21 nt; cDNA position: 1774) [40]. Comparison of the mutational burden in the heterozygous FLT3-ITD positive cell line MOLM-13 between instrument runs revealed a lower experimental variance in HTAS (0.17%, mean VAF ± standard deviation: 49.24 ± 0.36) compared to fragment analysis (4.92%, mean FLT3-ITD mRNA level ± standard deviation: 0.49 ± 0.02; Supplementary Figure 9), indicating high inter-run reproducibility and accuracy of HTAS.

**FLT3-ITD detection by HTAS requires controls to exclude ITD artifacts**

We detected ITD artifacts, which were present in the negative control cell line HL60 and the FLT3-ITD negative patient samples, at VAF levels below our cut-off of 0.5% (Supplementary Table 7). By manual inspection, these two ITD artifacts were therefore excluded from the analysis, even if occurring in patient samples at VAFs above the cut-off of 0.5%. This applied for the artifact ITD at cDNA position 1712 in 148 (61%) of analysed samples (median VAF: 0.99%; range: 0.5–4.71%). Furthermore, we identified and excluded a second ITD artifact within the primer region (cDNA position: 1831–1848), occurring at reference cDNA position 1837 exclusively. This ITD artifact occurred as subclone in 36 patients (15%), at a median VAF of 0.93% (range: 0.5–3.71%) and a median size of 68 nt (range: 35–107 nt). These ITD artifacts showed high sequence similarity to the ITD of the predominant clone, however, they were one nt shorter and displayed 1/20 of the VAF of the original FLT3-ITD (median length: 69 nt, range: 36–108 nt; median VAF: 20.57%, range: 6.38–79.49%, example shown in Supplementary Table 7). Thus, this points towards a PCR-based mispriming event, similar to those reported for false-positive calls in multiplex PCR-based NGS approaches [41].

**Mutations in other genes do not correlate with ITD position, length or clonality**

Individual patients were evaluated for mutations in NPM1, CEBPA, KIT, IDH1/2 and KMT2A-PTD. One hundred nineteen of 182 patients (65%) were positive...
for an NPM1 mutation, nine out of 112 patients (8%) were positive for a CEBPA mutation (three CEBPA-double positive), two out of 164 (1%) were positive for a KIT mutation, 14 out of 75 (19%) were positive for an IDH1/2 mutation, 19 out of 233 patients (8%) were positive for a KMT2A-PTD mutation. A point mutation in the TKD of FLT3 was found in six out of 208 patients (3%). Other mutations, in amongst others NRAS and RUNX1, occurred in up to four patients per affected gene. There was no correlation of FLT3-ITD clonality with other co-occurring mutations (Supplementary Figure 10). Mutations were equally distributed between the patients when clustered according to FLT3 domains (Table 3). Neither length nor position of FLT3-ITD correlated with the co-occurrence of other mutations. In agreement with published results [42], NPM1 mutations correlated with better clinical outcome in our study cohort (Supplementary Figure 11). Patients positive for FLT3-ITD and NPM1 mutation had a significantly increased RFS compared to FLT3-ITD positive and NPM1 negative patients (p-value: 0.049). For OS, a similar trend with borderline significance was observed. In line with published results [35, 43–45], an NPM1 mutation remained an independent favorable prognostic factor in multivariate analysis (Supplementary Table 6). In contrast, the other evaluated mutations did not show any correlations with clinical outcome in our FLT3-ITD positive study cohort.

**DISCUSSION**

In this study, we report the comparison of two different methods to detect FLT3-ITD mutations in 250 adult FLT3-ITD positive AML patients. Since FLT3-ITD parameters, including position and mutational burden, are of clinical relevance for risk stratification and therapy decision [1, 6, 9, 10, 16, 26, 46], a fast and reliable detection method for routine diagnostics is essential. Therefore, we established a high-throughput FLT3-ITD amplicon sequencing assay to gain information...
complementary to the results from routine fragment analysis. Although HTAS identified nearly all dominant ITD clones that were detected by routine diagnostics, we encountered technical limitations consistent with previous studies investigating smaller cohorts [47, 48]. In particular, we found methodological differences between HTAS and fragment analysis with respect to ITD length and the quantification of the FLT3-ITD mutational burden. In 5% of our patients the ITD length differed when comparing HTAS and fragment analysis results. In contrast to other studies with lower patient numbers [16, 22, 29], the ITD length and position did not correlate with clinical outcome in our study. Concordant with other reports [16, 17, 49, 50], the presence of ITDs with high mutational burden correlated with worse prognosis. In multivariate analysis, the FLT3-ITD level below 50% was an independent favorable prognostic factor. In our study, HTAS revealed more subclonal ITDs compared to fragment analysis and the additional clonal complexity uncovered by HTAS correlated with adverse clinical outcome. In line with our findings, it was recently shown that the number of driver mutations has prognostic relevance in MDS and AML [3, 51, 52]. It has already been suggested by others to consider the number of FLT3-ITD clones per patient

