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

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Materials and Methods

Patients and treatment

Between September 2010 and October 2015, 1086 consecutive patients were diagnosed with de novo AML based on WHO criteria at the Department of Hematology in the First Affiliated Hospital of Soochow University. Diagnostic bone marrow samples were collected and analyzed for genetic mutation in these patients. In the present study, out of 145 de novo adult AML patients with FLT3-ITD mutation (18-60 years, excluding acute promyelocytic leukemia), a total of 95 cases with only one ITD insertion in JMD region were chosen firstly. Standard induction chemotherapy comprising anthracycline and cytoarabine, 3+7 regimen and consolidation therapies were given to all these 95 patients. During induction chemotherapy, 6 of 95 (6.3%) cases were excluded from last analysis due to early death from indeterminate cause. If patients failed to achieve complete remission (CR) after two courses of standard induction therapy or relapsed, decitabine (20mg/m2×3 days[1] before reinduction regimen) combined with CAG regimen[2] were given for remission reinduction. And 8 of 13 cases took sorafenib[3] 400 mg orally twice daily continuously every cycle starting on day 1 of reinduction with CAG regimen. For all patients achieved first complete remission (CR1), allogeneic hematopoietic stem cell transplantation (allo-HSCT) was recommended as first-line consolidation treatment. Patients with matched related donor (MRD), matched unrelated donor (MUD) or haploidentical related donor (haplo) were allocated to Allo-HSCT. (Figure S1).

Detection of FLT3-ITD

All samples investigated in this study were obtained at the time of diagnosis. Genomic DNA was extracted from bone marrow samples according to the manufacturer's protocols (QIAGEN, Hilden, Germany). FLT3-ITD detection was performed using the method described by Meshinchi *et al*[4]. PCR products were sequenced using an ABI 3730 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA).

In order to detect the ITD sequences in mRNA, reverse transcriptase (RT)-PCR was

performed for all samples with intron contained ITDs.

Genescan analysis

Genescan analysis was carried out using an ABI 3730 automated sequencer. PCR setup and PCR conditions were the same as above except that PCR primer FLT3-ITDF was labeled with HEX, and amplified for 25 cycles. The areas under the curves were quantified for FLT3-ITD and wild-type allele, respectively, by using of the Genemapper version 4.0 software (Applied Biosystems, Foster City, USA). For allele ratio calculation, we used the value of mutant/(wt+ mutant) ratio. The allele ratio calculation method is the same as Gale's group[5].

Statistical analysis

Statistical analysis was performed by R software, version 3.33 (R Foundation for Statistical Computing, Vienna, Australia). To examine effect of ITD length on survival, other confounders contained as follows: patients' sex and age, allelic ratio was defined as ITD mutant to the sum of ITD and wild-type allelic ratio, and dichotomized at the median level (≥0.32 vs. < 0.32), classification of cytogenetic risk, logarithm of white blood cell (WBC) counts, received allo-HSCT in CR1, the molecular markers NPM1 mutation, CEBPA, and DNMT3A mutation. Chi-square or Fisher exact test was used to compare the categorical variables, while Mann-Whitney test was used for continuous variables. Correlations between continuous parameters were analyzed by Spearman rank correlation. Overall survival (OS) was defined as the time from study entry to death or the last observation. Progression free survival (PFS) was defined as time from study entry until relapse or death due to any causes. Kaplan Meier method with the log-rank test was used in analyzing the differences of OS and PFS. In Cox model, adjusted variables were chosen, when added to model, changed the matched hazard ratio (HR) by at least 10 percent[6]. Two-sided P values less than 0.05 were considered to be statistical significance. Finally, power simulation model was performed to evaluate the power for different sample size by pass software (PASS 11. NCSS, LLC. Kaysville, Utah, USA)[7,8]. The deadline for follow-up was June 30, 2017,

with a median follow-up time of 16.2 (0.6-76.4) months.

