Azacitidine as post-remission consolidation for sorafenib-induced remission of Fms-like tyrosine kinase-3 internal tandem duplication positive acute myeloid leukemia

Internal tandem duplication (ITD) of the fms-like tyrosine kinase 3 (*FLT3*) gene in acute myeloid leukemia (AML) confers an inferior prognosis.[1,2] Multi-kinase inhibitors against *FLT3*, including midostaurin, sorafenib and quizartinib, have been evaluated either as single agents or in combination with intensive chemotherapy.[3,4] Monotherapy often results in significant clearance of BM blasts but the response is uniformly transient and drug resistance invariably develops. Combination with intensive chemotherapy might improve event-free survival (EFS) in young[5] but not elderly patients.[6]

The use of azacitidine as maintenance therapy in myelodysplastic syndrome (MDS) and AML patients who are at risk of relapse has been evaluated.[7,8] Azacitidine has also been used in combination with sorafenib, resulting in a significant overall response in relapsed *FLT3*-ITD$^+$ AML.[9] Here, sorafenib monotherapy was effective as a re-induction agent and azacitidine consolidation prolonged response as compared with a historical cohort of patients treated with sorafenib monotherapy. Sorafenib and azacitidine synergistically inhibited leukemia cell growth *in vitro*, potentiated apoptosis and suppressed leukemia engraftment in xenotransplantation of *FLT3*-ITD$^+$ cell lines.

Patients with relapsed or refractory *FLT3*-ITD$^+$ AML after conventional chemotherapy and/or allogeneic HSCT were treated with sorafenib at 200 – 400 mg twice daily. BM examination was performed at three weeks and then every four weeks until best response was documented. Complete remission (CR) was defined as BM blasts < 5%, with absolute neutrophil count > 1×10^9/L and platelet count >100×10^9/L. Complete remission with insufficient hematological recovery (CRi) was defined as BM blasts < 5% with incomplete recovery of absolute neutrophil and/or platelet counts. Thereafter, azacitidine consolidation (100 mg/day x 4 days subcutaneously) was administered every four weeks until disease progression or allogeneic HSCT. The total dose of 400 mg per cycle was similar to that used for maintenance in AML.[7,8] The decision of a 4-day regimen was a practical one, taken in order to avoid drug wastage and to shorten the duration of administration. Data were censored on 30 July 2014. The study was approved by the institutional review board in accordance with the Helsinki Declaration. Determination of *FLT3*-ITD and *FLT3*-TKD and their allelic burden have been previously described.9 Categorical variables were analyzed with the chi-square test and the Fisher's exact test. Continuous variables were analyzed with the Mann-Whitney U test or the

