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Reactivating PP2A by FTY720 as a Novel Therapy for AML With C-KIT Tyrosine Kinase Domain Mutation

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ABSTRACT

The tyrosine kinase domain (TKD) mutations of receptor tyrosine kinase C-KIT are associated with a poor prognosis in acute myeloid leukemia (AML). However, the underlying mechanisms are not fully understood. We found the activity of protein phosphatase 2A (PP2A), a human tumor suppressor whose dysfunction contributes to malignant cell behavior, was significantly decreased in AML subgroups harboring C-KIT/D816V and AML cell line Kasumi-1 bearing C-KIT/N822K mutation. Primary AML cells and various AML cell lines were treated with PP2A activator FTY720. FTY720 showed a toxic effect in all leukemic cells, especially for cells harboring C-KIT/TKD mutation. Furthermore, FTY720-induced toxicity in AML leukemic cells was mediated by restoration of PP2A activity, via down-regulation of PP2A inhibitor SET, dephosporylation of PP2A-C^{TYR307}, and up-regulation of relevant PP2A subunit A and B55α. Our research indicates that the decreased PP2A activity in AML harboring C-KIT/TKD mutation may make the restoration of PP2A activity a novel therapy for AML patients with C-KIT/TKD mutation. J. Cell. Biochem. 113: 1314–1322, 2012. © 2011 Wiley Periodicals, Inc.

KEY WORDS: PP2A; C-KIT MUTATION; FTY720; KASUMI-1 CELL; ACUTE MYELOID LEUKEMIA

Although significant progress has been made in the treatment of acute myeloid leukemia (AML), most patients still can not achieve complete remission (CR), and about 40–50% of the patients who have reached CR eventually relapse and ultimately die from their disease [de la Rubia et al., 2002]. Recent studies have highlighted the importance of gene mutations in the relapse or resistance of AML, such as C-KIT mutation that frequently occurs in core-binding factor AML [Muller-Tidow et al., 2004; Wang et al., 2005; Boissel et al., 2006; Renneville et al., 2008; Shih et al., 2008]. C-KIT is a type 3 receptor tyrosine kinase, and gain-of-function mutations of the receptor cause constitutive, ligand-independent activation of the receptor and induce myeloid malignancies [Corbacioglu et al., 2006; Roberts et al., 2007; Xiang et al., 2007; Sritana and Auewarakul, 2008; Pedersen et al., 2009]. The most frequently occurring C-KIT mutation in AML is D816V mutation in its tyrosine kinase domain (TKD). In most cases, the presence of C-KIT with TKD mutations (C-KIT/TKD) in AML is associated with higher relapse rate and reduced survival compared with patients bearing the wild-type C-KIT (C-KIT/WT) receptor [Corbacioglu et al., 2006; Schnittger et al., 2006; Potenza et al., 2007; Advani et al., 2008; Li et al., 2008; Renneville et al., 2008; Malaise et al., 2009; Sun et al., 2009; Ustun et al., 2009; Luck et al., 2010; Roberts et al., 2010].

Protein phosphatase 2A (PP2A) is a ubiquitously expressed serine/threonine phosphatase which acts as a tumor suppressor and plays a crucial role in the regulation of cell cycle progression, survival, and differentiation [Silverstein et al., 2002; Epie et al., 2006; Guenin et al., 2008; Kolupaeva et al., 2008; Lu et al., 2009]. It is a heterotrimer consisting of a catalytic C subunit, a structural A subunit and one of at least 21 different regulatory B subunits. Recently it has been shown that functional loss of PP2A activity is important for some myeloid malignancies such as blast-crisis CML and AML [Chatfield and Eastman, 2004; Neviani et al., 2005; Eichhorn et al., 2009; Gallay et al., 2009; Cristobal et al., 2011]. Inhibition of PP2A activity can occur at different levels, either within the structure of the heterotrimeric enzyme itself, such as loss of expression or phosphorylation-induced inhibition of one or more subunits, or through the activity of external effectors, such as SET, a PP2A inhibitor.

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The TKD mutation of C-KIT indicates a poor prognosis in AML, and the functional loss of PP2A activity suggest a refractory clinical course or develop resistance to normal drug therapy in some myeloid malignancies. All these urged us to suppose whether there is an association between the TKD mutation of C-KIT and loss of PP2A activity in AML. In this research, we will compare the PP2A activity between AML cells with C-KIT/TKD and C-KIT/WT both in vivo and in vitro to reveal their association.

