Impact of FLT3-ITD Insertion Length on Outcomes in Acute Myeloid Leukemia: A Propensity Score-Adjusted Cohort Study

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Simple Summary: Acute myeloid leukemia (AML) is a complicated disease with well-studied genetic mutations that have been used in both risk stratification as well as targets for treatment. In this study, we examined the varying prognostic significance of different lengths of the special mutation, named internal tandem duplication or ITD, of a commonly mutated gene in AML named FLT3. We found that the longer the size of the ITD mutation, the better the clinical outcomes.

Abstract: The prognostic significance of the length of internal tandem duplication (ITD) insertions in mutant FLT3 genes in acute myeloid leukemia (AML) is controversial. We conducted a retrospective study to evaluate the correlation between the ITD base-pair (bp) insertion length and clinical outcomes. The mutational status of the FLT3 gene was evaluated in 402 of 467 consecutive AML patients treated at the University of Maryland Greenebaum Comprehensive Cancer Center between 2013 and 2020; 77 had FLT3-ITD mutations. Patients were divided into three cohorts based on bp insertion length (<30 (0–33rd percentile), 30–53 (34th–66th percentile), and >53 (>66th percentile)). The median overall survival (OS) of patients was 16.5 months (confidence interval (CI) 7.3-NA), 18.5 months (CI 7.3-NA), and 21.9 months (CI 19.1-NA) (p = 0.03) for the <30, 30–53, and >53 bp insertion length cohorts, respectively. The adjusted median event-free survival (EFS) for the ITD insertion lengths >30 (0–33rd percentile), 30–53 (34th–66th percentile), and >53 (>66th percentile)). The median overall survival (OS) of patients was 16.5 months (confidence interval (CI) 7.3-NA), 18.5 months (CI 7.3-NA), and 21.9 months (CI 19.1-NA) (p = 0.03) for the <30, 30–53, and >53 bp insertion length cohorts, respectively. The adjusted median event-free survival (EFS) for the ITD insertion lengths >30 (0–33rd percentile), 30–53 (34th–66th percentile), and >53 (>66th percentile)). The median overall survival (OS) of patients was 16.5 months (confidence interval (CI) 7.3-NA), 18.5 months (CI 7.3-NA), and 21.9 months (CI 19.1-NA) (p = 0.03) for the <30, 30–53, and >53 bp insertion length cohorts, respectively. The adjusted median event-free survival (EFS) for the ITD insertion lengths >30 (0–33rd percentile), 30–53 (34th–66th percentile), and >53 (>66th percentile)).

Keywords: acute myeloid leukemia; survival; prognosis; FLT3-ITD

1. Introduction

Mutations in the gene encoding the FMS-like tyrosine kinase 3 (FLT3) receptor tyrosine kinase occur in approximately 30% of acute myeloid leukemia (AML) cases, with internal tandem duplication (ITD) representing the most common type (25% of AML cases) [1]. Guidelines from organizations such as the World Health Organization (WHO), National Comprehensive Cancer Network (NCCN), and European LeukemiaNet (ELN) for AML
classification and risk stratification rely heavily on cytogenetic aberrations and gene mutations [1–4]. FLT3-ITD mutations are associated with high leukemic burden and poor prognosis in AML [5]. ITDs occur in the juxtamembrane domain of the FLT3 receptor. They cause disruption of the FLT3 signaling, which results in the loss of its autoinhibitory function and leads to FLT3 ligand-independent phosphorylation and activation of the FLT3 receptor [6,7]. FLT3-ITD mutations are heterogeneous in allelic burden, size, and location [8].

Currently, utilizing polymerase chain reaction (PCR) to amplify the 328 bp region within the FLT3 gene is achieved by using target-specific fluorescently labeled primers. This amplification method and fragment-size detection on the ABI 3730 genetic analyzer and analysis using GeneMappet software allows detection of the length of the insertions as well as the mutant allele burden. Currently, there are conflicting data about the relationship between the size of the FLT3-ITD insertion length and clinical outcome. FLT3-ITD insertion size does not currently affect clinical decision-making. Retrospective studies have variably shown worse overall survival (OS) with increasing insertion length [9–14], no impact on OS [15,16], poorer survival with medium-size ITD length [17], better OS with increased ITD insertion length [18], and differences by domain of the mutation [19]. Stirewalt et al. suggested [9] that the increasing ITD insertion length leads to a greater loss of the autoinhibitory function of FLT3, thus conferring higher risk with longer insertion; however, Kusec et al. [18] asserted that insertion length might not be related directly to the activation of these signaling pathways. In this study, we aimed to further elucidate the role of the FLT3-ITD base-pair (bp) insertion length in AML prognostication in a propensity score-adjusted retrospective study. We also aimed to investigate the correlation of the ITD insertion length with response to the FLT3 inhibitors midostaurin and gilteritinib.

2. Methods

2.1. Study Design

We conducted a single-site retrospective cohort study to compare OS, event-free survival (EFS), complete remission (CR), and complete remission with incomplete hematologic recovery (CRi) rates in adults with AML with different FLT3-ITD lengths from 2013 (the beginning of in-house PCR testing for FLT3 mutations at our institution) to 2020. All patients tested for FLT3-ITD mutation length using PCR were included. Patients not tested for FLT3-ITD mutations were excluded.

Treatment response was evaluated in accordance with the 2017 ELN criteria [11]. Composite CR rate (CCR) included CR+CRi. EFS was defined as the time from treatment initiation to induction failure, relapse, or death from any cause. OS was defined as the time from diagnosis to death from any cause. The study was approved by the University of Maryland Baltimore (UMB) Institutional Review Board (IRB).