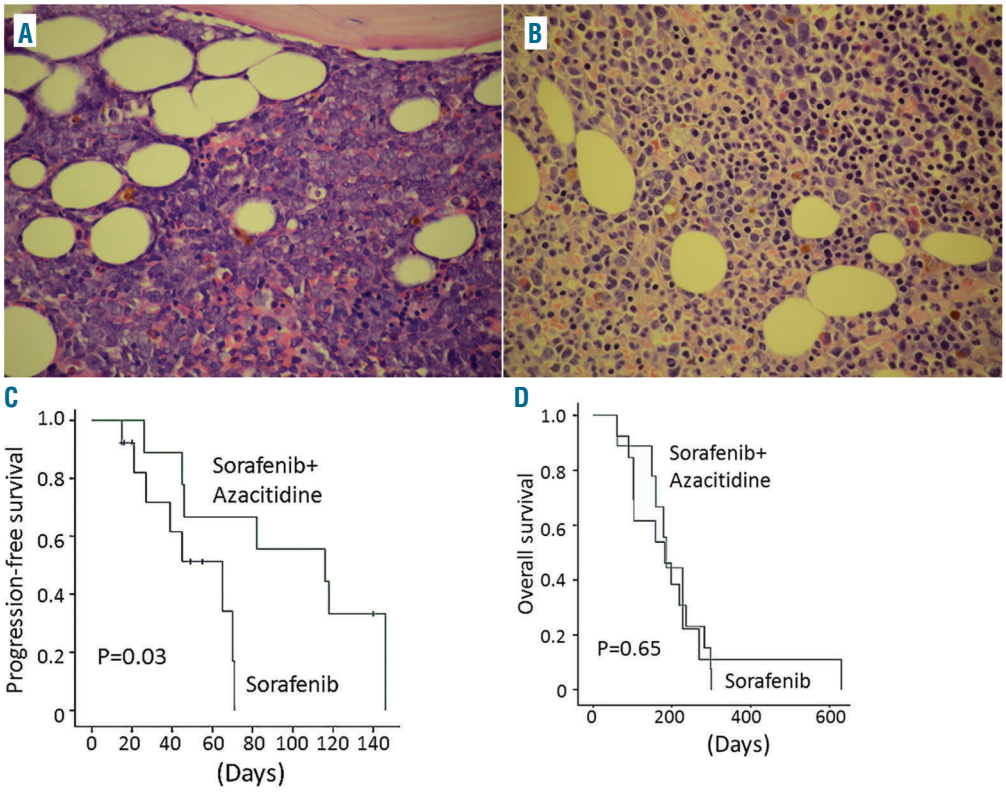

Figure 1. Response to sorafenib and azacitidine treatment in patients with *FLT3*-ITD$^+$ AML A. Bone marrow biopsy of a representative case with *FLT3*-ITD$^+$ AML before sorafenib treatment showing predominance of blasts. B. Bone marrow biopsy of the same case three weeks after sorafenib treatment, showing clearance of blast, and return of erythropoiesis and myeloid maturation. C. Progression-free survival (PFS) was measured from the time of best BM response to leukemia progression or last follow-up. Treatment with sorafenib and azacitidine significantly improved PFS as compared with patients treated with sorafenib alone. D. Overall survival was measured from the start of sorafenib until death or last follow-up and was comparable for the sorafenib and azacitidine cohort and the sorafenib alone cohort.