Cells, drugs and DNA constructs

Murine 32D cells (ATCC CRL-11346) were cultured in RPMI 1640 medium with 10% FBS and 1% penicillin-streptomycin in the presence murine recombinant IL-3 (Peprotech Inc., Rocky Hill, NJ, USA). pCDH-EF1A-T2A-GFP Lentiviral vectors were purchased from System Biosciences Inc (Palo Alto, CA, USA). Full length FLT3 variants were first cloned into pCDNA 3.1 (Thermo Fisher Scientific, Waltham, MA, USA) before being subcloned into pCDH-EF1A-T2A-GFP. Full-length FLT3 mutants including Mut1, Mut2, Mut3, and Mut4 were synthesized by Genscript. Inc (Piscataway, NJ, USA). Lentivirus was prepared as previously described[9].

Lentivirus transduction

In the case of lentiviral vectors expressing FLT3 variants, 32D cells were exposed to virus-containing supernatants (MOI = 5) via spinoculation and then sorted by flow cytometry based on GFP expression.

Analysis of viability, apoptosis

Cell growth was measured utilizing the Cell Titer-Glo Luminescent Cell Viability Assay Kit (Promega Corp. Madison, WI, USA). Briefly, cells were cultured under different treatment conditions and transferred to 89-well plates for 30 min before assay. Kit substrate and buffer reagents were added to each well and mixed to allow cell lysis. Addition of an additional solution generated a luminescent signal proportional to ATP levels present, which corresponds to the number of metabolically active cells in culture. Plates were read on a Microplate reader (Beckman Coulter DTX880). Control wells contained medium without cells to assess background luminescence. Results were expressed as a percentage of untreated or control cells for three replicates. Apoptosis was assessed by Annexin V/DAPI staining followed by flow cytometry.

Immunoprecipitation (IP) and Western Blotting

Cells were lysed in modified RIPA buffer with 1% Triton X-100 supplemented with protease and phosphatase inhibitors. Antibodies used for IP were conjugated to protein A/G beads (Millipore, Burlington, MA, USA) using an antibody cross-linking kit (Thermo Fisher). Boiled lysates were then resolved on 4-20% gradient gels (NuPAGE, Thermo Fisher) or 10% SDS-PAGE gels and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). Proteins of interest were sequentially probed with primary and secondary antibodies. Primary antibodies included rabbit anti-FLT3 S18 (Santa Cruz Biotechnology, Dallas, TX, USA), anti FLT3 Y589/Y591 (Cell Signaling Technology, Danvers, MA, USA), anti-STAT5 (Santa cruz, cat# sc-836), anti-Akt (Cell signaling, cat# 4691), anti-PSTAT5 Y694 (BD, cat#611965) , anti-PAKT s473 (Cell signaling, cat#4060), and anti-β-actin monoclonal antibody (AC-15) (Sigma-Aldrich, St. Louis, MO, USA). Horseradish peroxidase–conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories (Westgrove, PA, USA). Antibody detection was performed with SuperSignal™ West Pico or Femato kits (Pierce Biotechnology, Waltham, MA, USA).

Figure S1. Diagram of the patient selection process and treatment.

Abbreviation: AML, acute myeloid leukemia; FLT3-ITD: FLT3 internal tandem duplication mutation; APL, acute promyelocytic leukemia; TKD1: tyrosine kinase domain 1; JMD: juxtamembrane domain; +: 7+3 induction regimen were contained with intravenous idarubicin at 12 mg/m2 or daunorubicin 60mg/m2 daily on days 1 to 3 and IV cytarabine by continuous infusion at 100 mg/m2 on days 1 to 7. *: allogeneic hematopoietic stem cell transplantation (allo-HSCT) was recommended as first-line consolidation treatment for all patients after first CR; CR: complete remission; MRD: matched related donors; MUD: matched unrelated donors; Haplo: haploidentical donors; #: decitabine with or without sorafenib, combined with CAG regimen were given for remission reinduction; NR: non remission; HiDAC: high dose cytarabine for cases who still chose chemotherapy instead of allo-HSCT ($3g/m^2$ IV twice daily on days 1, 3, and 5×4 cycles).

