

***FLT3*-ITD DNA allelic burden, but not mRNA levels, influences the biological characteristics of AML patients**

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Abstract

FMS-like tyrosine kinase 3 gene internal tandem (FLT3-ITD) mutations represent one of the most frequent genetic lesions in acute myeloid leukemia (AML) and imparts a negative prognostic. For an optimal patient management, current clinical guidelines recommend the evaluation of the allelic ratio (AR), expressed as the DNA FLT3-ITD/WT mutational burden. We sought to evaluate the differences between the AR and FLT3-ITD/WT mRNA ratio (RR) and their respective impact on the biological characteristics of AML patients. A total of 32 DNA and mRNA samples from AML patients with FLT3-ITD were evaluated. There was a good correlation between the AR and RR (Spearman's $\rho = 0.652$, $P < 0.001$). None of the biological characteristics were influenced by the RR values, whereas patients with high AR values (≥ 0.5) had higher WBC counts (Mann-Whitney, $P = 0.01$), LDH levels (Mann-Whitney, $P = 0.037$), and circulating blasts levels (Mann-Whitney, $P = 0.023$) than patients with low AR values (< 0.5). Also, there was a good correlation between AR values and WBC count (Spearman's correlation, $P = 0.001$), and LDH levels (Spearman's correlation, $P = 0.007$). In our study population the AR, but not the RR, influenced the biological characteristic of patients suggesting a dose-independent effect of FLT3-ITD mutations.

Keywords: acute myeloid leukemia, mRNA, FLT3-ITD, Allelic-ratio

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Introduction

Acute myeloid leukemia is a heterogeneous disease characterized by a clonal expansion and accumulation of malignant myeloid precursor populations. One of the most frequent mutations is an internal tandem duplication insertion within the FMS-like tyrosine kinase 3 gene (*FLT3*-ITD), reported in around 20-25% of adult patients (1).

FLT3-ITD mutations are considered to confer an adverse prognostic (1,2) and the 2017 European LeukemiaNet (ELN) guidelines (3) recommend that every patient should be tested for the presence of a *FLT3*-ITD mutation with the determination of the mutant-to-wild-type allelic ratio (AR) to establish the genetic risk category. The AR is used as a proxy to determine the burden of *FLT3*-ITD positive mononuclear cells in a sample and, according to the ELN genetic risk stratification, patients with an $AR < 0.5$ have a better prognosis than patients with an $AR \geq 0.5$.

Although the current ELN risk stratification model is based on a number of retrospective studies (4–7), there are also several papers in which the AR does not allow for a clear-cut prognostic stratification (8–11). Furthermore, a couple of studies investigated the significance of *FLT3*-ITD mRNA mutant-to-wild-type ratio as an alternative biomarker for outcome in AML patients. Of these, the study by Schneider et al (12) showed that *FLT3*-ITD mRNA levels have a high prognostic impact on overall survival and relapse-free survival only in AML patients with coexisting Nucleophosmin 1 gene (*NPM1*) mutations, and no value in *NPM1*-WT AML cases. A more recent study based on a pediatric *FLT3*-ITD AML population (13) showed that a RNA-based mutant-to-wild-type ratio was better at predicting the outcome of these patients than the AR.

In this study we sought to evaluate if our current in-house *FLT3*-ITD assay could be adapted for *FLT3*-ITD mRNA qualitative and quantitative

analysis of primary adult AML samples. Also, we were able to characterize and compare different *FLT3*-ITD parameters (length, mutant-to-wild-type ratio, ITD populations) for both DNA and mRNA with different demographic and biologic characteristics within our patient population.

Materials and methods

Patients

Out of a total of 42 AML patients diagnosed with *FLT3*-ITD between March 2016 and June 2019, 32 had good quality RNA samples available for analysis. Demographic and biologic data from time of diagnosis, French-American-British (FAB) classification, cytogenetic findings, and molecular diagnostic results for recurrent fusion-genes and *NPM1* mutations were recorded. A control group was formed of 83 *FLT3*-ITD negative patients, matched for sex, age, FAB subtype, cytogenetics, and *NPM1* mutation status. All patients gave written informed consent for the sampling, testing, and storage of biological samples, and for the use and analysis of relevant medical information. The study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. The current observational study protocol was approved by the local ethics committee.