Figure 6: Impact of the number of FLT3-ITD clones on relapse-free and overall survival. (A) Relapse-free and (B) overall survival of patients according to number of FLT3-ITD clones detected by HTAS with cDNA template (n = 242; one FLT3-ITD per patient (n = 191), more than one FLT3-ITD per patient (n = 51)). (C) Relapse-free and (D) overall survival of patients according to number of FLT3-ITD clones detected by fragment analysis with cDNA template (n = 242; one FLT3-ITD per patient (n = 212), more than one FLT3-ITD per patient (n = 30). HTAS (high-throughput amplicon sequencing), ITD (internal tandem duplication).
for prognostic stratification [38, 49, 53]. Whereas in our analysis the FL3-ITD clonality (single versus multiple) did not reach statistical significance in multivariate analysis, we observed a trend for better outcome associated with single alterations when evaluated by HTAS. Although the scalable sensitivity of NGS approaches based on read depth seems attractive, the sensitivity might be limited by false-positive variant calls as observed in the present study. Therefore, the implementation of appropriate negative controls is essential. Moreover, NGS data has to be analyzed carefully to identify and to exclude ITD artifacts as detected at cDNA position 1837 in more than 15% of our patients.

Besides clonality, another important prognostic parameter is the FL3-ITD mutational burden. Although there was a significant correlation between FL3-ITD mRNA level detected by fragment analysis and variant allele frequency by HTAS, the prognostic relevance of FL3-ITD mutational burden was more pronounced when considering the results from fragment analysis. Of note, in fragment analysis the FL3-ITD mRNA levels were up to five-fold higher for patient samples compared to the VAF determined by HTAS. Consistent with results from a study utilizing Pindel software as well as a custom de-novo assembly approach for FL3-ITD detection [54], we found that HTAS underestimated the FL3-ITD mRNA levels of long ITDs, which are less likely to be mapped correctly compared to shorter ITDs. In our study, ITDs with a length > 75 nt showed inappropriate mapping to the reference sequence. Interestingly, ITD detection by Pindel was recently shown to be dependent on the length and on the relative position of the amplicon [47]. Thus, algorithms for read mapping and variant detection are currently limiting the detection of long ITDs. This limitation might be overcome with increasing read length, as in the present study 2 × 250 bp paired-end reads did not provide sufficient bi-directional coverage of long ITDs. Since the FL3-ITD/FL3-WT ratio, which is of prognostic value [4, 26, 28, 38, 49, 55], is currently measured more accurately by fragment analysis, ITD quantification by HTAS needs to be further optimized. On the other hand, detection of ITD subclones by NGS-based approaches with increased sensitivity contributes to the total FL3-ITD mutational burden and thus increases the FL3-ITD mutational burden.

![Figure 7: Sensitivity of FL3-ITD detection by HTAS compared to fragment analysis](image-url)