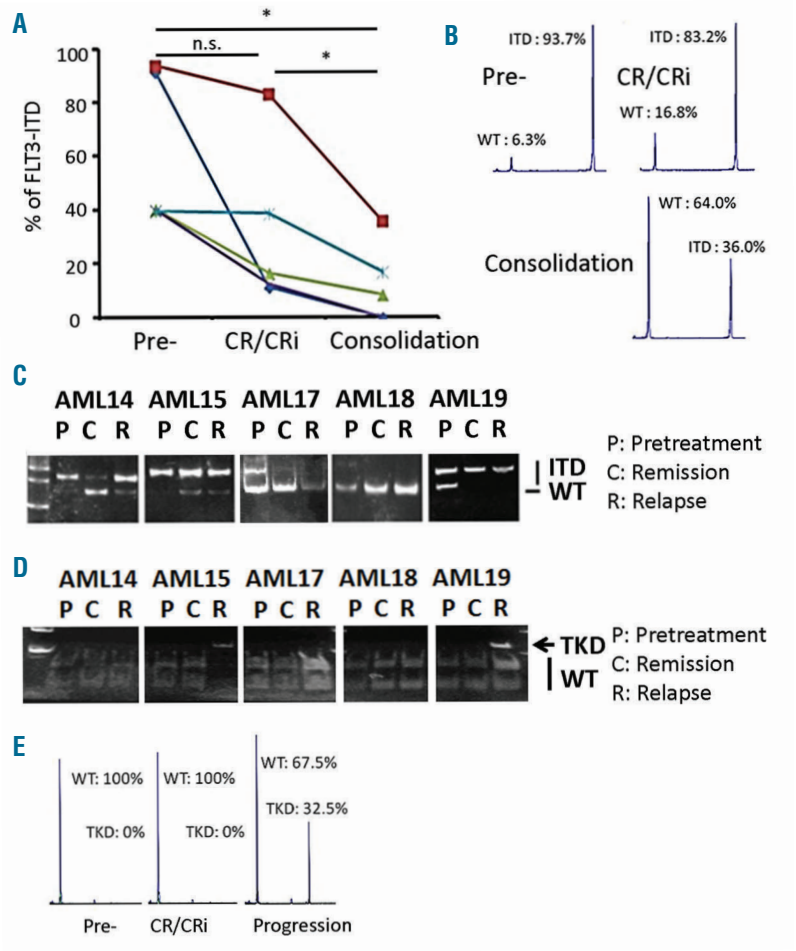

Figure 2. Molecular analysis of *FLT3* gene during sorafenib and azacytidine treatment in five cases of *FLT3*-ITD$^+$ AML. **A.** *FLT3*-ITD allele burden was only modestly decreased at CR/CRi, which was further reduced after consolidation with sorafenib and azacitidine. **B.** Representative gene scans of a case of *FLT3*-ITD$^+$ AML on presentation, at sorafenib-induced CR and during consolidation with sorafenib and azacitidine; showing progressive decrease of *FLT3*-ITD allele burden. **C.** PCR amplification of *FLT3* at presentation (P), on achieving sorafenib-induced CR/CRi (C), and at disease relapse (R). In the case AML17 and AML18, the *FLT3*-ITD clone was lost at C (AML 18) and R (AML 17 and 18). **D.** Emergence of *FLT3*-TKD D835Y mutant clone at relapse in AML15 and AML19, as shown by the loss of restriction site upon EcoRV digestion. **E.** Representative gene scans of a case, showing evolution of the allelic burden of wildtype *FLT3* (*FLT3*-WT) and *FLT3*-TKD mutation. *P<0.05.

Student's paired *t*-test. Survival analyses were performed using the Kaplan-Meier method. Differences in survival were determined with the log-rank test. All statistical analyses were performed with SPSS (version 18.0) (Chicago, IL, USA). A P-value of less than 0.05 was considered as statistically significant.

Nine patients were treated (*Online Supplementary Table S1*). Five patients (AML secondary to MDS, N=4; AML with MDS changes, N=1) had prior exposure to hypomethylating agents (decitabine, N=2; azacitidine, N=3). The overall response rate (ORR) to sorafenib was 100% (CR, N = 3; CRi, N = 6) (Figures 1 A,B). The median time to best bone marrow response (BBMR) was 26 days (range: 21–91 days). A median of 4 courses of azacitidine was administered (1–5 courses). To evaluate the benefits of azacitidine as a post-remission therapy, the outcome was compared with that of a historical cohort of 13 patients treated with sorafenib monotherapy, eight of whom had previously been reported.[10] The present cohort had significantly higher median hemoglobin, lower circulating leucocytes and PB/BM blasts percentages prior to treatment (*Online Supplementary Table S2*). The median *FLT3*-ITD allele burdens before sorafenib treatment in the current and historical cohorts were 48.8% (range: 11.2-93.7%) and 44% (range:28-100%) (P=0.99). With a median follow-up of 186 days (range: 61–630 days), the current cohort was superior to the historical sorafenib monotherapy cohort in median progression free survival (PFS) (116 *versus* 65 days; P=0.03, log-rank) (Figure 1C), but comparable in median overall survival (OS) (182 versus 186 days; P=0.65, log-rank) (Figure 1D). Despite an initial response, all nine patients ultimately progressed (emergence of PB and/or BM blasts of >20%).

To ascertain if azacitidine might increase the depth of response, *FLT3*-ITD allelic burden was examined serially in five patients. At CR/CRi after sorafenib monotherapy, the *FLT3*-ITD allelic burden was only modestly reduced, recapitulating our previous observations in patients treated with sorafenib monotherapy.10 However, azacitidine in conjunction with continued sorafenib treatment significantly reduced the allelic burden (Figure 2A,B), supporting our proposition that combined azacitidine and sorafenib treatment resulted in reduction of *FLT3*-ITD clone size. At disease progression, two patients showed loss of the *FLT3*-ITD clone present before sorafenib treatment (AML17 and AML18, Figure 2C). Emergence of *FLT3*-TKD D835Y mutant clone occurred in two patients (AML17 and AML19, Figure 2D,E). Other *FLT3* TKD mutations including F691, N841 and Y842 were not observed.

The anti-leukemia effects of combined sorafenib and azacitidine on *FLT3*-ITD$^+$ cell lines in vitro was evaluated. Sorafenib and azacitidine were synergistic in inhibiting cellular proliferation and inducing apoptosis (Figure 3A-C). Interestingly, myeloid differentiation, as reflected by increased CD11b expression, was not enhanced with combined treatment (Figure 3D). Furthermore, MV4-11 cells were treated for three days with sorafenib (1nM), azacitidine (0.1 μmol) or their combination before being