2.2. Analysis of FLT3 Mutation

Genomic DNA was extracted and amplified by PCR using primers targeted to the FLT3 gene that can identify ITD variants in exons 14 and 15. The wild-type FLT3 allele is detected as a 328 base-pair PCR product. The insertion length is determined by subtracting the base-pair size of the mutant peak from the base-pair size of the wild-type peak. The sensitivity threshold for detecting a FLT3-ITD variant is 3.1% allele burden relative to that of the normal DNA sequence, as per University of Maryland Translational Genomics laboratory validation protocols. NCBI reference sequences are NM_004119.2, NP_004110.2, and NG_007066.1. In order to determine the insertion site locations for comparison, we collected available Next-Generation Sequencing (NGS) data and mapped ITD mutations to domains by amino acid: juxtamembrane (JM), hinge region, and tyrosine kinase domain (TKD) I and II.

2.3. Data Source

We reviewed the medical records of patients diagnosed with AML at the University of Maryland Greenebaum Comprehensive Cancer Center (UMGCC) (2013–2020) using
the electronic medical records systems, including Epic and its features such as Care Everywhere and CRISP, a state-designed Health Information Exchange for a Maryland online database [20]. Data were collected and managed using Research Electronic Data Capture (REDCap) electronic data capture tools hosted at the University of Maryland [21,22].

2.4. Variables and Comparison Groups

Data extracted included age, gender, ethnicity, Eastern Cooperative Oncology Group (ECOG) performance status (PS), baseline comorbidities, AML categories (de novo, myelodysplasia-related, myeloproliferative-related, therapy-related), cytogenetics, available myeloid mutations, therapy received, and clinical outcomes. We compared patients with different ITD insertion length mutations. We compared insertion sites in different domains: JM, hinge region, and TKD I and II. Data were checked three times by independent data collectors. Patients were divided into three cohorts by percentiles: 0–33rd percentile (bp insertion < 30), 34–66th percentile (bp insertion 30–53), and >66th percentile (bp insertion > 53). These cut-offs were chosen to analyze the length as a continuous variable for a dose–response relationship, similar to previous studies [9,10,13,15].

2.5. Propensity Score Estimation

As we previously described [23], we conducted propensity score modeling to adjust for outcomes. This study obtained the Average Treatment Effect (ATE) as an estimand. We included the following variables in the propensity score model: age at diagnosis, gender, ethnicity, comorbidities, ECOG-PS, type of AML, cytogenetics at diagnosis, and ASXL1, FLT3, RUNX1, and TP53 mutational status. Inverse probability weighting method was used for the propensity score models. We used different methods to estimate weights, including multinomial regression, generalized boosted modeling, covariate balancing, and non-parametric covariate balancing [24]. The choice of weighting method was based on achieving the lowest standardized biases differences, smallest coefficients of variations, and largest estimated sample size [24]. Standardized bias score < 0.25 was used as a cutoff for model choice [25]. We used balance tables and Love plots to assess for covariate balance before and after matching. Robust variance estimator was used to account for within-person homogeneity [26]. Patients with relapsed or refractory AML receiving single-agent gilteritinib were analyzed without propensity score estimation.

2.6. Statistical Analysis

We used descriptive statistics to compare baseline characteristics of patients with various length FLT3-ITD mutations as well as FLT3-WT. Categorical variables were presented as absolute numbers and percentages. Continuous variables were presented as medians with interquartile ranges (IQR) or means with standard deviations (SD). Baseline characteristics were compared using t-test or analysis of variance (ANOVA) when continuous or Pearson chi-square or Fisher’s exact test when categorical. OS and EFS were compared using log-rank and Gehan–Breslow–Wilcoxon rank tests. Multivariable and univariable Cox proportional hazards models (CPH) were used to assess relative mortality. To test for effect modification of NPM1 mutation on outcomes, we estimated OS in ITD insertion categories, stratified by NPM1 mutational status. Moreover, we estimated a multivariable CPH model to predict mortality with NPM1 mutational status and an interaction term between NPM1 and ITD insertion length added to the model. We used regression diagnostics to evaluate model assumptions. All statistical tests were two-sided, and p-values < 0.05 were considered statistically significant. The R-statistical package “WeightIT” was used for propensity score weighting [25,26]. R-statistical software (version 4.1.1) was used for statistical analyses.
3. Results

3.1. Cohort Characteristics

A total of 467 AML patients were treated at UMGCCC during the study period (2013–2020). The FLT3 mutational status was evaluated in 402 patients. FLT3-ITD mutations were present in the AML cells of 77 patients (19%). The median age of the total cohort was 65.3 years [IQR 54.2–74.8], and 200 (44%) were female. The median follow-up for the whole cohort was 76.5 months (confidence interval (CI) 23.9–102.9). The median follow-up was 27 months (CI 13.2–76.3) for the <30 bp group, 29.3 months (CI 26-NA) for the 30–53 bp group, and 80.1 months (CI 78-NA) for the >53 bp group. The comparison groups included 25 patients with a <30 bp insertion, 28 patients with a 30–53 bp insertion, and 24 patients with a >53 bp insertion. Table 1 shows the baseline characteristics of patients with FLT3-ITD, categorized by insertion length. After weighting, all standardized biases scores were less than <0.25 (Figures S1–S3).