FLT3-ITD mutation assay

DNA and total RNA were extracted from bone marrow aspirate where available, or from peripheral blood samples. *FLT3*-ITD genotyping was performed as previously described, using the same primers (sequences can be found in the Supporting information text) for both gDNA and cDNA samples (14,15). *FLT3*-ITD mutations were considered present if amplicons longer than the *FLT3*-WT amplicon were detected. Later, RNA from *FLT3*-ITD positive patient sample were tested using the same method including .

Both gDNA and cDNA PCR products were assessed by fragment analysis using a CEQ8000 DNA Genetic Analysis System (Beckman Coulter UK, High Wycombe, United Kingdom). The *FLT3* mutant-to-wild-type ratio analysis was performed by determining the area under the peak (AUP) for *FLT3* amplicons and calculating the following ratio: $AUP^{FLT3-ITD}/AUP^{FLT3-WT}$ for both gDNA (allelic ratio – AR) and mRNA (mRNA ratio – RR) samples. In patients with multiple *FLT3*-ITD populations (≥ 2 *FLT3*-ITD amplicons) the mutant-to-wild-type ratio was calculated by pooling the AUP for each *FLT3*-ITD population and then dividing by the $AUP^{FLT3-WT}$. The result of DNA and mRNA analysis of *FLT3*-ITD mutations for an exemplary patient are presented in Figure S1. The assay details are presented in the Supporting Information.

Statistical analysis

Student's t-test was used to compare normally distributed data sets. For non-normal distributed data sets the Mann-Whitney U test was used. Kendall's tau-b correlation was used to evaluate the relationship between different continuous, non-parametric variables. Categorical variables were analyzed using Pearson's chi-square or Fischer's exact test where appropriate. P values of <0.05 were considered to be statistically significant. Statistical analysis was performed using SPSS software (version 23) and Graph-Pad Prism 8.

Results

Study patients and *FLT3*-ITD mutation characteristics

Clinical and biological characteristics of the patients are presented in Table 1. The main *FLT3*-ITD mutation parameters (Table S1) analyzed were: (i) the number of *FLT3*-ITD populations, (ii) mutation length, and (iii) the *FLT3*-ITD/WT ratio, for both DNA and mRNA samples.

In the DNA samples 5/32 patients (15.65%) presented more than 1 ITD population, 4 with 2 population, and 1 patient with 3 populations. In the mRNA samples 6/32 patients (18.75%) presented more than 1 ITD population, all 6 patients had 2 ITD populations. One patient, UPN-8334, presented an ITD mutation only on the DNA sample, whereas on the mRNA sample, only the *FLT3*-WT amplicon was detected. Patient UPN-8538 presented 1 ITD population on the DNA sample, whereas on the mRNA sample, the patient presented 2 ITD populations. Details are presented in the Supporting information – results.

There was a very strong correlation between DNA and mRNA *FLT3*-ITD mutation length for the first mutation populations (Kendall $\tau_b = 0.937$, $P < 0.001$) (Figure 1A), and the second mutation populations (Kendall $\tau_b = 1.0$, $P < 0.001$).

The RR values were generally higher than AR values – the median RR value was 0.72, while the median AR value was 0.56. Only 2 patients presented higher AR values than RR (details are presented in the Supporting information – results section, Table S1). There was a strong correlation between *FLT3*-ITD/WT AR and RR (Kendall's $\tau_b = 0.488$, $P < 0.001$) (Figure 1B). Two patients presented an $AR \geq 1.0$ indicative of *FLT3*-ITD homozygous mutant cells.

Further details regarding specific *FLT3*-ITD patient samples are presented in the Supporting Information – results.