**Figure 7: Sensitivity of FL3-ITD detection by HTAS compared to fragment analysis.** FL3-ITD from the heterozygous FL3-ITD positive cell line MOLM-13 detected after serial dilution in the FL3-WT cell line HL60 by HTAS or fragment analysis using cDNA (n = 3; log(10); 95% confidence interval). Cell line cDNA was derived from five million cells each. For amplification 1 µL cDNA template of each serial dilution was used. The dashed line represents the cut-off defined for ITD-analysis in patient samples. HTAS (high-throughput amplicon sequencing), ITD (internal tandem duplication).
ITD/FLT3-WT ratio. Superior sensitivity of NGS is also relevant for MRD monitoring in leukemia patients. In our serial dilution, HTAS reached a sensitivity of $10^{-3}$ for the heterozygous FLT3-ITD positive cell line MOLM-13, in a multi-sample sequencing setting of 96 samples per run, being superior to fragment analysis. Since sensitivity and coverage is scalable, depending on the number of samples per run, a higher sensitivity can be achieved, if required, as shown by others [30]. Although FLT3-ITD is a rather variable marker during therapy and disease progression, with mutational plasticity between diagnosis and relapse [56–59], several studies [27, 30, 33] have pointed out the advantages of NGS approaches for FLT3-ITD MRD assessment over conventional diagnostic applications. Especially, the recently approved TKI treatment with Midostaurin in combination with induction chemotherapy for FLT3-mutated AML [13] requires the reliable revascularion of FLT3 mutations for initial risk assessment, monitoring and therapeutic intervention. In a recent study, FLT3-mutation positive relapsed or refractory AML CHRYSLAS Phase I/II study patients were analysed for their TKI-response to Gilteritinib (ASP2215;clinical trial number: NCT02014558 [60]). Interestingly, the clinical response correlated with the post-treatment FLT3-ITD MRD levels, evaluated by NGS [61]. In addition, the identification of the ITD position by NGS approaches might be of prognostic relevance considering the impact of FLT3-ITD insertion site on therapy resistance and outcome – for conventional chemotherapy as well as for TKI treatment [10, 16–21]. However, the clinical relevance of ITD position still remains controversial [22, 24, 25, 38]. Furthermore, newly arising FLT3 point mutations during clonal evolution, as for example D835Y and D835G, have prognostic impact as being capable of mediating TKI resistance [33]. Thus, reads spanning the FLT3 regions JM to TKD2 should be used in future approaches as sequence read length steadily increases. With multiple parameters obtained from a single assay and hands-on time for sample preparation and analysis similar to FLT3-ITD fragment analysis or Sanger sequencing, NGS techniques become more and more attractive for diagnostic laboratories. Down-scaling of our 96-sample setting according to the needs in diagnostic routine is feasible when using recently introduced scalable sequencing instruments and reagents. HTAS may enable more precise therapy decisions based on the detection of small ITD clones and has a strong potential for monitoring of FLT3-directed interventions.

In summary, our study demonstrates the feasibility of HTAS for FLT3-ITD detection in AML. We show that the sensitive FLT3-ITD subclone detection by HTAS is of prognostic relevance and has the potential to shed light on the clonal architecture of AML. However, the detection of

Figure 8: FLT3-ITD mutational burden measured by fragment analysis and HTAS. Correlation of the FLT3-ITD mRNA level according to fragment analysis or according to the variant allele frequency in HTAS using cDNA template ($n = 220$). HTAS (high-throughput amplicon sequencing), ITD (internal tandem duplication).
long ITDs and the detection of ITDs in combination with deletions remain challenging. Increasing read length as well as improving variant detection algorithms will likely help to overcome these limitations. After methodological improvement, HTAS may serve as a robust and sensitive tool that could be implemented in future diagnostic routines, essential for a rapid risk stratification and therapeutic intervention.

**MATERIALS AND METHODS**

**Patient samples**

This study included 267 newly diagnosed patients with AML, of which 250 were FLT3-ITD positive according to routine diagnostics (median age: 59, range: 18–80 years; Figure 1). All patients were treated intensively according to the German Acute Myeloid Leukemia Cooperative Group (AMLCG) clinical trial study protocols with curative intent (AMLCG 1999 (ClinicalTrials.gov identifier: NCT00266136) and 2004 as published [35, 36], AMLCG 2008 (n = 38, ClinicalTrials.gov identifier: NCT01382147)), which were approved by the institutional review boards of the participating centers. Patients with acute promyelocytic leukemia (FAB M3) were not treated on these trials, and therefore not analysed in our study. Informed written consent was obtained from all patients in accordance with the Declaration of Helsinki. According to the ELN-classification [9] patients clustered into the following groups: intermediate I (70%), intermediate II (21%) or adverse (9%). A normal karyotype was observed in 70%, while 30% had a complex aberrant karyotype. After intensive induction chemotherapy, 142 (56.8%) patients achieved CR, 16 (6.6%) patients achieved CRi, while 34 (13.6%) patients had refractory disease, 35 (14.0%) patients died during induction therapy, and for 23 (9.1%) patients no remission status was available. Hematopoietic stem cell transplantation in first CR was performed in 36 (14.4%) patients. After a median follow-up of 50 months (range: 0–136), 97 (68.3%) patients that had reached CR eventually relapsed and 187 (74.8%) of all patients died. The median RFS and OS observed were seven and nine months, respectively. The two-year rates of RFS and OS were 20.0% and 25.2%, respectively. Samples were collected at the University Hospital LMU Munich at the time point of first diagnosis. Mononuclear cells were isolated from bone marrow aspirates or peripheral blood and subjected to routine diagnostics for conventional cytogenetic and routine mutational analysis of known molecular markers, including NPM1, CEBPA and FLT3-ITD, according to standard protocols [2, 26, 35, 62]. Patient characteristics and clinical parameters are provided in Table 1.