In addition, many drugs have shown success in treating AML patients without C-KIT/TKD. However, patients with C-KIT/TKD frequently occur resistance to these drugs [Corless et al., 2006; Kosmider et al., 2007; Piccaluga et al., 2007; Verstovsek et al., 2008; Chevallier et al., 2009; Ustun et al., 2009]. Herein we show that FTY720 can induce toxic effects in both primary leukemic cells from AML patients and the AML cell lines harboring C-KIT/TKD or C-KIT/WT. Interestingly, the cells harboring C-KIT/TKD are more sensitive to FTY720. Furthermore, FTY720-induced toxicity in AML leukemic cells may be mediated by the restoration of PP2A activity via down-regulation of SET, dephosphorylation of PP2A-C^{TYR307}, the phosphorylated form of PP2A-C which induces PP2A inactivation, and up-regulation of relevant PP2A subunit A and B55α which has been reported to be deregulated or associated with poor prognosis in human myeloid leukemia [Neviani et al., 2005; Cristobal et al., 2011; Ruvolo et al., 2011]. Here we present data supporting the investigation of FTY720 as a novel therapeutic approach for patients with refractory AML harboring C-KIT/TKD.

MATERIALS AND METHODS

CHEMICAL REAGENTS

FTY720 (Cayman, Ann Arbor) was dissolved in DMSO (Sigma, St. Louis) at 50 mM stock. Okadaic acid (OA; Sigma) was dissolved in DMSO at 1 mM stock. Both of them were used at indicated concentrations.

PATIENTS AND CELLS

Forty patients were enrolled in this study, including 8 AML with D816V-mutated C-KIT (C-KIT/D816V), 12 AML with C-KIT/WT, and 20 normal controls. The diagnosis was based on clinical data and examination of peripheral blood and bone marrow according to French–American–British classification. Blood was obtained from patients at Center for Stem Cell Research and Application and Department of Haematology, Union Hospital, Wuhan, China, after obtaining their informed consent. Leukemic cells were isolated from patients' blood samples using ficoll density gradient centrifugation (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). After separation, all samples contained at least 80% leukemic blast cells. These leukemic cells were used in the following PP2A activity assay experiment or cultivated in vitro.

For cell culture, isolated primary leukemic cells were incubated in RPMI 1640 media (Gibco, Carlsbad) supplemented with 30% heat-inactivated fetal bovine serum (FBS; Multicell, Woonsocket), 2 mM L-glutamine (Invitrogen, Carlsbad), and penicillin (100 U/ml)/streptomycin (100 μg/ml) (Sigma) at 37°C in an atmosphere of 5% CO_2. Freshly isolated leukemic cells were used in all experiments described herein. Human AML cell lines Kasumi-1 and HL-60 were obtained from American Type Culture Collection (ATCC, Manassas), and cells were grown in RPMI 1640 medium supplemented with 10% (for HL60) and 20% (for Kasumi-1) FBS, respectively. Cells were cultured with FTY720 and/or OA of various concentrations for indicated periods. The same volume of DMSO was used for the control culture and for dissolving reagents.

DETECTION OF WIDE-TYPE C-KIT AND C-KIT D816V MUTATION

Quantitative real-time PCR was performed on the Applied Biosystems 7500 FAST real-time PCR System using the following primers: C-KIT/D816V forward primer: ttgtgattttggtctagccagact, reverse primer: gtgccatccacttcacaggtag. C-KIT/WT forward primer: ttgtgattttggtctagccagaga; reverse primer: gtgccatccacttcacaggtag, as previously described [Lawley et al., 2005].

ASSESSMENT OF CELL VIABILITY

To determine the viability of AML cells, 10^4 AML cells were plated in 96-well plates in triplicate. Cells were treated with FTY720 (2.5, 5, 10, 20, and 40 μM) for 24 h. Then 10 μl of CCK-8 solution (Beyotime, Shanghai, China) was added to each well and the plate was incubated for 1 h. Next, the absorbance for each sample was collected at 450 nm (reference filter 650 nm) and viability for treated cells was compared to that of untreated control cells.