Clinical and biological characteristics of patients according to *FLT3*-ITD parameters

In order to analyze the relation between DNA and mRNA ITD length and clinical / biological parameters, only patients with one detectable *FLT3*-ITD population were considered. The patients were separated into 2 groups according to the median ITD length (Table S2) – an ITD <45 bp group, and an ITD ≥ 45 bp group for both DNA and mRNA samples. The only statistical-

Table 1. Clinical and biological characteristics of *FLT3*-ITD patients compared with control group

Feature	<i>FLT3</i> -ITD positive (n=32)	<i>FLT3</i> -ITD negative (n= 85)	
Age, y	Median 63.5 Range 24 – 80	Median 65 Range 24 – 81	NS
Sex M/F, n	13/19	42/41	NS
Rural/Urban Area, n	14/18	39/44	NS
WBC, x10 ⁹ /L	Median 63 000 Range 1860 – 287 000	Median 19 880 Range 300 – 309 000	0.007[†]
Hgb, g/dL	Median 8.1 Range 5.6 – 13.5	Median 8.3 Range 3.1 – 14.1	NS
PLT, x10 ⁹ /L	Median 36 000 Range 6 000 – 148 000	Median 53 000 Range 2 000 – 1 500 000	NS
LDH, IU/dL	Median 599 Range 288 – 1 617	Median 507 Range 156 – 2 200	0.049[†]
Peripheral blood blasts, %*	Median 57 Range 0 – 97	Median 35 Range 0 – 95	0.008[†]
BM blasts, %	Median 67 Range 32 – 96	Median 57 Range 20 – 94	NS
FAB subtypes included, n			NS
M1	18	52	
M2	2	6	
M4	6	11	
M5	6	14	
Cytogenetics, n			NS
Favorable	1	5	
Normal	17	37	
Intermediate	3	7	
Poor, of which w. complex karyotype	2, 0	6, 4	
Insufficient material	2	7	
Unknown	7	21	
<i>NPM1</i> mutations, n	17 (53.1%)	27 (32.5%)	0.54 [‡]

[†]Mann-Whitney U-test, [‡]Pearson's Chi-Square, range= min-max

ly significant difference was found between the PLT counts and the ITD DNA length, with higher PLT counts in the DNA ITD ≥ 45 bp group versus the ITD < 45 bp group ($P = 0.013$, Mann-Whitney U-test). However, when performing a bivariate correlation there was no significant correlation detected between the length of the DNA ITD insert and the PLT count in patients with only one detectable *FLT3*-ITD population (Kendall $\tau_b = 0.108$, $P = 0.418$). There were no significant dif-

ferences between the mRNA ITD length groups. For the analysis of DNA *FLT3*-ITD/WT AR, patients were separated into 2 groups according to the 2017 ELN genetic risk stratification AR cut-off value ($=0.5$) (3) into: an AR <0.5 group, and an AR ≥ 0.5 group. To analyze the mRNA *FLT3*-ITD/WT RR, patients were separated into 2 groups according to the median RR value ($=0.72$): a RR <0.7 group and a RR ≥ 0.7 group. According to the AR value, patients with