**Cell lines**

All cell lines were purchased from the German Collection of Microorganisms and Cell Culture (DSMZ, Braunschweig, Germany), with certified cell authentication using karyotyping and fluorescent in-situ hybridization, immunophenotyping, and testing of cancer-type specific mutations using RT-PCR analyses [63, 64]. AML cell lines positive for FLT3-ITD (MOLM-13, ACC-

![Figure 9: Mappability of FLT3-ITD sequence reads.](image-url) Mappability of sequence reads from HTAS according to length of ITD. *P*-value was computed using Mann-Whitney-U test. nt (nucleotide), HTAS (high-throughput amplicon sequencing), ITD (internal tandem duplication).
554) and negative for FLT3-ITD (HL60, ACC-3) were cultivated according to the supplier’s recommendations. Cell lines were tested for a mycoplasma contamination on a regular basis (MycoAlert Mycoplasma Detection Kit, Lonza Rockland Inc., Rockland, ME, USA).

**DNA and RNA isolation**

Extraction of DNA and RNA was performed using standard procedures. Per sample five million cells were used for DNA or RNA isolation each. gDNA and total RNA were extracted using the QIAamp DNA Mini Kit (51106, Qiagen) or RNeasy Mini Kit (74106, Qiagen, Hilden, Germany), respectively, utilizing a QIAcube (Qiagen) according to the suppliers’ recommendations. Purity of isolated RNA and DNA was verified by absorbance measurements (260/280 ratios) with a Nanodrop instrument (Peqlab Biotechnology, Erlangen, Germany).

**cDNA synthesis**

cDNA synthesis was performed by reverse transcription (RT) using total RNA extracted out of five million cells, the Superscript II Reverse Transcriptase and corresponding buffer (18064071, Invitrogen – ThermoFisher Scientific, Munich, Germany), 100 mM dNTPs Set (10297-117, Invitrogen–ThermoFisher Scientific), 25 µM Random Primer p(ND)6 (1034731, Roche Diagnostics, Penzberg, Germany) and RNase Inhibitor (N2615, Promega, Mannheim, Germany). Reactions were performed on a thermocycler (Peqlab Biotechnology), according to the technical protocol of the Superscript II Reverse Transcriptase (70° C 10 min, 37° C 120 min and 90° C 5 min, 1 cycle each).

**FLT3-ITD fragment analysis**

For fragment analysis, PCR (amplification for 28 cycles –1 min 95° C, 1 min 60° C, 1 min 72° C) with 1 µL patient or cell line cDNA or gDNA (10 ng/µL) template, respectively, was performed in a 25 µL reaction volume to amplify FLT3, utilizing fluorescently-labelled primers (10 pmol each, Table 2, Metabion, Planegg, Germany) as published [65, 66] and the Taq PCR Master Mix (201445, Qiagen). Thereafter, 0.5 µL fragment-length standard (GeneScan (500) ROX Size Standard, 401734, Applied Biosystems, Foster City CA, USA) and 13.5 µL PCR-grade water were added to 1 µL PCR-product. After initial denaturation of this mixture at 95° C, size-separation by capillary electrophoresis was performed on a Genetic Analyzer 3500xl (Applied Biosystems) using the separation matrix POP-6 polymer (4316357, Applied Biosystems). Data analysis was performed using the GeneMapper software (Version 3.5, Thermo Fisher Scientific).

**FLT3-ITD mutational burden (FLT3-ITD mRNA level respectively)** was calculated based on the WT to ITD ratio equation as previously published [26]. Comparative analysis of detected ITDs was based on cDNA fragment analysis, whilst gDNA fragment analysis, referred to as g(F), was performed as validation.

**FLT3 amplification and Sanger sequencing**

For Sanger sequencing PCR (amplification for 35 cycles – 1 min 95° C, 1 min 60° C, 1 min 72° C) with 1 µL patient cDNA template was performed in a 25 µL reaction volume to amplify FLT3, using the FLT3 primers 11F and 12R (10 pmol each, sequence as described [65]) and Taq PCR Master Mix (201445, Qiagen). After PCR-product purification with the Qiagen PCR Purification Kit (28106, Qiagen), bi-directional sequencing was performed.
with a second round of amplification using the Big Dye Terminator v1.1 kit (4337451, Life Technologies) and the FLT3 primer 11F and 12R (10 pmol each, sequence as described [65]). Samples were purified using the CentriSep 8 solution and Columns (CS-912, Princeton Separations) and subsequently sequenced using a Genetic Analyzer 3500xl (Applied Biosystems) according to the manufacturers’ instructions. Chromatograms were analyzed using Sequencher Software 5.1 (Gene Codes Cooperation, MI, USA) and the FLT3 cDNA reference sequence (NM_004119.2).