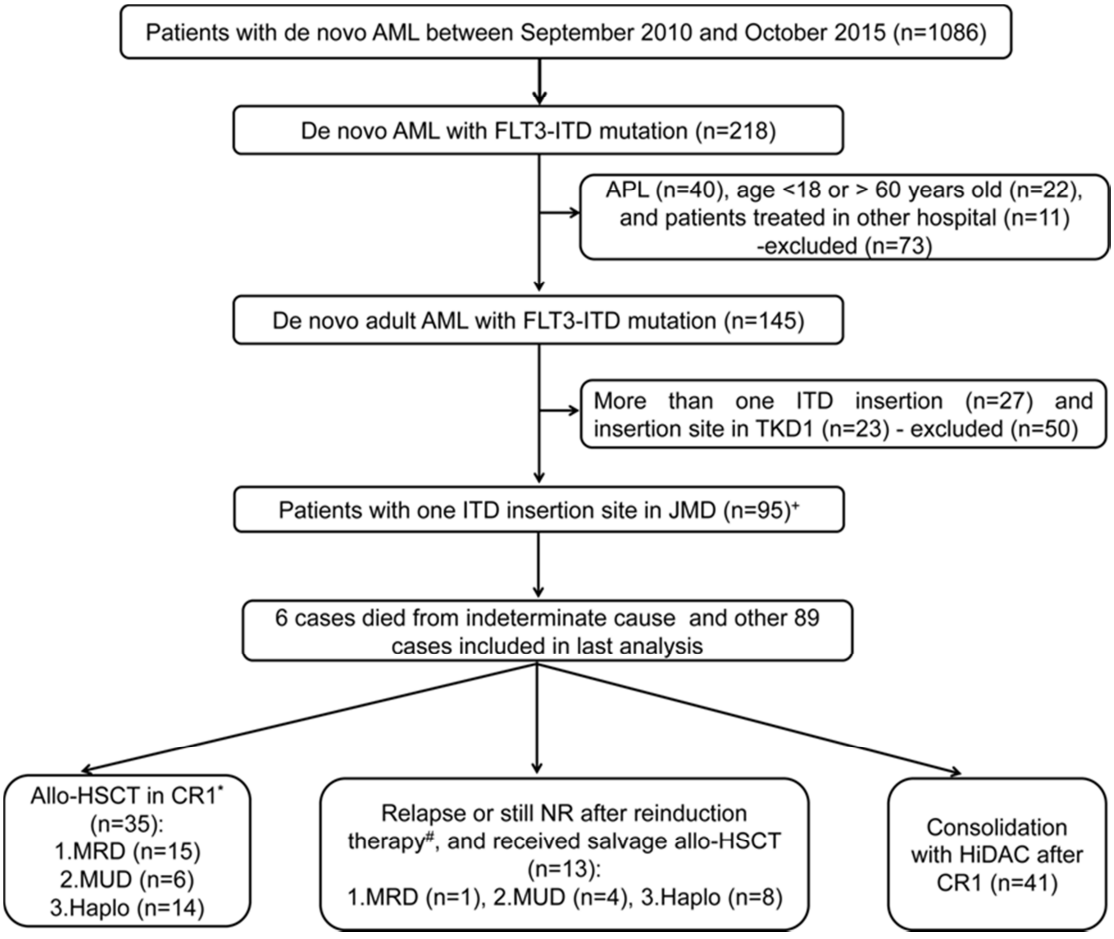


Table S1. Characteristics of 89 AML patients with FLT3-ITD mutations located in JMD

	Total (n=89)
Median age, y (range)	40 (19~59)