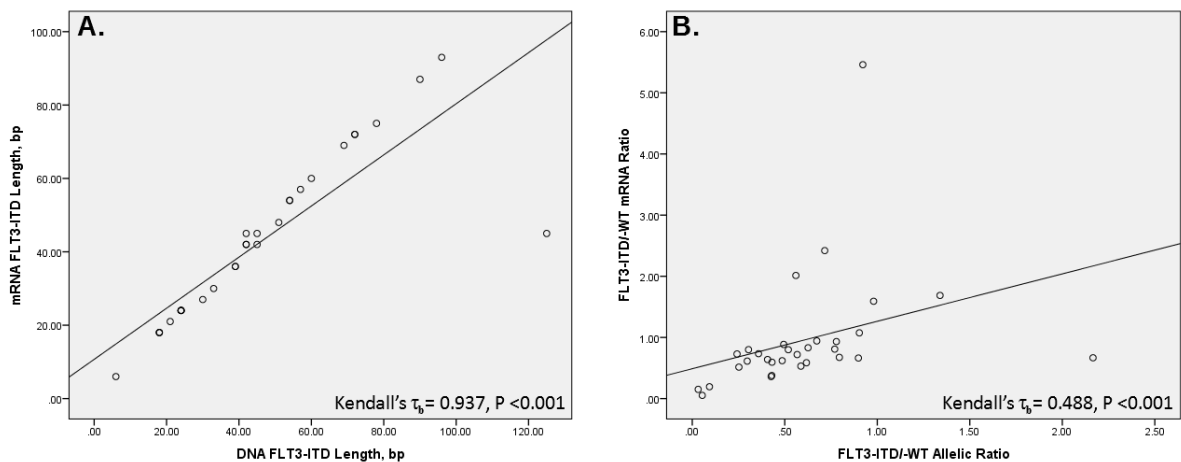


Figure 1. Correlation between *FLT3*-ITD DNA and RNA mutation characteristics. Dot-plot illustrating the correlation between: (A) mRNA *FLT3*-ITD and DNA *FLT3*-ITD mutation length (first population), and (B) *FLT3*-ITD/WT mRNA ratio and *FLT3*-ITD/WT allelic ratio.

AR ≥ 0.5 presented higher WBC counts (Mann-Whitney U-test, $P = 0.01$), higher LDH levels (Mann-Whitney U-test, $P = 0.037$), and higher circulating blast percentages (t-test, $P = 0.023$) than the AR < 0.5 group (Figure 2A). We further compared WBC count, LDH levels, and peripheral blood blasts percentages between the control *FLT3*-ITD negative group and the two AR groups. We observed that there were no significant differences between the AR < 0.5 group and the *FLT3*-ITD negative group (Figure 2A). There were no significant differences between the two RR groups. The detailed results are presented in Table S3.

We also performed a bivariate correlation analysis between AR levels and WBC count, LDH levels, and peripheral blood blasts percentages (Figure 2B). We observed moderate but significant correlations between AR levels and the three parameters: WBC count (Kendall $\tau_b = 0.384$, $P = 0.003$), LDH levels (Kendall $\tau_b = 0.348$, $P = 0.011$), and peripheral blood blasts percentages (Kendall $\tau_b = 0.300$, $P = 0.036$).

Concerning the relationship between *FLT3*-ITD and *NPM1* mutations described by Schneider et

al (12), we investigated the correlation between RR levels and biological parameters in *FLT3*-ITD and *NPM1* double-mutant cases. A total of 17 patients with *FLT3*-ITD and *NPM1* mutations were investigated. According to the RR level cut-off value ($= 0.72$), 9 patients were included in the RR < 0.7 group, and 8 patients were included in the RR ≥ 0.7 group. There were no statistically significant differences between these 2 RR groups (Table S4). Due to the low subject number of this specific patient population, we also performed a bivariate correlation between RR levels and biological parameters, without observing any significant correlations (data not shown).

Discussions

Our data indicate that our current *FLT3*-ITD assay can be confidently used to evaluate both DNA and mRNA samples. Furthermore, data regarding both DNA and mRNA *FLT3*-ITD mutation parameters were comparable with previously published results (13), with a very good correlation between DNA and mRNA ITD length, and a good correlation between RR and AR. Fur-