ANALYSIS OF CELL APOPTOSIS

Annexin V-FITC apoptosis detection Kit (Keygen, Nanjing, China) was used for apoptosis assay. According to the protocol, collected cells were washed with cold PBS twice and suspended in 500 μl binding buffer. Then 5 μl of Annexin V-FITC and 5 μl of propidium iodide (PI) were added and mixed. Suspending mixture was incubated in room temperature without light for 15 min before flow cytometry. The percentage of apoptotic cells was calculated as the sum of those stained with Annexin V alone and stained with both Annexin V and PI. All the experiments were repeated at least three times.

PP2A ASSAYS

PP2A assays were carried out using a PP2A immunoprecipitation phosphatase assay kit (Millipore, Bedford). Briefly, protein lysates (2×10^6 cells) in 50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1% NP-40 were incubated for 2 h at 4°C with 4 μg of PP2A-C antibody (clone 1D6) and protein A-agarose. After three washes, immunoprecipitates were used in a phosphatase reaction according to the manufacturer's instructions. As an internal control, the specificity of the reaction was assessed by inhibiting PP2A activity with 1 nM OA before titration. The amount of immunoprecipitated PP2A was also assessed using anti-PP2A-C Western blots.

WESTERN BLOTTING

Cells (4×10^6) were lysed in 400 μl of RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.1% SDS, 50 mM Tris (pH 8.0)) supplemented with protease and phosphatase inhibitors (1 mM PMSF, 25 μg/ml aprotinin, 10 μg/ml leupeptin, 100 μg/ml pepstatin A, 5 mM benzamidine, 1 mM Na_3VO_4, 50 mM NaF, 10 mM β-glycerol-phosphate). After incubation on ice for 30 min, lysates were clarified (12,000*g* for 15 min at 4°C) and quantified by the

bicinchoninic acid (BCA) method (Pierce, Rockford). Lysates with 50 μg of total protein were separated using 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and transferred to 0.2 μm nitrocellulose membranes (Beyotime). The blots were probed with indicated primary antibodies followed by horseradish peroxidase (HRP)-conjugated goat anti-rabbit or goat anti-mouse IgG (Beyotime). Rabbit anti-PP2A-C and anti-SET polyclonal antibody was purchased from Proteintech (Chicago). Rabbit anti-PP2A-C^{TYR307} monoclonal antibody, anti-PP2A-A, and anti-PP2A-B55α polyclonal antibody were generous gifts from Dr. Xiao (Huazhong University of Science and Technology, Wuhan, China). Mouse anti-β-actin polyclonal antibody was purchased from Beyotime.

STATISTICAL ANALYSIS

Statistical analysis was done by *t*-test to examine the effects of FTY720 and OA as well as the statistical significance of PP2A activity between different sample groups using SPSS 16.0. *P*-value <0.05 was considered statistically significant.

RESULTS

INVESTIGATION OF THE INCIDENCE OF C-KIT/D816V MUTATION IN AML PATIENTS IN CHINA

Two hundred eighty-six AML samples were collected in our research, and quantitative real-time PCR was used to examine C-KIT/D816V mutation.

Of the 286 AML samples studied, 8 were shown to have a point mutation at D816V, including 6 male cases and 2 female cases. This mutation was typically located within the M2 (five cases) and M4 (one cases) subclasses, with the other two cases not definitely diagnosed. In the present work, the D816V mutation occurred in 6.8% of M2, 4.3% of M4 subclasses, and the overall incidence of C-KIT/D816V was 2.7% in AML. The result supports that C-KIT/D816V mutation frequently occurs in AML patients in China (Table I).

STATUS OF PP2A ACTIVITY IN AML PATIENTS HARBORING C-KIT/D816V AND AML CELLS IN VITRO

We examined the PP2A activity in 8 C-KIT/D816V AML patients, 12 C-KIT/WT AML patients, and 20 healthy controls with PP2A immunoprecipitation phosphatase assay kit. All tested samples expressed comparable level of total PP2A as determined by immunoprecipitation. However, normal controls displayed highest PP2A activity (706.02 ± 154.22 pmol of released phosphate in 15 min, $n = 20$). This was reduced to 565.69 ± 122.56 pmol in C-KIT/WT samples ($n = 12$) and 419.01 ± 96.97 pmol in C-KIT/D816V samples ($n = 8$), respectively (Fig. 1A). Similarly, the primary AML cells harboring C-KIT/D816V cultivated in vitro and Kasumi-1, an AML cell line harboring C-KIT/N822K mutation derived from a M2 patient also showed lower PP2A activity than those harboring C-KIT/WT and HL60 bearing C-KIT/WT (Fig. 1B,C). Therefore, our data suggest that C-KIT mutations may play a role in inhibiting PP2A activity in AML cells.

