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SHORT REPORT

FLT3-targeted therapy restores GATA1 pathway function in NPM1/FLT3-ITD mutated acute myeloid leukaemia

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Abstract

One-third of newly diagnosed adult acute myeloid leukaemia (AML) carry *FLT3* mutations, which frequently occur together with nucleophosmin (*NPM1*) mutations and are associated with worse prognosis. *FLT3* inhibitors are widely used in clinics with limitations due to drug resistance. AML cells carrying *FLT3* mutations in both mouse models and patients present low expression of GATA1, a gene involved in haematopoietic changes preceding AML. Here, we show that FLT3 inhibition induces cellular responses and restores the GATA1 pathway and functions in *NPM1*/*FLT3*-ITD mutated AML, thus providing a new mechanism of action for this drug.

KEYWORDS
acute myeloid leukaemia, FLT3 inhibitors, FLT3-ITD, GATA1, heamatological malignancies, leukaemia therapy

1 | SHORT REPORT

FLT3 internal tandem duplications (*FLT3*/ITDs) are detected in about one-third of newly diagnosed adult acute myeloid leukaemia (AML) [1], resulting in constitutive FLT3 activation [2]. They frequently co-occur with *nucleophosmin* (*NPM1*) mutations [3, 4] and are associated with poor survival [5, 6]. We recently described a mouse model of *Npm1/Flt3-ITD* mutations [7] leading to leukaemia onset associated with GATA1 epigenetic deregulation [8].

GATA1 expression is frequently absent in AML patients with *FLT3*-ITD mutations [9], possibly as a consequence of epigenetic reprogramming of multipotent progenitor cells towards an immature myeloid lineage. The deregulation of GATA1 plays a key role in the haemopoietic changes preceding AML [10], therefore, we assessed the activity of FLT3 inhibitors (FLT3i) and whether they were able to modulate GATA1 expression both in an *Flt3-ITD* mutated mouse model of AML and in leukaemic patients' samples.

In vitro, the FLT3i AC220 (Quizartinib) significantly reduced viability and induced apoptosis of leukaemic cells from *Npm1/Flt3-ITD* mice compared to untreated cells (21.5% ± 3.6 vs. 65.9% ± 19.9 and 54.4% ± 6 vs. 16.3% ± 11.9 respectively, $p < 0.01$ and $p < 0.001$; (Figure 1A). In vivo (when administered daily for 28 days at 10 mg/kg by