Sex	
Male	41 (46.1%)
Female	48 (53.9%)
Localization of ITD length (bp)	39 (6~90)
Median ITD allelic ratio (range)	0.32 (0.01~0.99)
Localization of ITD integration	
JM-S	3 (3.4%)
JM-Z	69 (77.5%)
Hinge region	17 (19.1%)
Marrow blast (range, %)	73 (22~97)
Median WBC count, $\times 10^9$/L (range)	34 (2~441)
Median HB level, g/L (range)	82 (36~163)
Median PLT count, $\times 10^9$/L (range)	43 (4~432)
Cytogenetic risk group	
Favorable risk	13 (14.6%)
Intermediate risk	70 (78.7%)
High risk	6 (6.7%)
NPM1	
Unmutated	53 (59.6%)
Mutated	36 (40.4%)
CEBPA	
Wild-type or Single mutated	83 (93.3%)
Double mutated	6 (6.7%)
DNMT3A	
Unmutated	68 (76.4%)
Mutated	21 (23.6%)
Status pre-HSCT	
CR	35 (39.3%)
NR	13 (14.6%)
Chemotherapy only	41 (46.1%)

Table S2. Characteristics of the two groups of AML patients with FLT3-ITD mutations located in JMD based on ITD length

	Length < 39 bp (N=41)	Length ≥ 39 (N=48)	P value
Median age, y (range)	40 (19~59)	41 (20~58)	0.764
Sex			0.079
Female	18 (43.9%)	30 (62.5%)	
Male	23 (56.1%)	18 (37.5%)	
Median ITD allelic ratio (range)	0.24 (0.02~0.99)	0.38 (0.01~0.89)	0.344
ITD allelic ratio			0.174
< 0.32	23 (56.1%)	20 (41.7%)	
≥ 0.32	18 (43.9%)	28 (58.3%)	

Localization of ITD integration			0.913
JM-S	1 (2.4%)	2 (4.2%)	
JM-Z	33 (80.5%)	36 (75.0%)	
Hinge region	7 (17.1%)	10 (20.8%)	
Marrow blast (range, %)	67 (22~97)	76 (28~96)	0.150
Median WBC count, $\times 10^9$/L (range)	28.0 (2.0~243.0)	53.5 (2.0~441.0)	0.044
Median HB level, g/L (range)	83 (36~163)	80 (54~136)	0.827
Median PLT count, $\times 10^9$/L (range)	43 (4~432)	43 (13~273)	0.621
Cytogenetic risk group			0.496
Favorable risk	7 (17.0%)	6 (12.5%)	
Intermediate risk	30 (73.2%)	40 (83.3%)	
High risk	4 (9.8%)	2 (4.2%)	
NPM1			0.263
Unmutated	27 (65.9%)	26 (54.2%)	
Mutated	14 (34.1%)	22 (45.8%)	
CEBPA			0.527
Wild-type	35 (85.3%)	42 (87.5%)	
Single mutated	4 (9.8%)	2 (4.2%)	
Double mutated	2 (4.9%)	4 (8.3%)	
DNMT3A			0.870
Unmutated	31 (75.6%)	37 (77.1%)	
Mutated	10 (24.4%)	11 (22.9%)	
Allo-HSCT under CR1			0.957
No	25 (61.0%)	29 (60.4%)	
Yes	16 (39.0%)	19 (39.6%)	

Abbreviation: WBC, white blood cell; HB, hemoglobin; PLT, platelet.

Table S3. Effects of sample size based on a power simulation model[7,8]

Power	Length < 39 bp (N)	Length ≥ 39 bp (N)	Total sample (N)	Hazard ratio (HR)	Alpha	Beta
0.6389	21	24	45	2.06	0.05	0.3611
0.9003	41	48	89	2.06	0.05	0.0997
0.9771	62	71	133	2.06	0.05	0.0229
0.9953	83	94	177	2.06	0.05	0.0047
0.9991	103	118	221	2.06	0.05	0.0009
0.9998	124	141	265	2.06	0.05	0.0002
0.6892	21	24	45	2.16	0.05	0.3108
0.9300	41	48	89	2.16	0.05	0.0700
0.9872	62	71	133	2.16	0.05	0.0128
0.9980	83	94	177	2.16	0.05	0.0020
0.9997	103	118	221	2.16	0.05	0.0003
1.0000	124	141	265	2.16	0.05	0.0000