TABLE I. Patient Characteristics

	Total	C-KIT/WT	C-KIT/D816V
Total, no. (%)	286	278 (97.2)	8 (2.8)
Sex			
Male, no. (%)	154 (53.8)	148 (96.1)	6 (3.9)
Female, no. (%)	132 (46.2)	130 (98.5)	2 (1.5)
Median age (range), years	40 (6–80)	41 (6–80)	47 (29–56)
FAB subtype, no. (%)			
M2, no. (%)	74	69 (93.2)	5 (6.8)
M4, no. (%)	23	22 (95.7)	1 (4.3)
Other, no. (%)	34	34 (100)	0 (0)
Unknown, no. (%)	155	153 (98.7)	2 (1.3)

FAB, French–America–British classification.

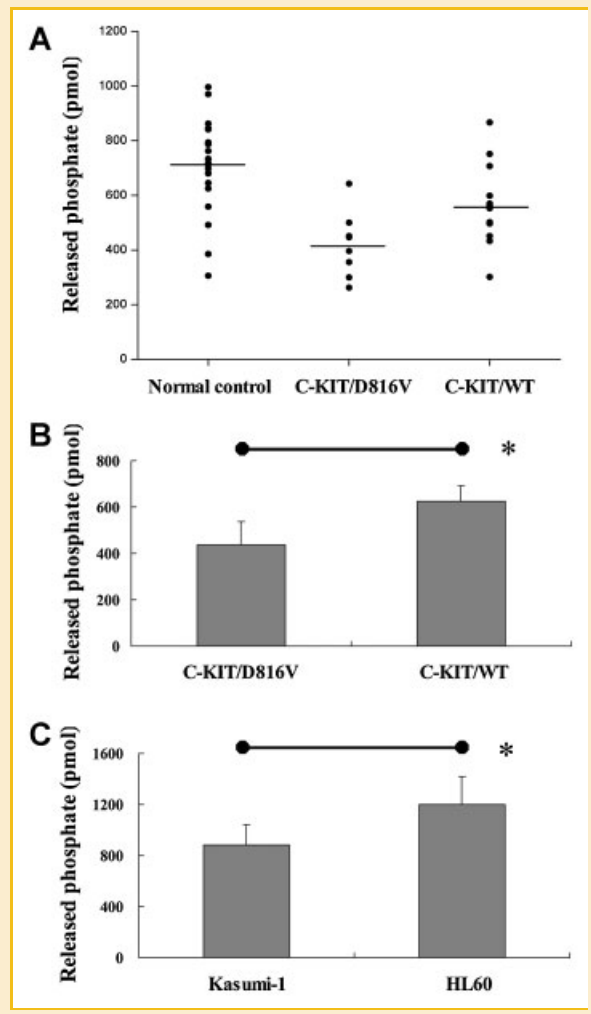

Fig. 1. PP2A activity status in AML patients and AML cells, results are expressed as pmol of released phosphate in 15 min. A: Status of PP2A in C-KIT/D816V AML, C-KIT/WT AML patients, and normal controls. Normal control group, $n = 20$; C-KIT/D816V group, $n = 8$; C-KIT/WT group, $n = 12$. The median values are depicted by the horizontal bars. B: PP2A assay on primary leukemic cells from C-KIT/D816V AML or C-KIT/WT AML. Data represent the mean ± SE of all samples in the group. Each group, $n = 3$. C: PP2A assay on Kasumi-1 and HL-60 cell lines. Data represent the means ± SE of four independent experiments. Bars above the graph refer to differences between groups. $^{*}P < 0.05$.