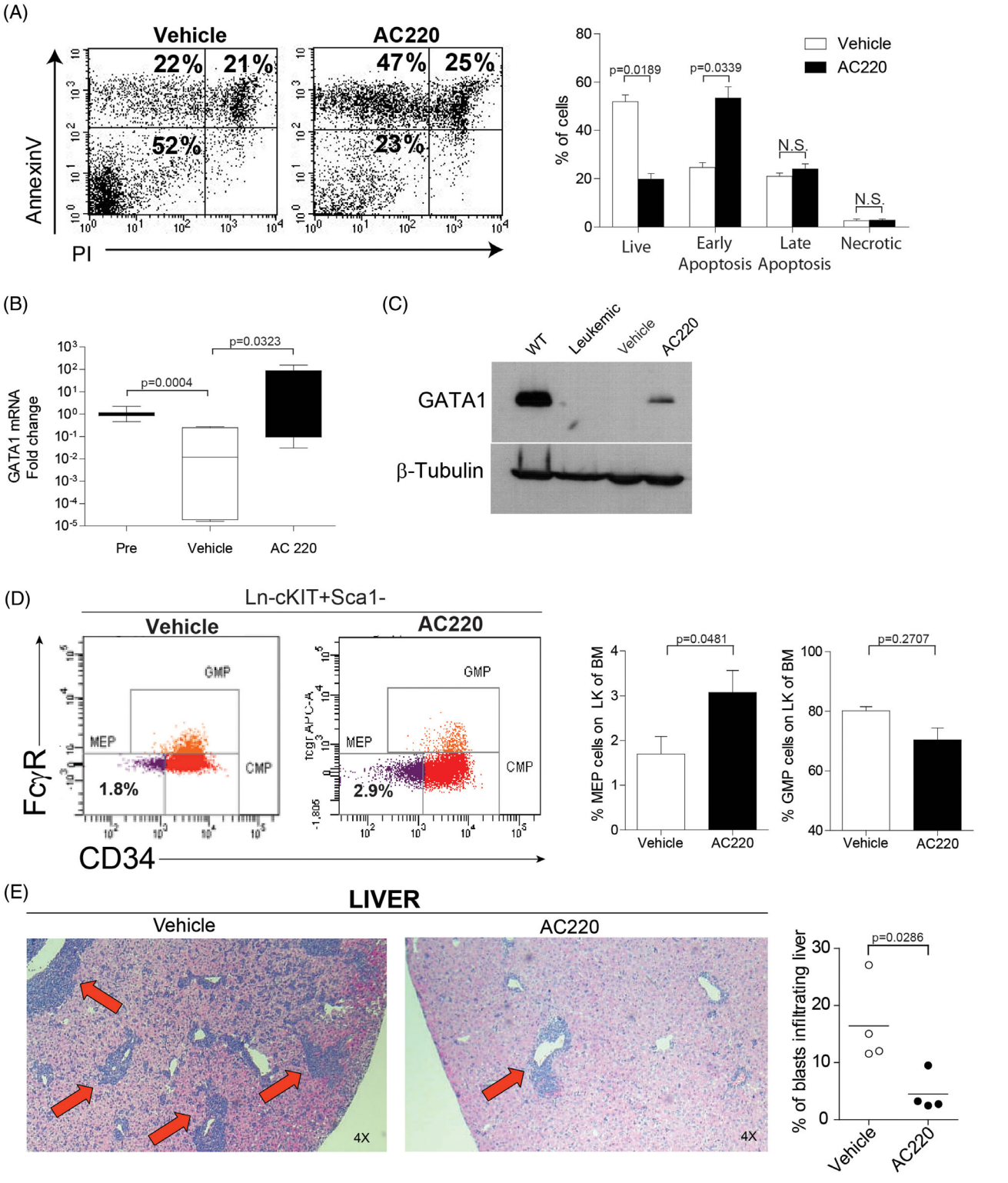

FIGURE 1 Antileukemic effect of Quizartinib. (A) Cell viability and apoptosis evaluated by flow cytometric analysis of annexin V/PI (left panel). Results (right panel) are presented as viable (AnnV- PI-), early apoptotic (AnnV+ PI–), late apoptotic (AnnV+PI+) and necrotic (AnnV-PI+). (B) mRNA levels of GATA1 evaluated by quantitative real-time PCR, normalized by GADPH and represented as fold change in BM of leukaemic, vehicle-treated and AC220-treated mice. (C) Western blot of GATA-1 was performed on BM of WT mice (positive control), leukaemic, vehicle-treated and AC220-treated mice. (D) Flow cytometric analysis of CMP, GMP and MEP progenitors, gated on Ln-cKIT+Sca1+ cells in leukaemic mice treated with vehicle or AC220 (left panel). Right panel, frequency of MEP cells in leukaemic mice treated with vehicle or AC220. (E) Hematoxylin and Eosin staining in the liver of vehicle and AC220 treated leukaemic mice. 4X of magnification, red arrows indicate the blasts infiltrating the area (left panel). Right panel, frequency of the area infiltrated by blasts in the vehicle and AC220 treated leukaemic mice in the liver. P values are indicated above each graph, according to paired t-test (A), and Mann–Whitney U-test (B, D and E).