High-throughput FLT3-ITD amplicon sequencing (HTAS) and genomic targeted sequencing

In case of HTAS, FLT3 amplicons were generated performing PCR (TD58, amplification for 30 cycles – 30 sec 95°C, 30 sec 58°C, 1 min 72°C) with 1 µL cDNA template in a 25 µL reaction volume, using the Taq PCR Master Mix Kit (201445, QIagen) and custom-designed FLT3 cDNA primers (10 pmol each, Table 2). The FLT3 cDNA primers, spanning the mutational hotspot region (spanning 366 base-pairs (bp)), included a barcode and Illumina-specific adapter-sequences (Supplementary Table 8), enabling a one-step PCR-protocol for barcoded FLT3-targeted amplification and multiplex-sequencing. As controls, cDNA from FLT3-ITD positive (MOLM-13) and negative (HL60) human cell lines were amplified. Correct amplicon fragment size was verified by agarose gel-electrophoresis. The PCR-product was purified utilizing NucleoFast 96 PCR clean-up plates (743100, Macherey-Nagel, Düren, Germany) according to the manufacturers’ instructions. DNA concentration was measured using the Quant-it dsDNA Broad Range High Sensitivity Kit (Q33120, Thermo Fisher Scientific) in compliance with the manufacturers’ protocol, following dilution to a final concentration of 4 nM (4 fmol/µL). Library preparation was performed according to the manufacturers’ instructions using the MiSeq Kit v2 500 cycles (MS-102–2003, Illumina, San Diego CA, USA), while adding PhiX control (PhiX Control v3 Kit, FC-110-3001, Illumina) in a ratio 4:1 (800 µL library, 200 µL PhiX control). Per library, up to 96 samples were pooled, adding 10 µL of each 4 nM sample. The cell lines HL60 and MOLM-13 were included in every instrument run, serving as inter-run FLT3-WT and FLT3-ITD control. Sequencing (2 × 250 bp paired-end) was performed on a MiSeq Personal Sequencer instrument (Illumina, San Diego CA, USA) in four independent runs, yielding a median of 79,110 reads per amplicon (range: 31,996–259,783). For validation purposes genomic targeted sequencing data, was obtained from a previous study, generated by utilization of an HaloPlex target enrichment system (Agilent Technologies, Santa Clara, USA) as described previously [3].

Next generation sequencing data analysis

Raw sequence reads were aligned to the FLT3 cDNA reference sequence (NM_004119.2) using BWA [67]. Parameters were adjusted in order to allow the incorporation of long insertions, i.e. minimized gap open and gap extension penalties, maximum number of gap extensions. Analysis of NGS-data was performed using the Galaxy platform [68, 69]. Tandem duplications were called using Pindel (version 0.2.5a7) [70] with a minimum size of 6 bp and a minimum of 10 supporting reads. Pindel reports left-aligned positions of variants, which is the leftmost possible position of an alteration. As tandem repeats are two identical and consecutive sequences, Pindel reports the starting position of the first sequence (i.e. the 5′-template) by default. In order to indicate the position of the inserted sequence (i.e. the 3′-duplicate), we added the length of the ITD to the position reported by Pindel. Furthermore, the 5′-UTR region (82 nucleotides), included in the FLT3 cDNA reference sequence, were substructed from the variant position. Thus, results from HTAS could be compared to results from Sanger sequencing, which refer to the coding sequence. The VAF was computed by dividing the number of supporting reads with the coverage at the insertion site. Minimum cut-off for VAF was set to 0.5% (HTAS), based on empirical analysis of FLT3-ITD negative control samples, to exclude any sequencing background noise (non-specific variants). Off-target ITDs and ITDs which appeared in the FLT3-WT cell line HL60 were excluded from further analysis. Per sample the median coverage per amplicon was 157,100× (range: 63,890× – 488,300×).

Statistical analysis

SPSS (IBM, version 21.0) or R (GNU GPL, version 3.4.1) software was used for statistical analysis. Spearman rank correlation was used to examine correlations between continuous parameters. Two-sided log rank test was used for Kaplan-Meier diagrams to compute survival curves. Mann-Whitney-U test was performed to test for differences between groups. Results were considered as significant with a p-value less than 0.05. Univariate and multivariate Cox-Regression analysis was performed to evaluate prognostic variables for OS and RFS. RFS was calculated from the time span between diagnosis and relapse [9, 71], failure to achieve a complete remission (CR) [35], last follow-up or death. OS was evaluated from initial diagnosis to last follow up or death. The criteria of relapse, resistant disease and CR were assigned according to ELN [9]. Cox proportional hazard model was used to estimate hazard ratios for multivariate analysis.

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CONFLICTS OF INTEREST

The authors have no conflict of interest to declare.

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