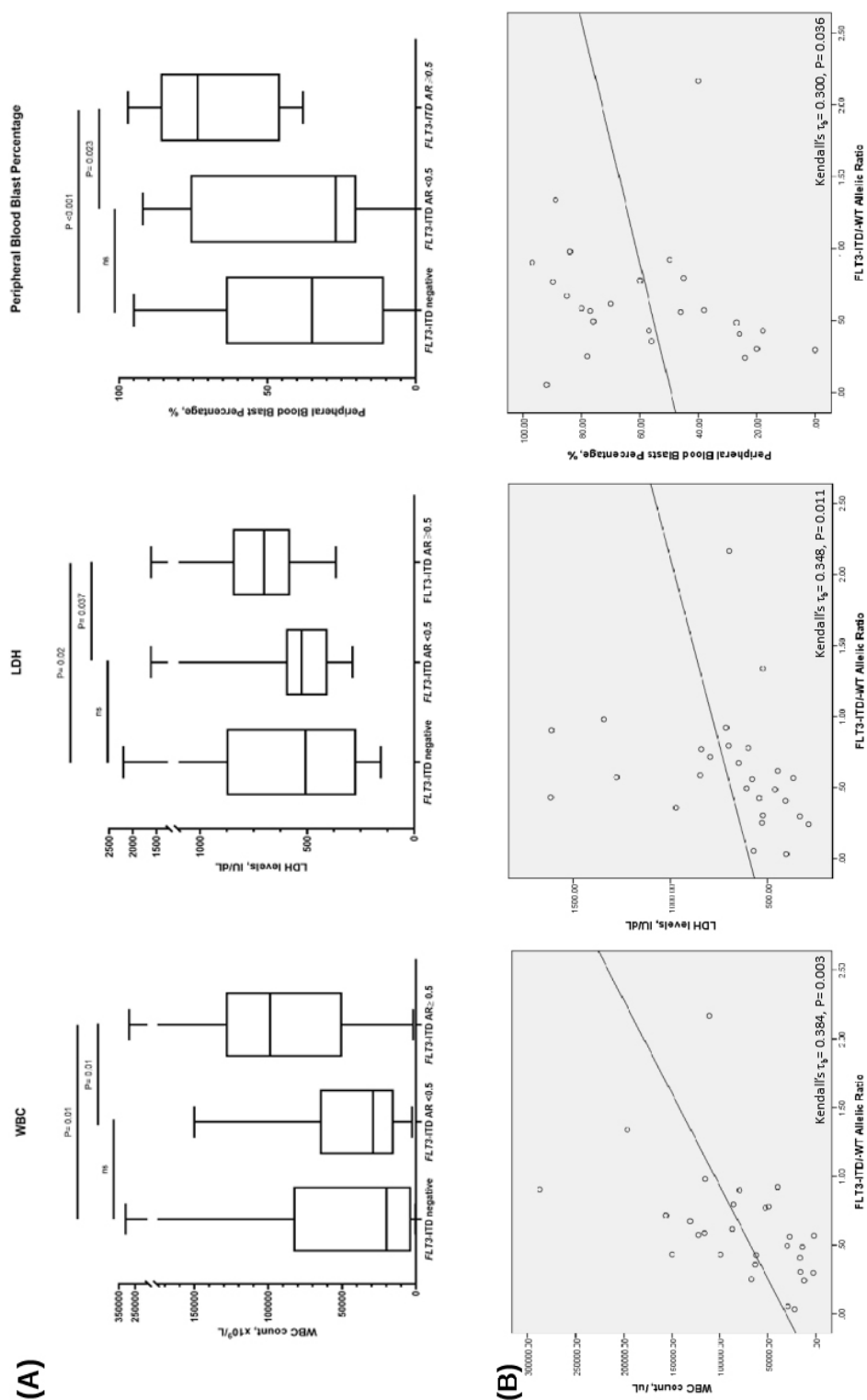


Figure 2. *FLT3*-ITD allelic ratio and biological parameters. (A) Box-plot graphs presenting the differences between the *FLT3*-ITD negative group, low AR <0.5 group, and high AR ≥ 0.5 group for WBC count, LDH levels, and peripheral blood blast percentage. (B) Dot-plot graphs presenting the correlation between *FLT3*-ITD allelic ratio and WBC count, LDH levels, and peripheral blood blast percentage.

thermore, RR levels were generally higher than AR values for the same patient, and in one case (UPN-8538) we were able to identify a supplementary ITD population on the mRNA sample (details in the Supporting information – results section).