Table 1. Baseline characteristics of patients with FLT3-ITD mutations, categorized by base-pair insertion length.

| Insertion Length | <30 bp | Percentage/SD/IQR | 30–53 bp | Percentage/SD/IQR | >53 bp | Percentage/SD/IQR | p-Value |
|------------------|-------|-------------------|----------|-------------------|--------|-------------------|---------|
| Number of patients | 25    | 28                | 24       | 24                |         |                   |         |
| Age (Average ± SD) | 63.9  | 63.8              | 58.6     | 15.2              | 15.2   | 0.43              |         |
| Age (Median, IQR) | 66.9  | 65.3              | 60.8     | 56.9–72.7         | 45.9–71.1 | 0.60          |         |
| Female           | 5     | 12                | 17       | 43                | 71     | 0.002             |         |
| Blood Counts at Diagnosis | | | | | | | |
| White Blood Cells (K/μL) (Median, IQR) | 18.5  | 75.9              | 70.7     | 13.2–130.3        | 0.03   |       |         |
| Hemoglobin (g/dL) (Median, IQR) | 8.4   | 8.6               | 8.2      | 7.3–9.5           | 0.75   |       |         |
| Platelets (K/μL) (Median, IQR) | 59.0  | 70.5              | 48.0     | 24–113            | 0.66   |       |         |
| Blast percentage (%) (Average ± SD) | 55.5  | 67.9              | 63       | 26.0              | 0.56   |       |         |
| Body Mass Index | 27.0  | 25.7              | 30       | 5.3               | 12.8   | 0.55   |         |
| Ethnicity        |       |                   |         |                   |        |       |         |
| Causian          | 18    | 20                | 17       | 71.4              | 0.99   |       |         |
| Other            | 7     | 8                 | 7        | 28.6              | 29.2   |       |         |
| Comorbidities    |       |                   |         |                   |        |       |         |
| Cardiovascular disease | 6     | 4                 | 4        | 14                | 0.57   |       |         |
| Diabetes mellitus | 7     | 4                 | 5        | 14                | 0.47   |       |         |
| Hypertension     | 11    | 11                | 9        | 39                | 0.89   |       |         |
| CKD stage III-V/ESRD | 1     | 2                 | 1        | 7                 | 0.84   |       |         |
| Active Cancer    | 0     | 1                 | 0        | 3.6               | 0.41   |       |         |
| AML type         |       |                   |         |                   | 0.37   |       |         |
| AML, de novo     | 17    | 22                | 19       | 78.6              | 0.41   |       |         |
| AML with MDS/CMML changes | 8    | 4                 | 4        | 14.3              | 16.7   |       |         |
| Therapy-Related AML | 0    | 2                 | 1        | 7.1               | 4.2    |       |         |
| ELN 2017 Cytogenetic Category |       |                   |         |                   | 0.680  |       |         |
| Favorable Risk   | 1     | 2                 | 0        | 7.1               | 0      |       |         |
| Intermediate Risk | 22    | 25                | 22       | 89.3              | 91.7   |       |         |
| Unfavorable Risk | 0     | 0                 | 0        | 0                 | 0      |       |         |
Table 1. Cont.

|                        | <30 bp | Percentage/SD/IQR | 30–53 bp | Percentage/SD/IQR | >53 bp | Percentage/SD/IQR | p-Value |
|------------------------|--------|-------------------|----------|-------------------|--------|-------------------|---------|
| Not performed/Poor banding, Inadequate | 2 8 | 1 3.6 | 2 8.3 |        |        |        |          |
| FLT3-ITD status        |        |        |          |        |        |        | 0.37     |
| FLT3-ITD-mutated allelic burden 1–49% | 18 72 | 15 53.6 | 15 62.5 |        |        |        |          |
| FLT3-ITD-mutated allelic burden 50–100% | 7 28 | 13 46.4 | 8 33.3 |        |        |        |          |
| FLT3 wild type         | 0 0   | 0 0   | 1 4.2   |        |        |        |          |
| FLT3-TKD mutated       | 10 40 | 4 14.3 | 7 29.2  | 0.11   |        |        |          |
| TP53 mutational status |        |        |          |        |        |        | 0.01     |
| TP53 mutated           | 1 4   | 0 0   | 0 0     |        |        |        |          |
| TP53 wild type         | 19 76 | 18 64.3 | 11 45.8 |        |        |        |          |
| TP53 untested          | 5 20  | 10 35.7 | 13 54.2 |        |        |        |          |
| RUNXI mutational status|        |        |          |        |        |        | 0.07     |
| RUNXI mutated          | 4 16  | 2 7.1 | 0 0     |        |        |        |          |
| RUNXI wild type        | 16 64 | 16 57.1 | 11 45.8 |        |        |        |          |
| RUNXI untested         | 5 20  | 10 35.7 | 13 54.2 |        |        |        |          |
| ASXL1 mutational status|        |        |          |        |        |        | 0.02     |
| ASXL1 mutated          | 3 12  | 0 0   | 0 0     |        |        |        |          |
| ASXL1 wild type        | 17 68 | 18 64.3 | 11 45.8 |        |        |        |          |
| ASXL1 untested         | 5 20  | 10 35.7 | 13 54.2 |        |        |        |          |
| NPM1 mutational status |        |        |          |        |        |        | 0.08     |
| NPM1 mutated           | 9 36  | 9 32.1 | 8 33.3  |        |        |        |          |
| NPM1 wild type         | 11 44 | 9 32.1 | 3 12.5  |        |        |        |          |
| NPM1 untested          | 5 20  | 10 35.7 | 13 54.2 |        |        |        |          |
| CEBPA mutational status|        |        |          |        |        |        | 0.1      |
| CEBPA mutated          | 1 4   | 4 11  | 1 4     |        |        |        |          |
| CEBPA wild type        | 19 76 | 15 53  | 10 42   |        |        |        |          |
| CEBPA untested         | 5 20  | 10 36  | 13 54   |        |        |        |          |
| ECOG-PS status         |        |        |          |        |        |        | 0.15     |
| I or II                | 23 92 | 23 82.1 | 24 100  |        |        |        |          |
| III or IV              | 2 8   | 4 14.3 | 0 0     |        |        |        |          |
| ECOG status unknown    | 0 0   | 1 3.6 | 0 0     |        |        |        |          |
| Types of first-line treatment |        |        |        |        |        |        | 0.28     |
| Anthracycline-based    | 10 40 | 12 42.9 | 16 66.7 |        |        |        |          |
| Non-anthracycline-based| 13 52 | 14 50  | 8 33.3  |        |        |        |          |
| None                   | 2 8   | 2 7.1 | 0 0     |        |        |        |          |
| Midostaurin with induction | 5 20  | 5 17.8 | 2 8.3  | 0.48   |        |        |          |

