

Impact of *FLT3*-ITD length on prognosis of acute myeloid leukemia

The FMS-like tyrosine kinase-3 internal tandem duplication (*FLT3*-ITD) is one of the most frequent mutations found in acute myeloid leukemia (AML) patients, with a frequency of 20-30%. The sizes of ITDs vary dramatically.¹ The *FLT3*-ITD consists of in-frame insertions of duplicated sequences, most of them (70%) are located in the juxtamembrane domain (JMD), while the remaining are in tyrosine kinase domain 1 (TKD1).^{2,3} Currently, it is well known that TKD1-ITDs, while significantly longer than JMD-ITDs, are associated with worse outcome.^{3,5} However, previous studies failed to exclude the TKD1-ITD⁺ patients for the correlation analysis of ITD length and prognosis, resulting in potential statistical interference.⁶⁻¹⁰ On the other hand, structural studies have demonstrated that only 15 base pair (bp) ITD insertions in JMD can disrupt the *FLT3* kinase auto-inhibition conformation, thus promoting ligand-independent *FLT3* receptor dimerization, and leading to constitutive auto-phosphorylation and activation of downstream survival and proliferation signaling.^{11,12} To date, there is no clear consensus on whether the length of ITD affects the clinical outcome of patients with JMD-ITD.⁶⁻¹⁰ Therefore, in this study, we specifically selected JMD-ITDs to examine the impact of the variability in ITD length on biological phenotype, and correlated this variability with clinical outcome in a cohort of adult AML patients with single ITD mutations.

We selected 89 adult *de novo* AML patients (excluding acute promyelocytic leukemia) with *FLT3*-ITD mutations retained in our tissue bank between September 2010 and October 2015 (Online Supplementary Figure S4). Every selected patient had single ITD insertion. All 89 ITDs were sequenced and quantified using an ABI 3730 automated DNA sequencer¹³ (Figure 1A). The lengths of the 89 ITDs ranged from 6 to 90 bp, with a median of 39 bp (13 amino acids) (Online Supplementary Table S4). In addition, the genomic localization of the ITD varied: most ITDs (77.5%) were located in the JM-Z region (n=69), 19.1% (n=17) of ITDs were located in the hinge region, only 3 ITDs (3.4%) were located in JM-S region.

We further explored whether ITD length impacted clinical outcome. Using the median ITD length of 39 bp as cut off, all 89 patients were divided into two groups. Interestingly, there was no relationship between ITD

length and the *FLT3*-ITD allelic ratio or age (Figure 1B and C). In our results, the group of larger (≥ 39 bp) ITDs had higher median white blood cell (WBC) counts at diagnosis ($P=0.044$) and a trend of more female patients (30 of 48 vs. 18 of 51; $P=0.079$) (Online Supplementary Table S2). Between these two groups, there were no statistical differences according to marrow blast counts, hemoglobin, platelet, age, treatment of allogeneic hematopoietic stem cell transplantation (allo-HSCT), cytogenetic distribution or mutation status of *NPM1*, *CEBPA*, and *DNMT3A* genes. We found that treatment outcome was poorer for patients with larger ITDs (≥ 39 bp) compared to patients with smaller ITDs (< 39 bp) [overall survival (OS) rate at 3 years: 27.1% vs. 61.2%; $P=0.004$] (Figure 1D). Likewise, shorter progression-free survival (PFS) was associated with increased ITD length ($P=0.006$). The 3-year PFS rates were 47.0% and 21.2% for patients with small and large ITDs, respectively (Figure 1E). Importantly, adjusted by other confounders (Table 1), multivariate Cox regression model analysis indicated that larger ITD size (≥ 39 bp) was an independent prognostic factor for inferior outcomes with Hazard Ratios (HR) of 2.27 ($P=0.014$) for OS, and 2.06 ($P=0.014$) for PFS. Based on the power simulation, the sample size was sufficient to draw statistical conclusions (Online Supplementary Table S3 and Online Supplementary Figure S2). In addition, ITD allelic ratio is considered to be an independent factor for worse outcome and is cited in the 2017 edition of European LeukemiaNet (ELN).¹⁴ Similar results were confirmed in our study (Online Supplementary Table S4 and Online Supplementary Figure S3), while age was excluded out of the multivariate analysis due to the equilibrium of age distribution for these two groups (Table 1 and Online Supplementary Tables S2 and S5).