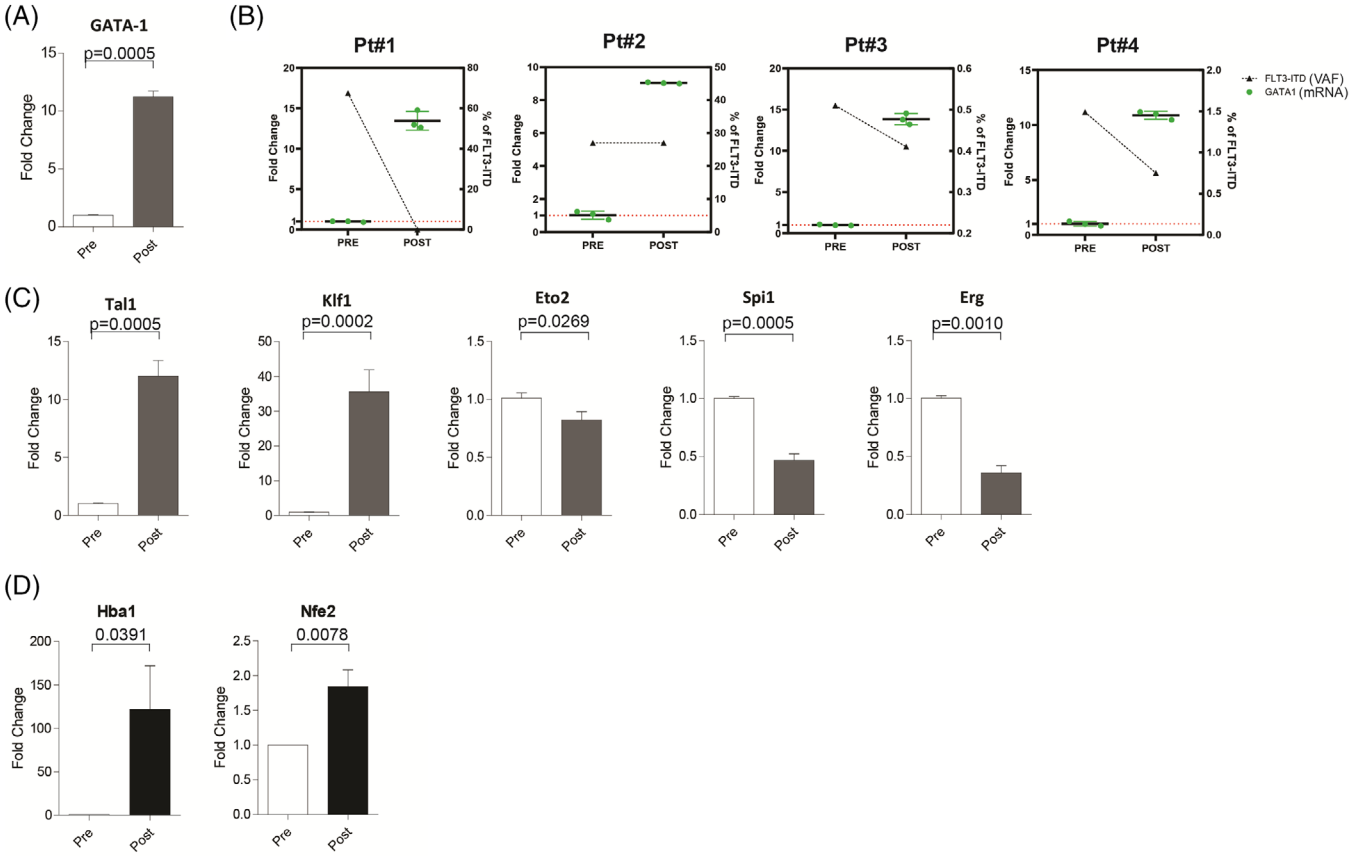

FIGURE 2 FLT3 inhibitor treatment restores the GATA-1 pathway and function. (A) mRNA levels of GATA-1 by quantitative real-time PCR, normalized by GADPH and represented as fold change in human leukaemic BM, pre and after FLT3 inhibitor treatment. (B) GATA-1 mRNA levels and concomitant variant allele frequency (VAF) of FLT3-ITD in human leukaemic patients pre and post-FLT3 inhibitor treatment. C) mRNA levels of TAL1, KLF1, ETO2, SPI1 and ERG by quantitative real-time PCR, normalized by GADPH and represented as fold change in human leukaemic BM, pre and post FLT3 inhibitor treatment. D) mRNA levels of Hba1 and Nfe2 by quantitative real-time PCR, normalized by GADPH and represented as fold change in human leukaemic BM, pre and post-FLT3 inhibitor treatment. P values are indicated above each graph, according to the Wilcoxon t-test.

oral gavage) Quizartinib significantly reduced the peripheral blood (PB) white blood cell count (especially the neutrophils count), as compared to vehicles (Figure S1A–C).

Notably, the improvement in the PB values in FLT3i-treated mice was associated with the re-expression of GATA1 mRNA (Figure 1B). Moreover, AC220 treated BM samples showed an increase of GATA1 protein level (Figure 1C) associated with a significant reduction of BM total number of cells and an improvement of the percentage of myelo-erythroid progenitors (MEP) population with concomitant reduction of granulocyte/monocyte progenitors (GMP) (Figure 1D). These findings are in keeping with the fact that GATA1 is necessary for erythroid lineage differentiation and antagonizes the activity of myeloid transcription factors [11].