0.7333	21	24	45	2.26	0.05	0.2667
0.9513	41	48	89	2.26	0.05	0.0487
0.9929	62	71	133	2.26	0.05	0.0071
0.9991	83	94	177	2.26	0.05	0.0009
0.9999	103	118	221	2.26	0.05	0.0001
1.0000	124	141	265	2.26	0.05	0.0000

Figure S2. Effects of different sample sizes, as evaluated using a power simulation model

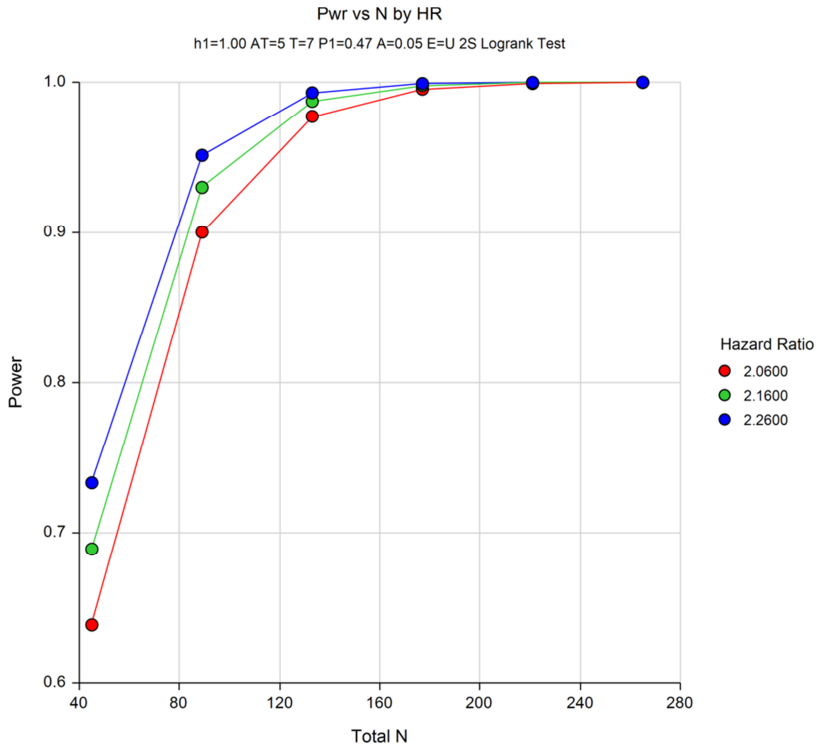


Figure S3. Survival analysis for *FLT3*-ITD mutated AML according to allelic ratio. A. Estimated 3 year OS rate for patients with mutant/(mutant+wt) ratio greater than 0.32 versus less than 0.32, the group differences of them were examined by a log-rank test. B. Estimated 3 year PFS rate for patients with mutant/(mutant+wt) ratio greater than 0.32 versus less than 0.32, the group differences of them were examined by a log-rank test.