Next, we examined the biological function of different ITD lengths. From the 89 ITD⁺ patients, we chose the median size 39 bp as the cut-off value to screen the "long ITD" and "short ITD" sequences, and we chose two pairs of ITD sequences from the JM-Z and hinge regions. Each pair of ITDs differed in length, but was located in the same or adjacent insert site (Figure 2A). Each of the four ITDs were then amplified and individually sub-cloned into a lentiviral vector co-expressing green fluorescent protein (GFP). Murine myeloid 32D cells stably expressing wild-type *FLT3* or each of the four *FLT3*-ITD constructs were generated by lentiviral transduction, and comparable surface expression of *FLT3* in each clone was confirmed by FACS analysis (*data not shown*). As expect-

Table 1. Multivariate Cox regression models of association between length of internal tandem duplication (ITD) and survival.

	Overall survival		Progression-free survival	
	Hazard ratio (95%CI)	P	Hazard ratio (95%CI)	P
ITD length (≥ 39 vs. < 39 bp)	2.14 (1.12~4.08)	0.022	2.06 (1.18~3.61)	0.012
ITD ratio (≥ 0.32 vs. < 0.32)	1.94 (1.03~3.67)	0.041	1.43 (0.83~2.49)	0.199
Sex (male vs. female)	0.61 (0.33~1.15)	0.127	0.70 (0.40~1.24)	0.221
Cytogenetic risk group				
Favorable risk	Reference group		Reference group	
Intermediate risk	7.14 (0.96~53.03)	0.055	3.78 (1.15~12.42)	0.029
High risk	12.18 (1.19~124.89)	0.035	4.54 (0.95~21.74)	0.058
<i>DNMT3A</i> (mut vs. unmut)	2.29 (1.16~4.50)	0.017	2.21 (1.22~4.02)	0.009
HSCT under CR1 (yes vs. no)	0.30 (0.15~0.61)	<0.001	0.24 (0.13~0.45)	<0.001

HSCT: hematopoietic stem cell transplantation; CR1: first complete remission; CI: Confidence Interval; mut: mutated; unmut: unmutated.

ed, the four ITD infected 32D lines but not the wild-type FLT3 control, proliferated and survived under IL-3 deprivation; this is consistent with previous studies showing ITD mutations conferring growth factor-independent cell growth.^{11,12} Interestingly, cells expressing the long FLT3-ITD sequences (Mut 2 and Mut 4) had higher growth factor-independent proliferative capacity regardless of the insertion localization compared with the short FLT3-ITD clones (Figure 2B).

Next, we analyzed the constitutive tyrosine phosphorylation of FLT3-ITDs, and their downstream genes like STAT5, Akt, using specific phosphorylation antibodies. As we expected, the longer FLT3-ITDs showed stronger

phosphorylation signals, regardless of whether the insertions were located in JM-Z or hinge regions (Figure 2C). We assessed the effect of ITD length on sensitivity to the second-generation FLT3-kinase inhibitor quizartinib (AC220). AC220 is a promising compound which is currently under evaluation in a multicenter phase III clinical trial.¹⁵ 32D cells transformed with the Mut1, Mut2, Mut3, and Mut4 FLT3-ITDs were exposed to various doses of AC220 for three days. Although nanomolar concentration of AC220 could effectively inhibit growth of 32D cells harboring any of the ITD variations, cells harboring the longer ITDs were less sensitive than those with shorter ITDs to AC220 treatment, as shown by