However, restoring GATA1 expression did not result in a strong antiblastic effect on AC220-treated mice. In fact, there was no significant reduction in the BM and spleen immature Mac1+ cKIT+ cells (Figure S2A) and only a modest reduction in the ratio of spleen/body weight in AC220 treated mice as compared to vehicle (Figure S2B). Consequently, FLT3 inhibition had no significant impact on the survival of *Npm1/Flt3-ITD* mice (Figure S2C). Quizartinib-treated mice, displayed a strong reduction in blast infiltration in peripheral organs (Figure 1E), compared to vehicle-treated leukaemic mice but the clearance of these blasts was not complete, causing leukaemia relapse.

A 5 days Quizartinib treatment was further performed in a xenotransplant model of human *NPM1/FLT3-ITD* mutated AML. Mice receiving the FLT3i displayed a significant reduction in the size of the subcutaneous AML tumors compared to vehicles (Figure S3A). Molecular analysis of masses showed that GATA1 mRNA levels were significantly up-regulated in Quizartinib-treated mice compared to vehicles (Figure S3B) but the treatment did not result in complete eradication of AML cells. This is in keeping with previous reports and suggests that the treatment with single-agent FLT3i prolonged the overall survival, as is demonstrated in the QuANTUM-r trial but [12] is unable to achieve a complete anti-leukaemic effect [2, 13].

In order to understand the clinical relevance of molecular observations, BM samples from patients with *FLT3-ITD/NPM1* mutated AML were analyzed for the GATA1 mRNA levels at diagnosis and after treatment with second-generation FLT3i (i.e., Quizartinib or Gilteritinib)

(Table S1). As shown in mice, patients who received FLT3i displayed an increased level of GATA1 mRNA in BM cells, compared to GATA1 levels at diagnosis (Figure 2A and Table S1). This was associated with a concomitant decrease of the *FLT3-ITD* allelic ratio, supporting the concept that FLT3i treatments restore GATA1 levels in the clinical setting (Figure 2B).

We also evaluated the mRNA expression of Tal1 and Klf1 (known positive GATA1-interactors) and Eto2, Erg and Spi1, which negatively interfere with GATA1 activity [14]. Patients receiving FLT3i showed a significant up-regulation of Tal1 and Klf1, while Eto2, Erg and Spi1 were down-regulated following FLT3i (Figure 2C), indicating that FLT3 inhibition improve the GATA1 network activity.

Accordingly, in human AML samples (Table S1), we measured the levels of GATA1-controlled genes such as nuclear factor erythroid 2 (Nfe2) and Hemoglobin Alpha1 (Hba-a1), fundamental genes for erythroid differentiation. FLT3i treatment in patients, led to upregulation of Nfe2 *and* Hba-a1 (Figure 2D), demonstrating that the anti-leukaemic treatment interferes also with GATA1 functions and sustains GATA1-dependent erythroid differentiation in vitro.

In conclusion, we demonstrate a new molecular effect of FLT3 inhibition on the erythroid master regulator GATA1 resulting in erythroid lineage differentiation and myeloid transcription factors antagonism with mild anti-leukaemic effects. GATA1 targeting using ERG and ETO2 inhibitors could represent a novel approach for *FLT3*-mutated AML since they functionally interfere with GATA1 activity and restrain GATA1-dependent erythroid differentiation [14]. Therefore, the inhibition of ERG and/or ETO2 may result in the increase of GATA1 and its targets, supporting FLT3i treatment in order to avoid drug resistance and improve the duration of response.

AUTHOR CONTRIBUTIONS

P.S and B.F designed, conceived and coordinated the study D.S and A.S performed research and were responsible for data collection, statistical analysis and interpretation, and manuscript preparation. A.S, S.S and A.M performed the in vivo experiments. R.R, F.D.F, F.M.A and C.R performed in vitro experiments. C.G and R.A performed cytometric analysis. E.D and A.E contributed to performing experiments. L.M and M.G.M performed RT–PCR analysis. M.P.M reviewed the manuscript and provided critical data and suggestions.

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None.

CONFLICT OF INTEREST STATEMENT

B. Falini applied for a patent on the clinical use of NPM1 mutants. The other authors declare no conflict of interest.

FUNDING INFORMATION

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DATA AVAILABILITY STATEMENT

All data and information concerning this study will be made available from the corresponding authors upon reasonable request.

ETHICS STATEMENT AND PATIENT CONSENT STATEMENT

The study was conducted following the Declaration of Helsinki and Good Clinical Practice. All patients signed an informed consent form, and the study was approved by the Ethics Committee of the University of Perugia (protocol #88471).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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