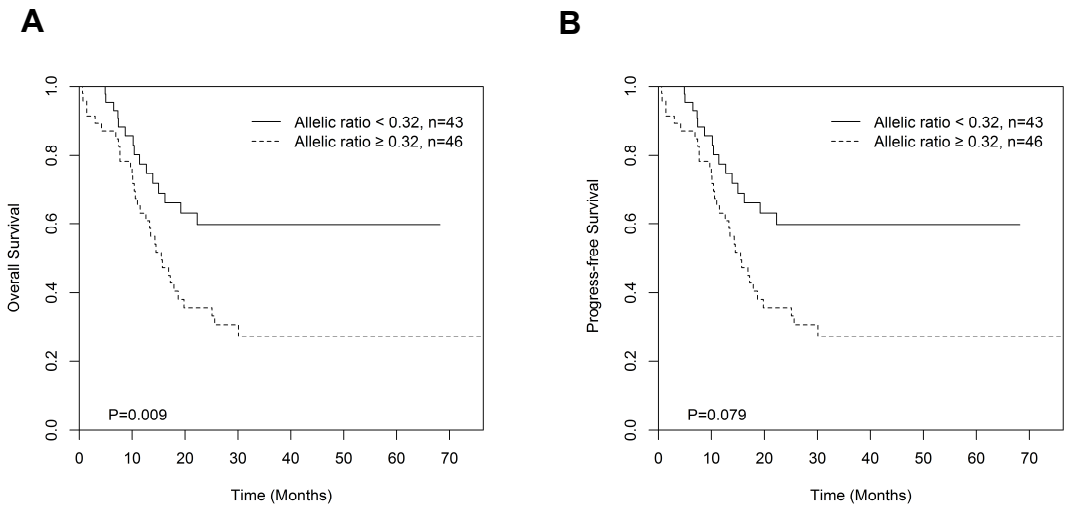


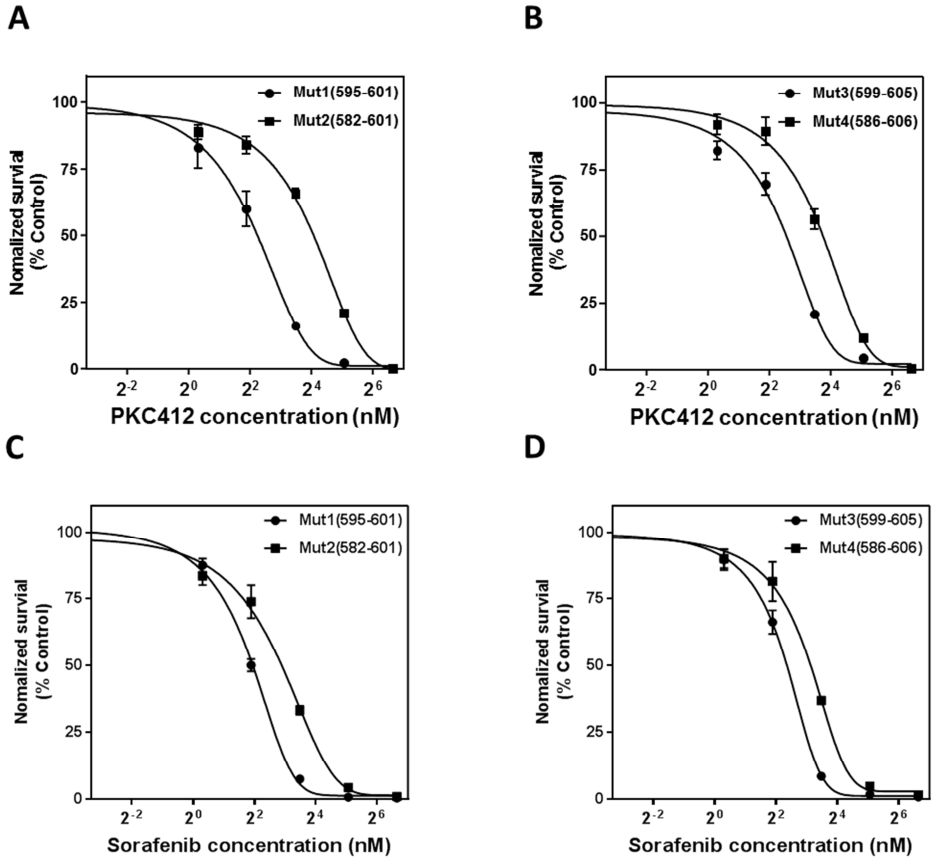
Table S4. Univariate Cox regression models about association between clinical characteristics and survival