3.2. Outcomes of FLT3-ITD-Mutated AML, Categorized by Base-Pair Insertion Length

On the weighted propensity score analysis, the adjusted CCR rates for the 73 treated patients with insertion lengths <30, 30–53, and >53 bp were 64, 55, and 79%, respectively, \( p = 0.23. \) The covariate balances before and after propensity score weighing for OS are shown in Figure S1. The adjusted median OS between groups increased with the bp insertion length: 16.5 months (CI 7.3-NA) for the <30 bp cohort compared to 18.5 months.
(CI 7.3-NA) for the 30–53 bp cohort and 21.9 months (CI 19.1-NA) for the >53 bp cohort ($p = 0.03$). The unadjusted median OS for the three cohorts was 13.8 months (CI 7.2-NA), 12.1 months (CI 7.3-NA), and 20.6 months (CI 19.1-NA) ($p = 0.2$), respectively. Compared to the <30 bp insertion cohort, the weighted mortality hazard ratios (HR) for the bp 30–53 and bp >53 cohorts were 0.79 (CI 0.37–1.65; $p = 0.52$) and 0.56 (CI 0.29–1.09; $p = 0.09$), respectively. On the unweighted multivariable Cox proportional hazard, patients with an insertion length of >53 bp had a relative mortality of 0.44 (CI 0.24–0.81, $p = 0.05$) or >53 bp (HR 0.70, CI 0.35–1.39, $p = 0.5$). The adjusted OS at years 1 and 2 for all three cohorts showed no statistically significant differences (Table S2). The adjusted median EFS for the FLT3-ITD mutation insertion length: <30, 30–53, and >53 bp were 11.1 months (CI 2.8–16.5), 5.2 months (2.9–12.6), and 9.1 months (5.4-NA), respectively, ($p = 0.5$). The adjusted EFS at years 1–3 for patients in all three bp insertion length cohorts showed no statistically significant differences (Table S2). Compared to patients with insertion lengths of <30 bp, the relative mortality or progression were not statistically significantly different in patients with an insertion length of 30–53 bp (HR 1.22, CI 0.69–2.17, $p = 0.5$) or >53 bp (HR 0.70, CI 0.35–1.39, $p = 0.31$), using the weighted univariable Cox proportional hazards regression. Figure 2 demonstrates the propensity score-adjusted OS for patients in all three cohorts. The adjusted OS at years 1 and 2 for all three cohorts are shown in Table S1.

![Image](image-url)

**Figure 1.** Propensity score-adjusted overall for patients with FLT3-ITD-mutated AML, categorized by insertion length.

The EFS was assessed in 73 of the 77 patients with FLT3-ITD mutations who received treatment. The covariate balances before and after propensity score weighing for the EFS are shown in Figure S2. The adjusted median EFS for the FLT3-ITD mutation insertion lengths >30, 30–53, and <53 bp were 11.1 months (CI 2.8–16.5), 5.2 months (2.9–12.6), and 9.1 months (5.4-NA), respectively, ($p = 0.5$). The adjusted EFS at years 1–3 for patients in all three insertion length cohorts showed no statistically significant differences (Table S2). Compared to patients with insertion lengths of <30 bp, the relative mortality or progression were not statistically significantly different in patients with an insertion length of 30–53 bp (HR 1.22, CI 0.69–2.17, $p = 0.5$) or >53 bp (HR 0.70, CI 0.35–1.39, $p = 0.31$), using the weighted univariable Cox proportional hazards regression. Figure 2 demonstrates the propensity score-adjusted EFS for patients in all three bp insertion lengths cohorts.

### 3.3. Outcomes of FLT3-ITD-Mutated and Wild-Type (WT) AML, Categorized by Insertion Length

To compare the outcomes of insertion lengths categories with FLT3-WT AML, regardless of other cytogenetics or mutational status, another inverse probability weighting model was estimated. The unadjusted median OS for patients with FLT3-WT versus FLT3-ITD AML (lengths: <30, 30–53, >53 bp) was 12 months (CI 10.2–15.9) compared to 16.5 months...
(CI 12.5-NA), 21.8 months (CI 9.8-NA), and 23.6 months (CI 20.2-NA) (p < 0.001). The covariate balances before and after propensity score weighing for OS are shown in Figure S3. Figure S4 demonstrates the propensity score-adjusted OS for patients with FLT3-ITD AML versus FLT3-WT AML.

