Title: Concomitant monitoring of *WT1* and *FLT3-ITD* expression in *FLT3-ITD* Acute Myeloid Leukemia patients: which should we trust as a minimal residual disease marker?

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To the Editor:

FLT3-ITD mutations (FMS-like tyrosine kinase 3) are the most common molecular alterations observed in Acute Myeloid Leukemia (AML). The presence of these mutations has an unfavorable prognostic significance and is the target of specific *FLT3-ITD* inhibitor drugs, currently in advanced stage clinical trials **[1,2]**. In recent years minimal residual disease (MRD) detection in AML has taken a very important prognostic role, becoming essential for the choice of the most appropriate consolidation strategy **[1-4]**. A recent study by Gaballa et al has shown how *FLT3-ITD*

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molecular status at the time of transplant is a key predictor of disease relapse and survival in patients with *FLT3-ITD* AML **[3]**. Similarly, a study by our group has demonstrated the high predictive value of MRD assessment by the Wilms' tumor (*WT1*) gene expression at the time of stem cell transplant in this subset of patients **[4]**. However, to date, there are no studies comparing the effectiveness of *FLT3-ITD* and *WT1* detection on MRD monitoring in *FLT3-ITD* AML.

The reliability of the *FLT3-ITD* mutation as a MRD marker has not yet been clarified and the use of polymerase chain reaction (PCR) techniques for its detection do not seem to guarantee high sensitivity ("PCR bias") **[1,2]**. Therefore, in order to verify the usefulness of serial *FLT3-ITD* tests along the course of the disease, we compared its expression level with the quantitative analysis of *WT1,* a known pan leukemic marker available for MRD assessment in AML **[5]**.

We analyzed 24 consecutive *FLT3-ITD* positive AML patients (diagnosed at the University of Udine-Hematology Department), all with *WT1* overexpression at diagnosis. The bone marrow samples were collected at three specific time-points for each AML case: at diagnosis, post-induction, and finally either post consolidation therapy (in cases in cytological remission), post salvage therapy (in primary refractory cases) or at cytological relapse (in cases of relapse after a previous remission). Of note, none of these patients received therapy with *FLT3-ITD* inhibitors. The expression level of *FLT3-ITD* mutation was determined by PCR (Applied Biosystem, Foster City, CA), followed by capillary gel electrophoresis to determine allelic burden **[6]**. Concomitantly, at the same time points, we performed quantitative PCR for *WT1* gene expression, in accordance with the LeukemiaNet guidelines and the detection of *WT1* copies > 250/104 copies of the control gene Abelson (*Abl*) was defined as WT1 overexpression (Ipsogen *WT1* ProfileQuant Kit, Qiagen, EU) **[5]**.

At diagnosis, the median bone marrow blast cells (BC) count was 90% (range 20-100%), with a 37% median *FLT3-ITD* allelic burden (range 3% -83%) and 9970 copies of WT1 (range 481-36423). As reported in **TABLE 1**, patients were divided into three groups according to the cytologic remission status of their AML after induction therapy: 1) patients with disease refractory (RES) to induction chemotherapy (AML-RES, 8 patients, median bone marrow BC after chemotherapy of 30%, range 15%-80%); 2) patients in cytologic Complete Remission (CR) post Induction therapy (AML-CR, 10 patients, BC<5%); 3) patients in relapse (REL) post CR (AML-REL, 6 patients, median bone marrow BC at relapse of 35%, range 20% -100%). We found that in those patients who achieved and maintained a cCR after induction (10 AML-CR, group 1), both *WT1* and *FLT3-ITD* became negative in a concordant manner: 8/10 cases (80%) were double negative after induction and 2/10 (20%) were double positive (concordance 100%); after consolidation 10/10 cases (100 %) were double negative. In cases with resistant AML (8 AML-RES, group 2) or at relapse (6 AML-REL, group 3) post chemotherapy, *WT1* was overexpressed in the totality of cases (14/14, 100%), while *FLT3-ITD* was positive only in 9/14 cases (64%), being negative in 5/14 (36%) cases, even in the presence of cytologically evident bone marrow disease. We have therefore observed a discrepancy between *WT1* and *FLT3-ITD* expression levels in relapsed (AML-REL) and disease-resistant (AML-RES) patients, with WT1 showing greater stability as a marker of MRD and a stronger concordance with cytology.