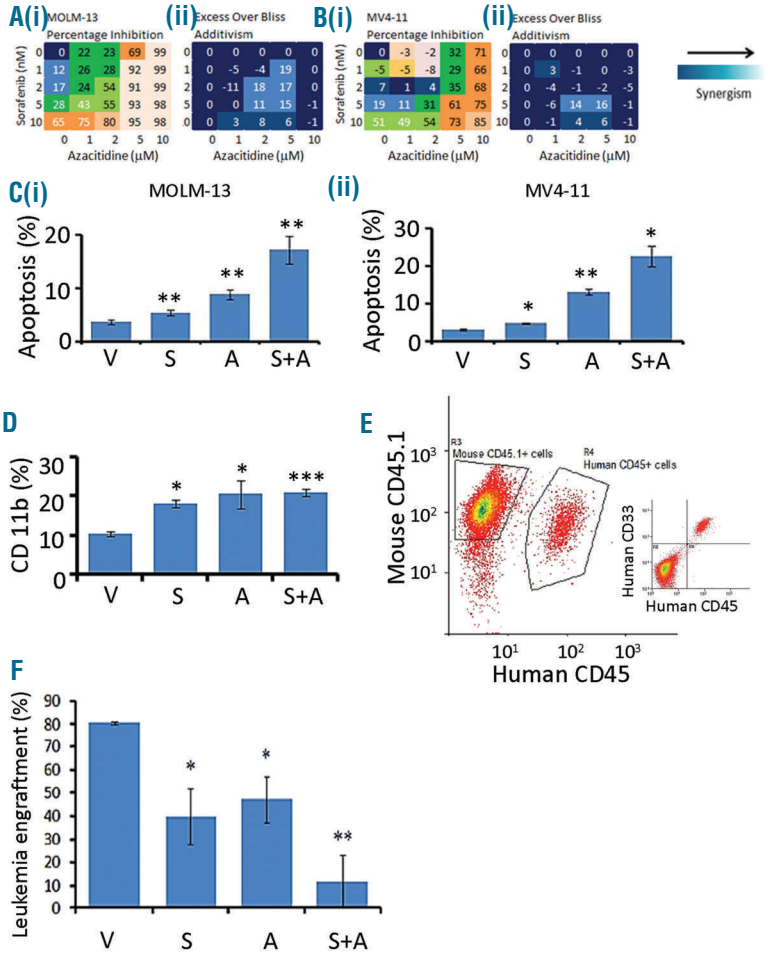

Figure 3. Targeting of *FLT3*-ITD$^+$ AML by sorafenib and azacitidine *in vitro* and *in vivo*. A and B. Sorafenib and azacitidine targeted AML synergistically *in vitro*. Azacitidine and sorafenib inhibited cell growth in MOLM-13 (Ai) and MV4-11 (Bi). Viable cell number was enumerated by AccuCheck Counting Beads (Life Technologies, Grand Island, NY, USA) after PI exclusion. The number in each box represented the mean percentage inhibition in triplicates. Difference in percentage growth inhibition between combination treatment and the multiplication product of growth inhibition by each treatment alone (excess over Bliss additivism) in MOLM-13 (Aii) and MV4-11(Bii). Positive values indicated synergism between sorafenib and azacitidine. C. Combination of sorafenib and azacitidine potentiated apoptosis in MOLM-13(i) and MV4-11(ii) as detected using phycoerythrin (PE) Annexin V Apoptosis Detection Kit I (BD Bioscience) based on flow cytometry. D. Sorafenib and azacitidine significantly induced differentiation of MOLM-13 cells as enumerated by the increase in CD11b$^+$ expression but their combination had not resulted in synergism. E. Representative diagram showing human engraftment in NSG mice as shown by the human CD45$^+$ and mouse CD45.1$^-$ population. After treatment *in vitro*, MOLM-13 and MV4-11 cells were injected intravenously (i.v.) into sublethally irradiated (250 cGy) 6 to 8 week-old NOD/SCID/IL2-R$\gamma^{-/-}$ (NSG) mice. At 6 weeks, mouse BM was evaluated after staining with antibodies against human CD45, and mouse CD45.1 (BD Bioscience). The inset showed a representative flow cytometric analysis of double human CD45$^+$ and human CD33$^+$ cells in mice transplanted with MV4-11 cells. Xenogeneic transplantation was approved by the Committee on the Use of Live Animals for Teaching and Research (CULATR 837-03, 1587-07) of the University of Hong Kong. F. Sorafenib and azacitidine significantly reduced AML engraftment in NSG mice (Paired *t*-test: *:*P*<0.05, **:*P*<0.01).

transplanted into NSG mice to assess leukemia engraftment, as represented by the presence of myeloblasts expressing human CD45$^+$ and CD33$^+$ at 6 weeks post-transplantation. Both sorafenib and azacitidine treatment significantly reduced leukemia engraftment, which was further enhanced on combined treatment. (Figure 3E).