Figure 2. Propensity score-adjusted event-free survival for patients with FLT3-ITD-mutated AML, categorized by insertion length.

3.4. Outcomes of FLT3-ITD-Mutated AML Categorized by Domain Insertion Expansion Categories

Clinical outcomes were analyzed for patients for whom NGS data were available according to ITD insertion site location. Twenty-five patients had insertions in the JM domain, three patients had insertions in the hinge region, and six patients had insertions in the TKD domain. The unadjusted median OS for patients with FLT3-ITD insertions in the hinge region was 28.1 months (CI 16.8-NA) compared to 18.5 months (CI 12.1-NA) for patients with FLT3-ITD insertions in the JM domain versus 20.5 months for patients with FLT3-ITD insertions in the TKD domain (CI 9.5-NA) (p = 0.6, with the reference as the hinge domain). Figure S5 demonstrates the propensity score-adjusted OS for patients with FLT3-ITD AML versus FLT3-WT AML. Compared to the hinge domain insertion cohort, the weighted mortality hazard ratios for the JM domain and TKD domain cohorts were 1.99 (CI 0.46–8.67; p = 0.36) and 1.26 (CI 0.23–6.95; p = 0.79), respectively.

3.5. Outcomes after Using Midostaurin plus Induction Chemotherapy in FLT3-ITD AML, Categorized by Base-Pair Insertion Length

Patients who received midostaurin plus cytarabine and daunorubicin induction therapy were analyzed according to the ITD bp insertion length. Five patients had an insertion length of <30 bp, five had an insertion length of 30–53 bp, and two had an insertion length of >53 bp. The unadjusted CCR rates for insertion lengths <30, 30–53, and >53 bp were 80%, 100%, and 100% (p = 0.56).

The unadjusted median OS was 19.1 months (CI 19.1-NA) for the <30 bp cohort, 21.8 months (CI 21.8-NA) for the 30–53 bp cohort, and not reached for the >53 bp cohort (p = 0.8). Figure S6 demonstrates OS curves for patients in all three groups. Using linear
regression, the average change in the FLT3 allelic burden after midostaurin treatment with induction was $-28.3\%$ for the $<30$ bp cohort (standard error (SE) $28.3\%$, $p = 0.16$), $-49.9\%$ for the $30–53$ bp group (SE $25.8\%$; $p = 0.44$), and $-7.29\%$ for the $>53$ bp group (SE $45\%$; $p = 0.65$), respectively.

3.6. Outcomes after Using Gilteritinib Monotherapy in Relapsed or Refractory FLT3-ITD AML, Categorized by Base-Pair Insertion Length

Patients with relapsed or refractory AML receiving single-agent gilteritinib were analyzed for outcomes according to bp insertion length. Eight patients had a $<30$ bp insertion length, nine patients had an insertion length of $30–53$ bp, and nine patients had an insertion length of $>53$ bp. The unadjusted CR+CRi rates were $88\%$, $67\%$, and $44\%$, respectively ($p = 0.18$). The unadjusted median OS in the insertion length groups was 29.9 months (CI 16.3-NA) for the $<30$ bp cohort, 18.9 months (CI 4.7-NA) for the $30–53$ bp cohort, and 11.7 months (CI 5.13-NA) for the $>53$ bp cohort; however, this finding was not statistically significant ($p = 0.3$). Figure S7 demonstrates unadjusted OS curves for patients with relapsed or refractory AML treated with gilteritinib.

Using linear regression, the average change in the FLT3 allelic burden after the initial use of gilteritinib treatment was $-37.4\%$ for the $<30$ bp cohort (SE $17.5\%$; $p = 0.054$), $-6.64\%$ for the $30–53$ bp cohort (SE $24.7\%$; $p = 0.24$), and $-4.98\%$ for the $>53$ bp cohort (SE $24.7\%$; $p = 0.21$), respectively.

3.7. Outcomes for Patients with FLT3-ITD and Mutated Nucleophosmin (NPM1) AML

We tested outcomes for NPM1-mutated compared to NPM1 wild-type AML with different ITD lengths and the results are presented in Tables S3–S5 and Figure S8. We excluded patients who did not have their NPM1 status checked. There were 26 patients with both NPM1 and FLT3-ITD mutations: 9 in the $<30$ bp cohort, 9 in the $30–53$ bp cohort, and 8 in the $>53$ bp cohort. There were 23 controls (NPM1 WT with FLT3-ITD mutation). The median OS of patients in different insertion length groups are presented in Table S3, stratified by the NPM1 mutational status. The NPM1 mutational status was not associated with a statistically significant change in relative mortality (Tables S4 and S5). In the Cox proportional hazards model shown in Table S4, we added an interaction term between the ITD bp insertion length, modeled as a continuous variable, and the NPM1 mutational status. The interaction coefficient indicates that NPM1-WT was associated with higher mortality when the length of the ITD insertion increased; however, this was not statistically significant.

In the Cox proportional hazards model shown in Table S5, we added an interaction term between the ITD bp insertion length, modeled as a categorical variable, and the NPM1 mutational status. The interaction coefficient indicates that NPM1-WT is associated with higher mortality when the length of the ITD was $>53$ bp and lower mortality when the length was between 30 and 53 bp, both compared to length $<30$ bp; however, this was not statistically significant.