EXPRESSION OF SET, PP2A-C^{TYR307} AND PP2A-A, B55α, AND C IN AML PATIENTS HARBORING C-KIT/D816V AND AML CELL LINES IN VITRO

Deregulation of SET and phosphorylation of PP2A-C on TYR307 have been reported to inhibit PP2A activity [Chen et al., 1992; Li et al., 1996]. In addition, the deregulated expression of PP2A subunits is associated with poor prognosis of leukemia [Neviani et al., 2005; Ruvolo et al., 2011]. To evaluate the potential factors reducing PP2A activity in AML, we compared the expression of SET, PP2A-C^{TYR307}, PP2A-A, B55α, and C subunits in AML cells harboring C-KIT/TKD mutation or C-KIT/WT. As shown in Figure 2, the expression levels of PP2A-A and PP2A-B55α were significantly lower in C-KIT/TKD AML cells. By contrast, SET and PP2A-C^{TYR307} expression was markedly increased in these samples.

FTY720 PREFERENTIALLY SUPPRESSES THE GROWTH OF AML CELLS HARBORING C-KIT/TKD

To investigate the potential relationship between PP2A and C-KIT, PP2A activator FTY720 was used to treat the AML cells bearing C-KIT/TKD or C-KIT/WT.

Kasumi-1 and HL60 cell lines were incubated with different concentrations of FTY720 (2.5, 5, 10, 20, and 40 μM) for 24 h. A dose-dependent growth inhibition effect of FTY720 was observed in both Kasumi-1 and HL60 cells (Fig. 3A and Table II). Nevertheless, the IC_{50} of FTY720 in Kasumi-1 (13.5 μM) was significantly lower than that in HL60 (27.3 μM).

We also treated primary leukemic cells with various concentration of FTY720 (2.5, 5, 10, 20, and 40 μM) for 24 h. As shown in Figure 3B and Table II, leukemic cells with C-KIT/D816V mutation were more sensitive to FTY720.

FTY720 INDUCES APOPTOSIS IN AML CELLS

To further investigate the nature of FTY720-induced growth inhibition, we examined the apoptosis profile in AML cells treated with FTY720. Kasumi-1 and HL60 was incubated with FTY720 (10 μM) for 36 h. This treatment induced the occurrence of significant apoptosis, as determined by Annexin V and PI staining (Fig. 4A). Incubation of primary C-KIT/D816V and C-KIT/WT AML cells with 10 μM FTY720 also resulted in a decrease in viable cells (Fig. 4B; $P < 0.05$, untreated vs. 10 μM treated). Consistent with growth inhibition data, more AML cells harboring C-KIT/TKD underwent apoptosis after FTY720 treatment when compared with the AML cells carrying C-KIT/WT (Fig. 4C).