Our group of *FLT3*-ITD patients presented higher WBC count, LDH levels, and blasts count when compared with *FLT3*-ITD negative cases as previously reported (14–16). Based on this, we investigated the association of various *FLT3*-ITD parameters to the biological characteristics of patients, and found that only the AR had a significant impact on the WBC count, LDH levels, and blasts count (Figure 1, Table S3). Furthermore, the results we obtained when comparing the biological characteristics of patients with *FLT3*-ITD grouped according to the AR cut-off value of <0.5 , as per the ELN 2017 genetic risk stratification (3), the *FLT3*-ITD $AR < 0.5$ group was not statistically different from the *FLT3*-ITD negative control (Figure 1A). To note that in a number of studies – *FLT3*-ITD mutations with an $AR < 0.5$ have a comparable evolution with a *FLT3*-ITD negative control (4–6).

An unexpected result was that *FLT3*-ITD mRNA levels were not associated with the analyzed biological parameters, not even within the prognostically relevant *FLT3*-ITD and *NPM1* double-mutant patients (12). From this we can conclude that, in our study population, the biological characteristics of *FLT3*-ITD AML cases (WBC, LDH levels, circulating blasts) are not correlated with the level of *FLT3*-ITD mRNA expression, expressed as the RR, but with the burden of *FLT3*-ITD positive cells, expressed as the AR. The fact that, in our study group, WBC counts, LDH levels, and circulating blasts levels are higher in patients with an $AR \geq 0.5$, is a probable indication that the *FLT3*-ITD mutant cell population is predominant, leading to the characteristic biological landscape of these patients. One of the main limitations of this paper is that

we did not analyze survival data to evaluate the impact of *FLT3*-ITD mutational parameters on the evolution and outcome of our study patients. This is due to the fact that we disposed mainly of diagnostic data, and that the patients were not treated uniformly. Other limitations were the small patient cohort and that we were not able to perform experiments either on cell-lines or on patient-derived primary cultures to validate and further investigate our results.

Recent publications report that *FLT3*-ITD mRNA levels have a clear functional impact. In a study evaluating the expression and localization of the *FLT3*-ITD receptor, samples with high mRNA levels of *FLT3*-ITD after exposure to FLT3 tyrosine kinase inhibitors (TKI) lead to a higher surface expression of the mutant receptor than samples with low *FLT3*-ITD mRNA expression (17). In another study, the mRNA *FLT3*-ITD/WT ratio – apart from being a better prognostic marker than the AR in a pediatric AML population, was also shown to predict the sensitivity to gilteritinib, a third generation FLT3-TKI (13). In this situation, patient-derived primary cell cultures from patients with a mRNA *FLT3*-ITD/WT ratio < 0.5 showed little susceptibility to gilteritinib, whereas cultures from patients with a ratio of ≥ 0.5 showed a significant decrease in clonogenic capacity in the presence of gilteritinib.

Further prospective clinical studies, and studies on mouse models are needed in order to evaluate the differential effect of AR versus RR on functional and survival parameters. Single-cell approaches would also be better suited than the current populational studies (i.e. AR and RR) to shed light on the behavior of individual *FLT3*-ITD positive leukemic cells.

To conclude, our study presents new data regarding the utility of mRNA-based *FLT3*-ITD analysis, and on the correlation of the *FLT3*-ITD mutational burden and mRNA expression with the biological characteristics of patients with this mutation. From our results, a possible dose

independent effect of *FLT3*-ITD mutations upon biological parameters can be sketched, where the studied parameters are only influenced by the proportion of *FLT3*-ITD cells and not on the level of *FLT3*-ITD expression.

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Authors' contributions

DSS: Conceptualization, Methodology, Investigation, Validation, Formal analysis, Data curation, Writing original draft preparation, Writing review and editing

ER: Conceptualization, Funding acquisition, Methodology, Validation, Formal analysis, Resources, Writing original draft preparation, Writing review and editing

ID: Methodology, Investigations

AA: Methodology, Investigations

CE: Methodology, Investigations

HB: Conceptualization, Project administrator, Funding acquisition, Validation, Supervision, Writing review and editing

AMV: Conceptualization, Funding acquisition, Supervision, Project administrator, PhD supervisor

Conflict of interest

There are no conflicts of interests to declare.

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