These data, within the limitations of a small series, suggests that *WT1* could be a reliable MRD marker and highlight the instability of *FLT3-ITD*, with possible switch from *FLT3-ITD* positivity at diagnosis to *FLT3-ITD* negativity at the time of cytological relapse or during post-chemotherapy resistance. This probably reflects the fact that the AML is constituted by leukemic subclones heterogeneous for *FLT3* expression and that a FLT3-ITD negative subclone may be responsible for the recurrence or resistance of AML **[1,2]**.

Conversely, overexpression of the pan leukemic marker *WT1* seems to be more stable along the course of the disease and more homogeneous in the different leukemic subclones, making this an appropriate and reliable marker for MRD detection; furthermore, overexpression of *WT1* appears to have a high degree of concordance with cytology (100% in this series).

In summary, these data suggest that *FLT3-ITD* is not, by itself, an optimal MRD marker and that the current available technique for its detection has lower sensitivity compared to the one in use for *WT1*. More sensitive molecular methods, such as new PCR based tests or next-generation sequencing (NGS) assays, could increase *FLT3-ITD* sensitivity and its use as MRD marker [**2,7**]. However, even with the current technique, 2 patients in our series were negative for *WT1* and positive for *FLT3-ITD* after induction and later relapsed. It might therefore be useful, until more efficient methods are routinely available, to test both *WT1* and *FLT3-ITD* markers at diagnosis and throughout the AML course to better evaluate the depth of remission and to guide MRD driven therapy in *FLT3-ITD* positive AML.

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TABLE 1. Comparative analysis of FLT3 and WT1 according to AML remission status after induction chemotherapy.

GROUP 1 AML-cCR (10 CASES)	Diagnosis	Post Induction (in cCR)	Post Consolidation
FLT3 POSITIVE (allelic burden-%)	10/10 48,5 (29-76)	2/10 (20%)* (3; 6)	0/10
WT1 POSITIVE (number of copies/10^4 Abl)	10/10 11488 (4164-26393)	2/10 (20%)* (1154; 438)	0/10
GROUP 2 AML-RES (8 CASES)	**Diagnosis**	**Post Induction (RES)**	**Post Salvage CHT (RES)**
FLT3 POSITIVE (allelic burden-%)	8/8 38 (3-83)	**6/8 (75%)** 30,5 (3,4-89)	**6/7° (85%)** 44,5 (16-98)
WT1 POSITIVE (number of copies/10^4 Abl)	8/8 10733 (481-36423)	8/8 (100%) 5266 (752-17047)	7/7° (100%) 6081 (757-28371)
GROUP 3 AML-REL (6 CASES)	**Diagnosis**	**Post Induction (in cCR)**	**Relapse**
FLT3 POSITIVE (allelic burden-%)	6/6 34 (4,7-39)	2/6 (33%)** (5; 10,5)	**3/6 (50%)** (43; 37; 48,4)
WT1 POSITIVE (number of copies/10^4 Abl)	6/6 7497 (1328-13719)	2/6 (33%)** (1451; 447)	6/6 (100%) 11613 (651-21589)

cCR, Cytologic Complete Remission; RES, Refractory; REL, relapse; CHT, chemotherapy; Abl, Abelson gene.

° 1 AML-RES patient died after CHT.

* MRD positive=**2/2** (with both FLT3-ITD positive and WT1 positive).

** MRD positive =**4/6** (2/6 only FLT3-ITD positive and 2/6 only WT1 positive); MRD negative=2/6 (with both FLT3-ITD negative and WT1 negative).