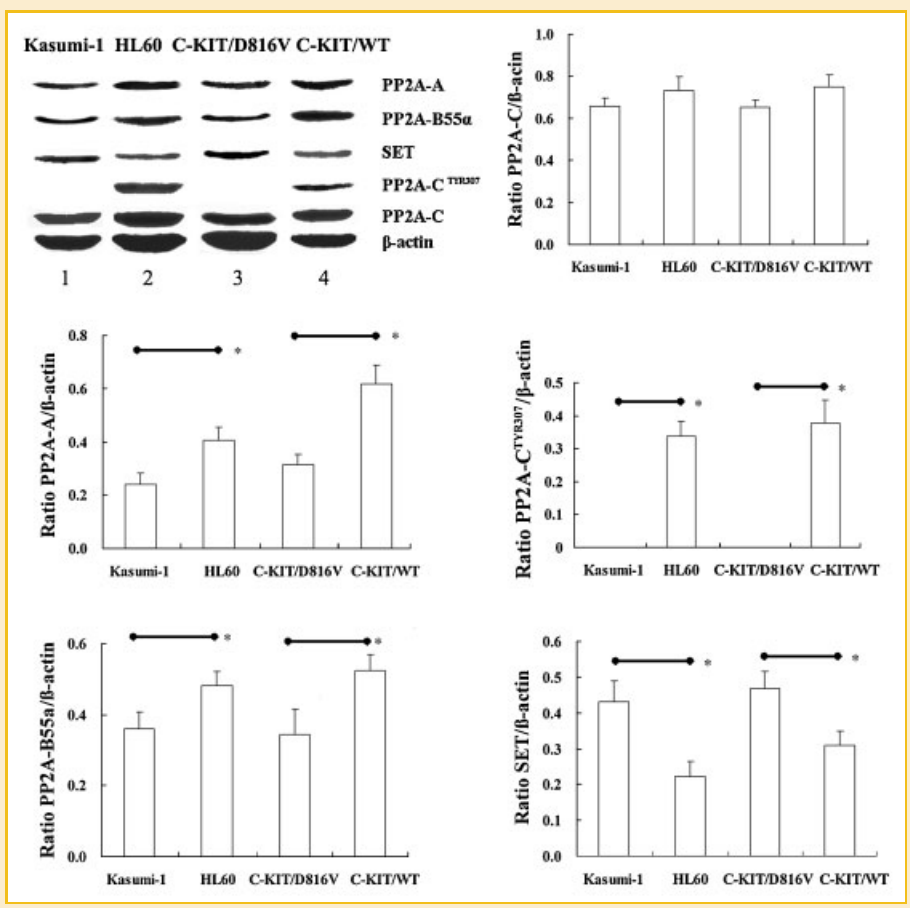

Fig. 2. PP2A-A, B55α, C subunit, PP2A-C^{TYR307}, and SET expression in AML patients harboring C-KIT/D816V and C-KIT/WT, Kasumi-1, and HL60, including a densitometric analysis of each protein. β-Actin was used as a loading control. Blots are a representative of three independent experiments. Bars above the graph refer to differences between groups. $^{*}P < 0.05$.

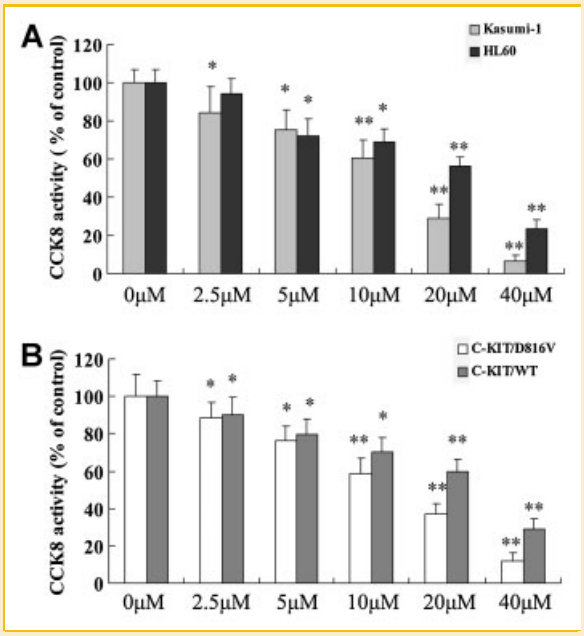

Fig. 3. Effects of FTY720 on proliferation of AML cells. A: FTY720 suppressed growth of Kasumi-1 and HL60 cells in a dose-dependent manner and it more effectively suppressed the growth of Kasumi-1 than HL60. Data represent the means ± SE of three independent experiments. B: FTY720 suppressed growth of primary leukemic cells from C-KIT/D816V AML patient more efficiently than that from C-KIT/WT patients. Cells were cultured for 24 h with indicated concentrations, n = 3. Asterisks above columns indicate that significance was calculated with respect to normal controls. $^{*}P < 0.05$ and $^{**}P < 0.01$.