4. Discussion

Prognostic models for AML primarily rely on cytogenetic aberrations and molecular abnormalities, such as FLT3-ITD, NPM1, and CEBPA mutations in patients with a normal karyotype [27]. Here, we aimed to elucidate the prognostic significance of a commonly reported laboratory finding: FLT3-ITD insertion size in AML with this molecular abnormality. When patients were divided into three cohorts based on bp insertion length percentile, we found that the adjusted median OS improved with a longer bp insertion length. This finding remained consistent on analysis when we compared the patient outcomes of all three FLT3-ITD length cohorts to FLT3-WT. This may be due to a high proportion of poor-risk features, such as complex karyotype and TP53 mutations in AML with FLT3-WT. As shown in Table 1, none of the FLT3-ITD groups had high-risk cytogenetics.
Previous studies have theorized that increasing the insertion size leads to a greater loss of autoinhibitory function of the FLT3 receptor, thus conferring higher risk with longer insertion [9–12]. These studies have hypothesized that small ITDs may better preserve the autoinhibitory function of the juxtamembrane domain, whereas larger ITDs completely disrupt this function, leading to uninhibited proliferation. Our study, using propensity score adjustment, suggests that size may play a different role than previously thought. It could be hypothesized that the size of the ITD insertion plays a role in autoinhibitory downstream effects and is not directly tied to disruption. One study reported no prognostic significance of size, but rather that mutations that occurred more toward the 5′ end were associated with a better outcome than mutations closer to the 3′ end [16]. This also suggests that perhaps location plays a role in autoinhibitory function. Our study directly identified a larger size as more favorable. Upon review of the effect of the insertion location, we observed that the closer the expansion to the membrane, the poorer the survival. However, we only had small numbers of patients with this sequencing information, underpowering the results, which was statistically insignificant. It is possible that location along the domain and size may be more directly linked than previously thought, and in vitro studies examining correlations between size and insertion location may further elucidate the complex interaction between the two. In a brief report, Kayser et al. showed similar findings to ours, using a multivariate Cox proportional hazard model that demonstrated that increasing ITD size was associated with a better prognosis [14].

The overall survival for patients with relapsed or refractory disease treated with gilteritinib decreased with an increasing insertion length: 29.9 months (CI 16.3-NA) for the <30 bp cohort, 18.9 months (CI 4.7-NA) for the 30–53 bp cohort, and 11.7 months (CI 5.1-NA) for the >53 bp cohort. This trend may be explained by better mechanistic inhibition of FLT3-ITD with the shorter insertion length by gilteritinib, with less efficacy in long insertions. To date, the Phase 3 clinical trial of gilteritinib in relapsed or refractory FLT3-mutated AML has not reported any negative prognostic effect of the insertion length on survival outcomes [27]. The efficacy of gilteritinib against relapsed or refractory AML with certain base insertion lengths is independent of the prognostic significance of the insertion length in the total FLT3-mutated population of this study. Though these results were not statistically significant, the small sample size and thus power may have led to a Type II statistical error.

Upon testing for an NPM1 and FLT3-ITD length interaction, we found that NPM1 did not predict outcomes independently and did not modify the effect of bp insertion lengths on outcomes. This was either because of our small number of patients (N = 26), or because though the two have previously been shown to be associated [28–32], the NPM1 mutation may not be biologically affected by different FLT3 insertion lengths. One other retrospective study has shown a consistent positive prognostic effect of NPM1 mutations regardless of the FLT3-ITD mutation status [15].

The major limitation of this study is that it was a retrospective single-site model with a limited number of patients. Because retrospective studies are inherently confounded, we used propensity score weighting, weighted multivariable regression, and unweighted multivariable regression to control for observable confounding. After weighting, the standardized mean difference was less than 0.2 in all variables.

5. Conclusions

Though previous studies have been conflicting, we found that increased FLT3-ITD length was associated with better outcomes in a propensity score-adjusted cohort. Future studies, both laboratory and clinical, should be performed to elucidate the link between FLT3-ITD size aberration and outcome as a means of enhancing prognostication and clinical decision-making.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/biology11060916/s1, Figure S1: Loveplot describing covariate balance before and after
matching for different insertion lengths of FLT3 mutated AML for OS; Figure S2. Loveplot describing covariate balance before and after matching for different insertion lengths of FLT3 mutated AML for EFS; Figure S3. Loveplot describing covariate balance before and after matching for different insertion lengths of FLT3 mutated and WT AML, categorized by insertion length; Figure S5: Unadjusted OS for patients with FLT3-ITD mutated AML treated with Midostaurin, categorized by insertion length; Figure S7: Unadjusted OS for patients with FLT3-ITD mutated and NPM1 mutated and WT AML, categorized by insertion length; Table S1. Adjusted OS at years 1–2 for patients with FLT3-ITD mutated AML, categorized by insertion length; Table S2. Adjusted EFS at years 1–2 for patients with FLT3-ITD mutated AML, categorized by insertion length; Table S3. Unadjusted median OS in months for patients with FLT3-ITD and NPM1 mutated AML, categorized by insertion length; Table S4. Unadjusted median OS in months for patients with FLT3-ITD and NPM1 mutated AML, categorized by domain insertion expansion categories; Table S5. Unadjusted median OS in months for patients with FLT3-ITD and NPM1 mutated AML, categorized by domain insertion expansion categories.

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Conflicts of Interest: 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.