	Overall survival		Progression-free survival	
	Hazard ratio (95%CI), P-value		Hazard ratio (95%CI), P-value	
ITD length (continuous variable)	1.06 (1.01~1.10)	0.010	1.04 (1.00~1.08)	0.054
ITD length (≥ 39 bp vs. < 39 bp)	2.45 (1.31~4.59)	0.005	2.10 (1.22~3.61)	0.008
Allelic ratio (continuous variable)	5.25 (1.44~19.16)	0.012	3.91 (1.04~14.69)	0.043
Allelic ratio (≥0.32 vs. < 0.32)	2.22 (1.20~4.11)	0.011	1.60 (0.94~2.71)	0.083
Sex (Male vs. Female)	0.45 (0.24~0.82)	0.009	0.55 (0.32~0.95)	0.030
Age (continuous variable)	1.01 (0.98~1.04)	0.566	1.00 (0.98~1.03)	0.751
Cytogenetic risk group				
Favorable risk	Reference group		Reference group	
Intermediate risk	9.53 (1.31~69.27)	0.026	4.30 (1.34~13.83)	0.014
High risk	9.24 (0.96~89.12)	0.054	3.95 (0.88~17.71)	0.072
NPM1 (mut vs. unmut)	1.36 (0.77~2.42)	0.290	1.38 (0.82~2.33)	0.224
CEBPA (double-mut vs. unmut)	0.43 (0.10~1.76)	0.240	0.53 (0.17~1.70)	0.285
DNMT3A (mut vs. unmut)	1.71 (0.92~3.18)	0.089	1.75 (1.00~3.07)	0.051
LogWBC (continuous variable)	1.06 (0.92~1.22)	0.439	0.99 (0.87~1.13)	0.936
HSCT under CR1 (yes vs. no)	0.37 (0.20~0.71)	0.003	0.31 (0.17~0.57)	<0.001

Table S5. Multivariate Cox regression models with age about association between length of ITD and survival

	Overall survival		Progression-free survival	
	Hazard ratio (95%CI), P value		Hazard ratio (95%CI), P value	
ITD length (≥ 39 vs. < 39 bp)	2.16 (1.12~4.16)	0.021	2.02 (1.15~3.56)	0.015
ITD ratio (≥0.32 vs. < 0.32)	1.93 (1.02~3.66)	0.043	1.47 (0.85~2.56)	0.173
Sex (male vs. female)	0.61 (0.33~1.15)	0.126	0.67 (0.37~1.19)	0.172
Cytogenetic risk group				
Favorable risk	Reference group		Reference group	
Intermediate risk	7.08 (0.95~52.69)	0.056	3.89 (1.18~12.81)	0.025
High risk	12.23 (1.19~125.67)	0.035	4.40 (0.92~21.08)	0.064
DNMT3A (mut vs. unmut)	2.29 (1.16~4.51)	0.017	2.25 (1.23~4.10)	0.008
HSCT under CR1 (yes vs. no)	0.31 (0.15~0.61)	<0.001	0.24 (0.13~0.44)	<0.001
Age (continuous variable)	1.00 (0.98~1.03)	0.815	1.00 (0.96~1.02)	0.410

Figure S4. Comparison of drug sensitivity of FLT3-ITDs with long and short ITD insertion length. A/B. Dose response curves of mutant FLT3-ITD clone cell proliferation in the presence of PKC412 (Midostaurin); C/D. Dose response curves of mutant FLT3-ITD clone cell proliferation in the presence of Sorafenib;


A
Mut1(595-601)
Mut2(582-601)
Nomalized survial
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PKC412 concentration (nM)
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Mut3(599-605)
Mut4(586-606)
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Sorafenib concentration (nM)
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Nomalized survial
(% Control)
100
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25
0
2^{-2} 2^{0} 2^{2} 2^{4} 2^{6}
Sorafenib concentration (nM)

Reference

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