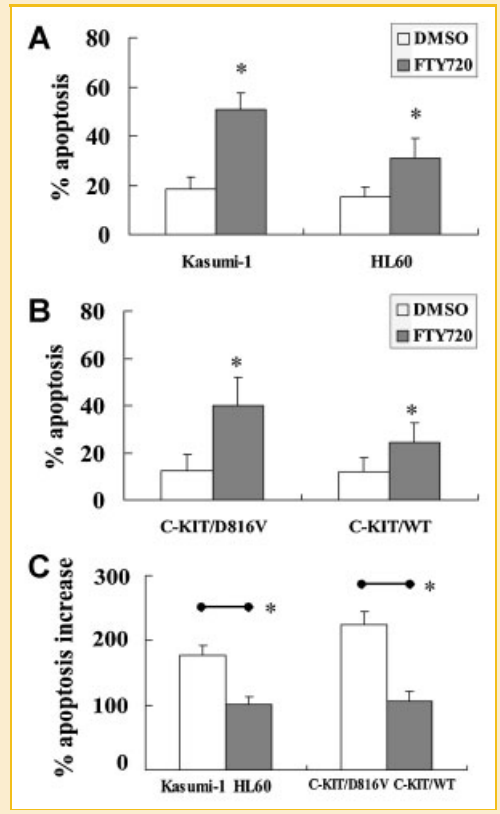

Fig. 4. Effects of FTY720 on apoptosis of AML cells. Cells were incubated for 36 h with 10 μM FTY720. FTY720 (gray columns) greatly increased the Annexin V (+) fraction of Kasumi-1 and HL60 cells (A) and primary leukemic cells from C-KIT/D816V and C-KIT/WT AML patients (B). Apoptosis was measured by the Annexin V test and the % of apoptotic cells is reported. C: Apoptosis increases with respect to the % of apoptotic cells in the control are reported. Data represent the means ± SE of independent experiments. Primary cells and cell lines, n = 3. Asterisks above columns indicate that significance was calculated with respect to untreated cells; bars above the graph refer to differences between indicated groups. $^{*}P < 0.05$.

FTY720-MEDIATED TOXICITY IS DEPENDENT ON THE INCREASED PP2A ACTIVITY IN AML CELLS

We examined whether FTY720 could increase PP2A activity in AML cells. Kasumi-1, HL60, and primary leukemic cells were treated with 5 μM, 10 μM FTY720 for 5 h, and PP2A activity was assayed as described above. Consistent with previous studies [Liu et al., 2008], FTY720 induced an increase in PP2A activity (Fig. 5A,B). Of note, a more significant up-regulation of PP2A activity was observed in C-KIT/TKD AML cells after FTY720 treatment (Fig. 5D). We went further to show that FTY720-induced PP2A activation was inhibited by specific PP2A inhibitor OA when used at nM concentration (Fig. 5C). To examine the contribution role of PP2A activation to FTY720-mediated cellular toxicity, we pretreated the cells with 5 nM OA for 2 h. This treatment partially rescued the cells from FTY720-induced apoptosis (Fig. 5E), which indicates PP2A activation is required for FTY720-induced apoptosis in AML cells.

Next, we detected the expression level of PP2A subunits and SET after FTY720 treatment. As shown in Figure 6, FTY720 did not enhance the expression of PP2A-C. However, it significantly increased PP2A-A and PP2A-B55α expression and decreased SET expression. Furthermore, PP2A-C^{TYR307} was down-regulated in C-KIT/WT AML cells after FTY720 treatment, suggesting FTY720 may modulate the phosphorylation of catalytic components to regulate PP2A activity. However, in Kasumi-1 and primary leukemic cells harboring C-KIT/D816V, PP2A-C^{TYR307} was barely detected even before FTY720 exposure.

TABLE II. Comparison of IC_{50} (24 h) Values of FTY720 Derived From the Growth Inhibition Curve

	IC_{50} (μM)	*P*-value
Kasumi-1	13.5	<0.05
HL60	27.3	
Primary C-KIT/D816V cells	16.9	<0.05
Primary C-KIT/WT cells	24.5	

The IC_{50} of FTY720 in Kasumi-1 (13.5 μM) and primary C-KIT/D816V cells(16.9 μM) was significantly lower than that in HL60 (27.3 μM) and primary C-KIT/WT cells(24.5 μM) respectively. $P < 0.05$.
[a]IC_{50} values are the means of three independent experiments.

DISCUSSION

The efficacy of current therapy for refractory AML is far from satisfactory because of the existence of some clinical problems, such