References
1. O’Donnell, M.R.; Tallman, M.S.; Abboud, C.N.; Altman, J.K.; Appelbaum, F.R.; Arber, D.A.; Bhatt, V.; Bixby, D.; Blum, W.; Coutre, S.E.; et al. Acute myeloid leukemia, version 3.2017: Clinical practice guidelines in oncology. [NCCN J. Natl. Compr. Cancer Netw. 2017, 15, 926–957. [CrossRef] [PubMed]
2. Levis, M. FLT3 mutations in acute myeloid leukaemia: What is the best approach in 2013? Hematology/Educ. Program Am. Soc. Hematol. Am. Soc. Hematol. Educ. Program 2013, 2013, 220–226. [CrossRef]
3. Schetelig, J.; Rollig, C.; Kayser, S.; Stoelzel, F.; Schaefer-Eckart, K.; Haenel, M.; Roesler, W.; Einsele, H.; Kaufmann, M.; Serve, H.; et al. Validation of the ELN 2017 Classification for AML with Intermediate Risk Cytogenetics with or without NPM1-Mutations and High or Low Ratio FLT3-ITD s. Blood 2017, 130, 2694. [CrossRef]
4. Döhner, H.; Estey, E.; Grimwade, D.; Amadori, S.; Appelbaum, F.R.; Büchner, T.; Dombret, H.; Ebert, B.L.; Fenaux, P.; Larson, R.A.; et al. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. Blood 2017, 129, 424–447. [CrossRef]
5. Ding, L.; Ley, T.J.; Larson, D.E.; Miller, C.A.; Koboldt, D.C.; Welch, J.S.; Ritchey, J.K.; Young, M.A.; Lamprecht, T.; McLellan, M.D.; et al. Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing. Nature 2012, 481, 506–510. [CrossRef] [PubMed]
6. Griffith, J.; Black, J.; Faerman, C.; Swenson, L.; Wynn, M.; Lu, F.; Lippke, J.; Saxena, K. The Structural Basis for Autoinhibition of FLT3 by the Juxtamembrane Domain. Mol. Cell 2004, 13, 169–178. [CrossRef]
7. Parcells, B.W.; Ikeda, A.K.; Simms-Waldrip, T.; Moore, T.B.; Sakamoto, K.M. FMS-Like Tyrosine Kinase 3 in Normal Hematopoiesis and Acute Myeloid Leukemia. *Stem Cells* 2006, 24, 1174–1184. [CrossRef]

8. Pratz, K.W.; Sato, T.; Murphy, K.M.; Stine, A.; Rajkhowa, T.; Levis, M. FLT3-mutant allelic burden and clinical status are predictive of response to FLT3 inhibitors in AML. *Blood* 2010, 115, 1425–1432. [CrossRef]

9. Stirewalt, D.L.; Kopecky, K.; Meshinchi, S.; Engel, J.H.; Pogosova-Agidjanyan, E.L.; Linsley, J.; Slovak, M.L.; Willman, C.L.; Radich, J.P. Size of FLT3 internal tandem duplication has prognostic significance in patients with acute myeloid leukemia. *Blood* 2006, 107, 3724–3726. [CrossRef]

10. Kim, Y.; Lee, G.D.; Park, J.; Yoon, J.H.; Kim, H.J.; Min, W.S.; Kim, M. Quantitative fragment analysis of FLT3-ITD efficiently identifying poor prognostic group with high mutant allele burden or long ITD length. *Blood Cancer J.* 2015, 5, e336. [CrossRef]

11. Meshinchi, S.; Stirewalt, D.L.; Alonzo, T.A.; Boggon, T.J.; Gerbing, R.B.; Rocnik, J.L.; Lange, B.J.; Gilliland, D.G.; Radich, J.P. Structural and numerical variation of FLT3/ITD in pediatric AML. *Blood* 2008, 111, 4930–4933. [CrossRef] [PubMed]

12. Liu, S.B.; Dong, H.J.; Wang, J.; Qiu, Q.C.; Xue, S.L.; Li, L. Effect of FLT3-ITD Length on 32D Cell Proliferation, Apoptosis and Sensitivity to FLT3 Inhibitor. *Zhonggguo Shi Yan Xue Ye Xue Za Zhi* 2021, 29, 1034–1038. [CrossRef] [PubMed]

13. Liu, S.B.; Dong, H.J.; Bao, X.B.; Qiu, Q.C.; Li, H.Z.; Shen, H.J.; Ding, Z.X.; Wang, C.; Chu, X.L.; Yu, J.Q.; et al. Impact of FLT3-ITD length on prognosis of acute myeloid leukemia. *Haematologica* 2019, 104, e9–e12. [CrossRef] [PubMed]

14. Kayser, S.; Schlenk, R.F.; Londono, M.C.; Breitenbuecher, F.; Wittke, K.; Du, J.; Groner, S.; Späth, D.; Krauter, J.; Ganser, A.; et al. Insertion of FLT3 internal tandem duplication in the tyrosine kinase domain-1 is associated with resistance to chemotherapy and inferior outcome. *Blood* 2009, 114, 2386–2392. [CrossRef]

15. Gale, R.E.; Green, C.; Allen, C.; Mead, A.J.; Burnett, A.K.; Hills, R.K.; Linch, D.C. The impact of FLT3 internal tandem duplication mutant level, number, size, and interaction with NPM1 mutations in a large cohort of young adult patients with acute myeloid leukemia. *Blood* 2008, 111, 2776–2784. [CrossRef]

16. Schnittger, S.; Bacher, U.; Haferlach, C.; Alpermann, T.; Kern, W.; Haferlach, T. Diversity of the juxtamembrane and TKD1 mutations (Exons 13-15) in the FLT3 gene with regards to mutant load, sequence, length, localization, and correlation with biological data. *Genes Chromosomes Cancer* 2012, 51, 910–924. [CrossRef]