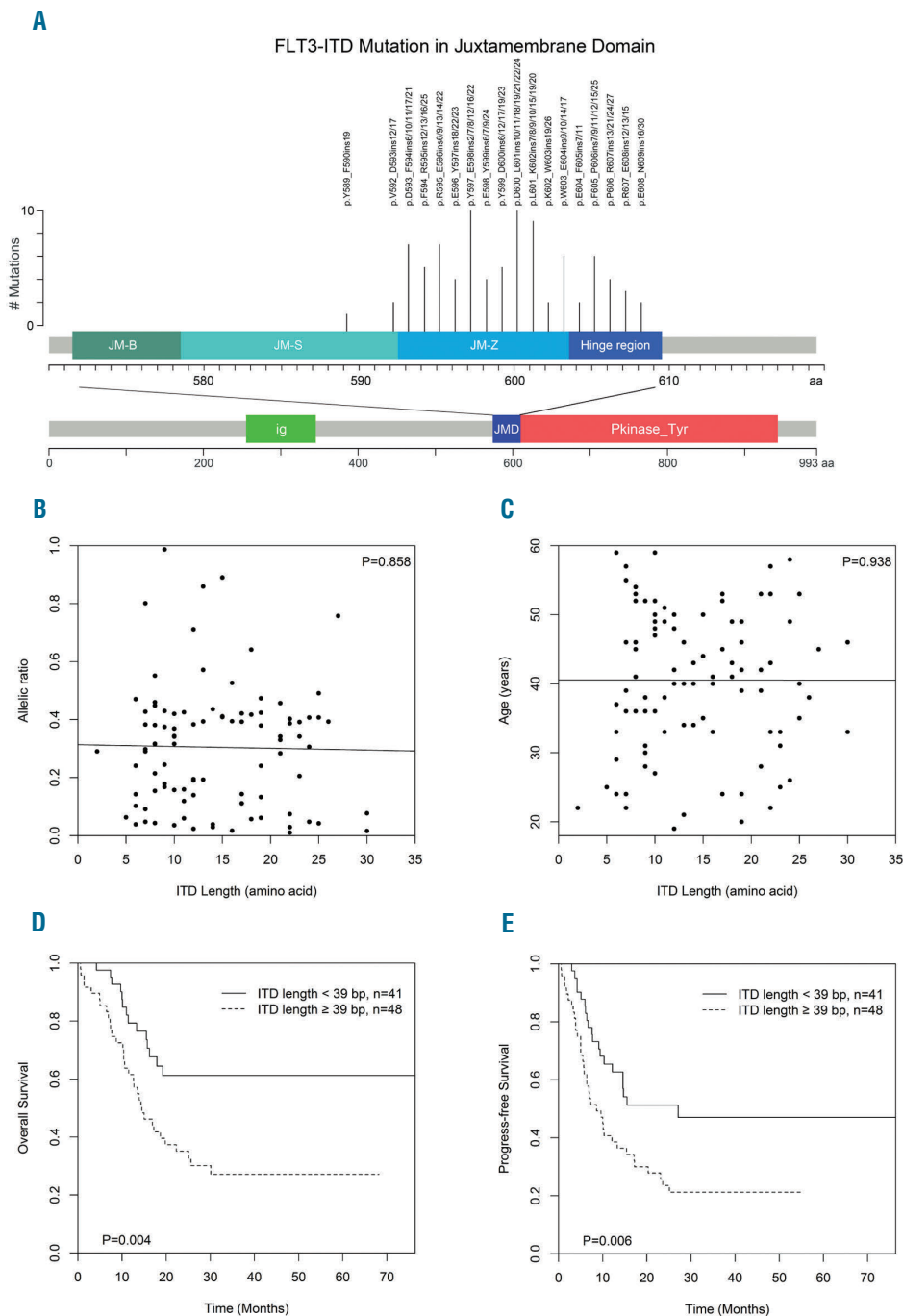


Figure 1. Survival analysis for FLT3-ITD-mutated acute myeloid leukemia (AML) according to length of internal tandem duplication (ITD). (A) FLT3 and the locations of ITD mutations in the juxtamembrane domain (JMD). (B) ITD length and allelic ratio. No correlation was observed between ITD length and allelic ratio by Spearman correlation coefficient ($r=0.018$, $P=0.858$). (C) ITD length and age. No correlation was observed between ITD length and age by Spearman correlation coefficient ($r=0.008$, $P=0.938$). (D) Estimated 3-year overall survival (OS) for ITD length equal to or longer than 39 bp versus shorter than 39 bp. (E) Estimated 3-year progression-free survival (PFS) for ITD length equal to or longer than 39 bp versus shorter than 39 bp. Group differences for both OS and PFS were analyzed by log-rank tests.

both proliferation ($Mut1_{IC50} = 0.446 \text{ nM}$ vs. $Mut2_{IC50} = 0.836 \text{ nM}$, $Mut3_{IC50} = 0.530 \text{ nM}$ vs. $Mut4_{IC50} = 0.847 \text{ nM}$) (Figure 2D) and apoptosis (Figure 2E); the same conclusion was also made for sorafenib or midostaurin treatment (*Online Supplementary Figure S4*). These results indicated that longer ITDs are associated with higher FLT3 kinase activity. This is in line with the proliferative advantage (Figure 2B) and higher FLT3 autophosphorylation (Figure 2C).

Taken together, although there is considerable disagreement regarding the prognostic impact of FLT3-ITD length; our results established a biological link between

the ITD length in JMD and FLT3 kinase activity. Mechanistically, it is possible that the longer insertion in the JMD may cause more significant disruption of its autoinhibitory function.^{11,12} In addition, our clinical data showed that patients with longer ITDs had significantly worse OS and PFS rates, and ITD longer than 39 bp was an independent prognostic factor in multivariate Cox regression models. These findings may help to stratify patients when considering therapeutic combinations based on tyrosine kinase inhibitors.