In this study, sorafenib induced a remarkable response resulting in CR/CRi in all nine patients. It is noteworthy that these patients were either elderly or refractory to conventional treatment, signifying that alternative therapeutic options were very limited. Azacitidine added to sorafenib treatment after sorafenib-induced CR/CRi further increased the depth of response, as shown by a significant reduction in *FLT3*-ITD allelic burden. This was translated into a significant increase in PFS, as compared with a historical cohort treated with sorafenib monotherapy.

We have further provided insights that might improve the treatment outcome of these patients. Firstly, the ability of sorafenib to induce CR/CRi in all four cases of AML secondary to antecedent MDS was unexpected. Importantly, *FLT3* was wild-type during the MDS phase (unpublished data), but *FLT3*-ITD developed at leukemic transformation. An increased incidence of acquisition of *FLT3*-ITD in AML transforming from MDS has been reported, but the relevant therapeutic implication has not been explored.[11] To our knowledge, our results provide the first demonstration that *FLT3*-ITD is a valid therapeutic target when it is acquired during leukemic transformation of MDS. Hence, *FLT3*-ITD should be sought in AML arising from underlying MDS, even if initially absent. This is particularly pertinent in MDS cases transforming to AML while on hypomethylating agents (as in our cases), because otherwise the prognosis for patients is extremely poor.

Secondly, PFS was significantly prolonged with combined sorafenib and azacitidine treatment, as compared with a historical cohort of sorafenib monotherapy. Previous reports of combination treatment of a tyrosine kinase inhibitor (TKI) with induction chemotherapy in newly-diagnosed AML had largely been negative[12-14], excepting one recent study that showed prolongation of LFS.[5] However, in relapsed or refractory *FLT3*-ITD$^+$ AML treated with sorafenib, a high response rate with acceptable toxicity was observed, suggesting that addiction to *FLT3* signaling might be more pronounced in relapsed or refractory states.

Thirdly, we showed that sorafenib and azacitidine both induced apoptosis and partial differentiation of *FLT3*-ITD$^+$ AML cells *in vitro* and their combined treatment potentiated leukemia cell apoptosis but not differentiation. Combined sorafenib and azacitidine treatment also

suppressed leukemia cell engraftment in immunodeficient mice upon xenotransplantation, providing a laboratory correlation to the improved leukemia free survival of patients receiving azacitidine consolidation. However, its clinical relevance remains to be determined, and *in vivo* treatment of animals may provide a closer resemblance to the clinical scenarios.

There were a number of limitations in this study. It included a relatively small number of patients, who were over-represented by AML transforming from underlying MDS. Moreover, the pre-clinical studies were based primarily on leukemia cell lines. The benefits to AML patients with wild-type *FLT3* are also unknown. However, the outcome of transformed MDS in elderly patients is uniformly fatal, and any novel strategies that might induce remission, which occurred at a high frequency in our cohort, should be further investigated. The mechanistic link between sorafenib and azacitidine is unclear but could be related to their concerted effects on signal transduction and promoter demethylation of tumor suppressors. Previous reports on this combination predicated response on azacitidine not inducing an increase in the Flt3 ligand, which might hamper the effects of sorafenib.[9] In this study, we observed that sorafenib as monotherapy achieved a remarkable response as a single agent in relapsed *FLT3*-ITD$^+$ AML, and that low-dose azacitidine in combination with sorafenib achieved a durable remission. Furthermore, the enhanced anti-leukemia effects between sorafenib and azacitidine *in vitro* implied potential synergisms at a cellular level. However, despite a significant increase in PFS, there was no improvement in OS as all patients eventually relapsed. Hence, a more effective consolidation strategy has to be identified.

In conclusion, the results in this study provide proof-of-principle evidence that consolidation treatment after TKI-induced remission in AML is potentially beneficial. This concept should further be tested in future clinical trials of TKI in the treatment of acute leukemia.

Harinder Gill,[1] Cheuk-Him Man,[1*] Alvin H.W. Ip,[2] William W.L. Choi,[2] Howard C.H. Chow,[1] Yok-Lam Kwong,[1] and Anskar Y.H. Leung[1]*

**GH and MCH contributed equally to the manuscript writing*

[1]Division of Haematology, Medical Oncology and Bone Marrow Transplantation, Department of Medicine, the University of Hong Kong; and [2]Department of Pathology, the University of Hong Kong, China.