17. Koszarska, M.; Megggesi, N.; Bors, A.; Batai, A.; Csacsovszki, O.; Lehoczky, E.; Adam, E.; Kozma, A.; Lovas, N.; Sipos, A.; et al. Medium-sized FLT3 internal tandem duplications confer worse prognosis than short and long duplications in a non-elderly acute myeloid leukemia cohort. *Leuk. Lymphoma* 2014, 55, 1510–1517. [CrossRef]

18. Kusec, R.; Jaksic, O.; Ostojic, S.; Kardum-Skelin, I.; Vrhovac, R.; Jaksic, B. More on prognostic significance of FLT3/ITD size in acute myeloid leukemia (AML). *Blood* 2006, 108, 405–406. [CrossRef]

19. Rucker, F.G.; Du, L.; Luck, T.J.; Benner, A.; Krzykalla, J.; Gathmann, I.; Voso, M.T.; Amadori, S.; Prior, T.W.; Brandwein, J.M.; et al. Molecular landscape and prognostic impact of FLT3-ITD insertion site in acute myeloid leukemia: RATIFY study results. *Leukemia* 2021, 36, 90–99. [CrossRef]

20. CRISP. 2021. Available online: https://www.crisphealth.org (accessed on 4 October 2021).

21. Harris, P.A.; Taylor, R.; Minor, B.L.; Elliott, V.; Fernandez, M.; O’Neal, L.; McLeod, L.; Delacqua, G.; Delacqua, F.; Kirby, J.; et al. The REDCap consortium: Building an international community of software platform partners. *J. Biomed. Inform.* 2019, 95, 103208. [CrossRef] [PubMed]

22. Harris, P.A.; Taylor, R.; Thielke, R.; Payne, J.; Gonzalez, N.; Conde, J.G. Research electronic data capture (REDCap)-A metadata-driven methodology and workflow process for providing translational research informatics support. *J. Biomed. Inform.* 2009, 42, 377–381. [CrossRef] [PubMed]

23. Corley, E.M.; Mustafa Ali, M.K.; Alharthy, H.; Kline, K.A.F.; Sewell, D.; Law, J.Y.; Lee, S.T.; Niyongere, S.; Duong, V.H.; Baer, M.R.; et al. Impact of IDH1 c.315C>T SNP on Outcomes in Acute Myeloid Leukemia: A Propensity Score-Adjusted Cohort Study. *Front. Oncol.* 2022, 12. Available online: https://www.crisphealth.org/applications/clinical-data/#health-records (accessed on 16 May 2022). [CrossRef] [PubMed]

24. Greifer, N. WeightIt. 2021. Available online: https://github.com/ngreifer/WeightIt (accessed on 1 November 2021).

25. Harder, V.S.; Stuart, E.A.; Anthony, J.C. Propensity score techniques and the assessment of measured covariate balance to test causal associations in psychological research. *Psychol. Methods* 2010, 15, 234–249. [CrossRef] [PubMed]

26. Austin, P.C. Variance estimation when using inverse probability of treatment weighting (IPTW) with survival analysis. *Stat. Med.* 2016, 35, 5642–5655. [CrossRef] [PubMed]

27. Döhnner, H.; Estey, E.H.; Amadori, S.; Appelbaum, F.R.; Büchner, T.; Burnett, A.K.; Dombret, H.; Fenaux, P.; Grimwade, D.; Larson, R.A.; et al. Diagnosis and management of acute myeloid leukemia in adults: Recommendations from an international expert panel, on behalf of the European LeukemiaNet. *Blood* 2010, 115, 453–474. [CrossRef] [PubMed]

28. Smith, C.C.; Levis, M.J.; Perl, A.E.; Hill, J.E.; Rosales, M.; Bahceci, E. Molecular profile of FLT3 -mutated relapsed/refractory patients with AML in the phase 3 AIM2 study of gilteritinib. *Blood Adv.* 2022, 6, 2144–2155. [CrossRef] [PubMed]

29. Thiede, C.; Koch, S.; Creutzig, E.; Steudel, C.; Illmer, T.; Schaich, M.; Ehninger, G. Prevalence and prognostic impact of NPM1 mutations in 1485 adult patients with acute myeloid leukemia (AML). *Blood* 2006, 107, 4011–4020. [CrossRef]

30. Verhaak, R.G.W.; Goudswaard, C.S.; van Putten, W.; Bijl, M.A.; Sanders, M.A.; Hugens, W.; Uitterlinden, A.G.; Erpelinck, C.A.J.; Delwel, R.; Löwenberg, B.; et al. Mutations in nucleophosmin (NPM1) in acute myeloid leukemia (AML): Association with other gene abnormalities and previously established gene expression signatures and their favorable prognostic significance. *Blood* 2005, 106, 3747–3754. [CrossRef]
31. Döhner, K.; Schlenk, R.F.; Habdank, M.; Scholl, C.; Rücker, F.G.; Corbacigolu, A.; Bullinger, L.; Fröhling, S.; Döhner, H. Mutant nucleophosmin (NPM1) predicts favorable prognosis in younger adults with acute myeloid leukemia and normal cytogenetics: Interaction with other gene mutations. Blood 2005, 106, 3740–3746. [CrossRef]

32. Schnittger, S.; Schoch, C.; Kern, W.; Mecucci, C.; Tschulik, C.; Martelli, M.F.; Haferlach, T.; Hiddemann, W.; Falini, B. Nucleophosmin gene mutations are predictors of favorable prognosis in acute myelogenous leukemia with a normal karyotype. Blood 2005, 106, 3733–3739. [CrossRef]