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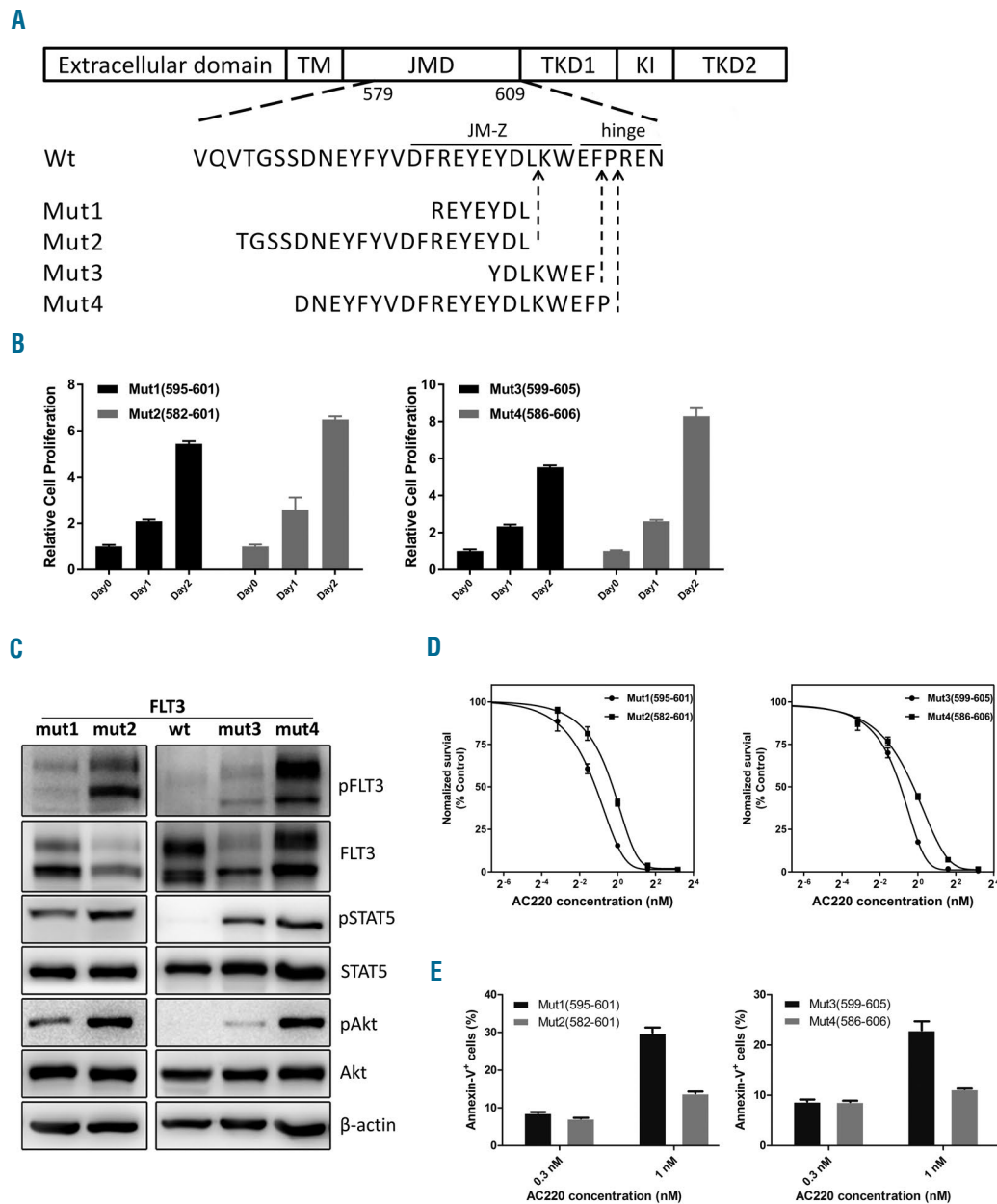


Figure 2. Comparison of biological properties of FLT3-ITDs with long and short internal tandem duplication (ITD) insertion length. (A) Localization and inserted amino acids of the 4 ITD mutations in JMZ (Mut1, Mut2) and hinge region (Mut3, Mut4). (B) Cell proliferation capacity of FLT3-ITD mutations in JMZ (left) and hinge region (right) without IL-3 assayed by Cell Titer-Glo. The relative cell proliferation was normalized to the value of each group at day 0. (C) Phosphorylation of FLT3 (Y589/591), STAT5, and Akt of 4 FLT3-ITD cell strains with ITD insertions in JMZ (left) and hinge region (right), was determined by immunoblotting. (D) Dose response curves of mutant FLT3-ITD clone cell proliferation in the presence of AC220. (E) Induction of apoptosis in FLT3-ITD transduced 32D cell strains with AC220. Survival rate was determined by flow cytometry of DAPI and Annexin-V stained cells.

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