Funding: the authors would like to thank the S.K. Yee Medical Foundation for the funding. AYH Leung is the Li Shu Fan Medical Foundation Professor in Haematology and receives funding from its Endowment.

Correspondence: ayhleung@hku.hk
doi:10.3324/haematol.2014.123034

Key words: acute myeloid leukemia, FLT3-ITD, Sorafenib, azacitidine.

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

References

1. Schnittger S, Schoch C, Dugas M, et al. Analysis of FLT3 length mutations in 1003 patients with acute myeloid leukemia: correlation to cytogenetics, FAB subtype, and prognosis in the AMLCG study and usefulness as a marker for the detection of minimal residual disease. Blood. 2002;100(1):59-66.
2. Frohling S, Schlenk RF, Breitruck J, et al. Prognostic significance of activating FLT3 mutations in younger adults (16 to 60 years) with acute myeloid leukemia and normal cytogenetics: a study of the AML Study Group Ulm. Blood. 2002;100(13):4372-4380.
3. Fischer T, Stone RM, Deangelo DJ, et al. Phase IIB trial of oral Midostaurin (PKC412), the FMS-like tyrosine kinase 3 receptor (FLT3) and multi-targeted kinase inhibitor, in patients with acute myeloid leukemia and high-risk myelodysplastic syndrome with either wild-type or mutated FLT3. J Clin Oncol. 2010;28(28):4339-4345.
4. Cortes JE, Kantargian H, Foran JM, et al. Phase 1 study of quizartinib administered daily to patients with relapsed or refractory acute myeloid leukemia irrespective of FMS-like tyrosine kinase 3-internal tandem duplication status. J Clin Oncol. 2013;31(29):3681-3687
5. Röllig C, Müller-Tidow C, Hüttmann A, et al. Sorafenib versus placebo in addition to standard therapy in younger patients with newly diagnosed acute myeloid leukemia: results from 267 patients treated in the randomized placebo-controlled SAL-Soraml Trial. 2014 56th ASH Annual Meeting (Abstract).
6. Serve H, Krug U, Wagner R, et al. Sorafenib in combination with intensive chemotherapy in elderly patients with acute myeloid leukemia: results from a randomized, placebo-controlled trial. J Clin Oncol. 2013;31(25):3110-3118.
7. de Lima M, Giralt S, Thall PF, et al. Maintenance therapy with low-dose azacitidine after allogeneic hematopoietic stem cell transplantation for recurrent acute myelogenous leukemia or myelodysplastic syndrome: a dose and schedule finding study. Cancer. 2010; 116(23):5420-5431.
8. Goodyear OC, Dennis M, Jilani NY, et al. Azacitidine augments expansion of regulatory T cells after allogeneic stem cell transplantation in patients with acute myeloid leukemia (AML). Blood. 2012; 119(14):3361-3369.
9. Ravandi F, Alattar ML, Grunwald MR, et al. Phase II study of azacytidine plus sorafenib in patients with acute myeloid leukemia and FLT-3 internal tandem duplication mutation. Blood. 2013; 121(23):4655-4662.
10. Man CH, Fung TK, Ho C, et al. Sorafenib treatment of FLT3-ITD(+) acute myeloid leukemia: favorable initial outcome and mechanisms of subsequent nonresponsiveness associated with the emergence of a D835 mutation. Blood. 2012;119(22):5133-5143.
11. Daver N, Strati P, Jabbour E, et al. FLT3 mutations in myelodysplastic syndrome and chronic myelomonocytic leukemia. Am J Hematol. 2013;88(1):56-59.
12. Leung AY, Man CH, Kwong YL. FLT3 inhibition: a moving and evolving target in acute myeloid leukaemia. Leukemia. 2013; 27(2):260-268.
13. Ravandi F, Cortes JE, Jones D, et al. Phase I/II study of combination therapy with sorafenib, idarubicin, and cytarabine in younger patients with acute myeloid leukemia. J Clin Oncol. 2010; 28(11):1856-1862.
14. Stone RM, Fischer T, Paquette R, et al. Phase IB study of the FLT3 kinase inhibitor midostaurin with chemotherapy in younger newly diagnosed adult patients with acute myeloid leukemia. Leukemia. 2012;26(9):2061-2068.