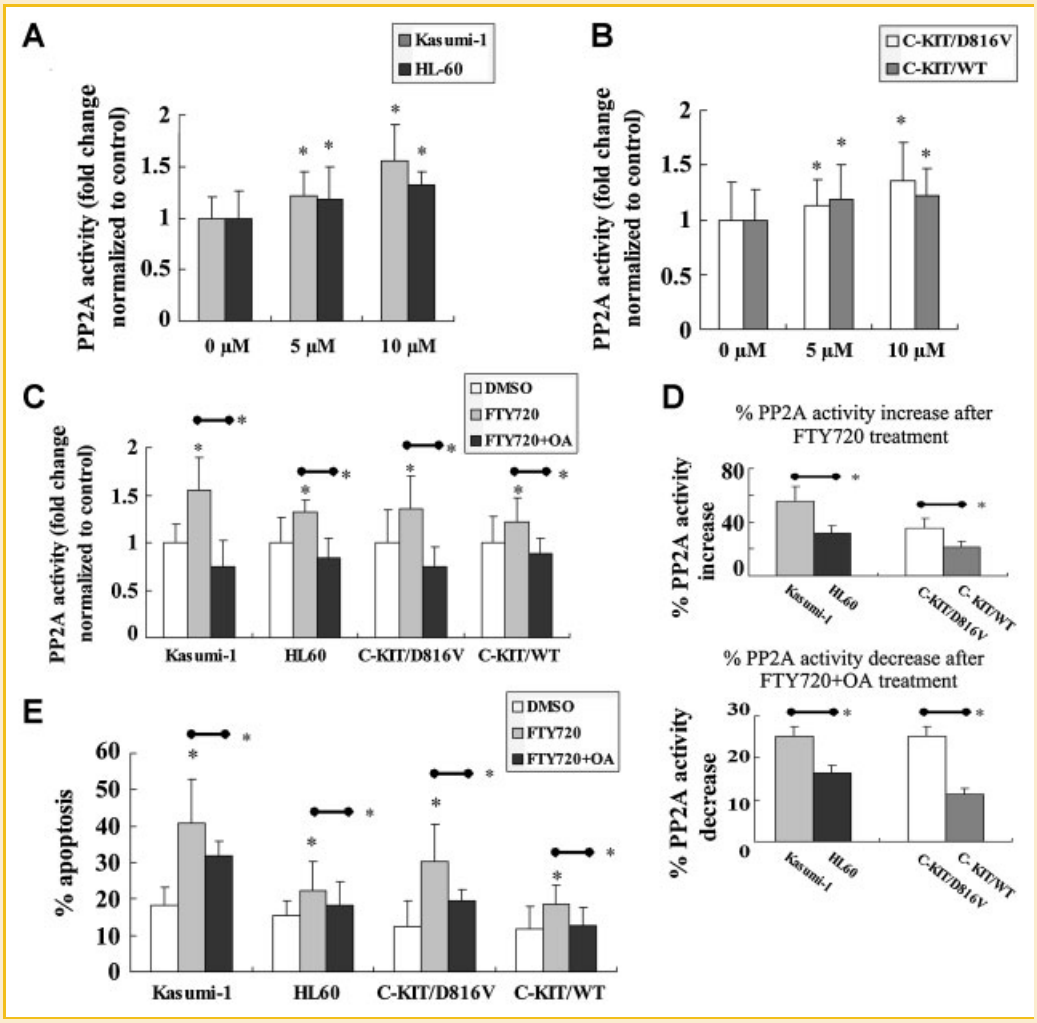

Fig. 5. FTY720-induced toxicity in AML cell lines and primary leukemic cells is dependent on the activation of PP2A. A: FTY720 increased PP2A activity in Kasumi-1 and HL60. B: FTY720 increased PP2A activity in primary leukemic cells from C-KIT/D816V and C-KIT/WT AML. Cells were incubated with DMSO, 5 μM, 10 μM FTY720 for 5 h. C: The increased PP2A activity induced by FTY720 in Kasumi-1, HL60 cell line, and primary leukemic cells from C-KIT/D816V and C-KIT/WT AML was inhibited by OA. Cells were pretreated with DMSO or 5 nM OA for 2 h, followed by incubation with DMSO or 10 μM FTY720 for 5 h. D: PP2A activity increase after FTY720 treatment or decrease after FTY720 + OA treatment with respect to the % of PP2A activity in the control are reported. E: FTY720-induced cellular toxicity was partially rescued by OA in Kasumi-1, HL60, and primary leukemic cells from C-KIT/D816V and C-KIT/WT AML. Cells were pretreated with DMSO or 5 nM OA for 2 h, followed by incubation with DMSO or 10 μM FTY720 for 5 h. The apoptosis was analyzed by flow cytometry and measured by the Annexin V test and the % of apoptotic cells was reported. Data represent the means ± SE of independent experiments. Primary cells and cell lines, n = 3. Asterisks above columns indicate that significance was calculated with respect to untreated cells; bars above the graph refer to differences between indicated groups. $^{*}P < 0.05$.

as resistance to conventional chemotherapeutic drugs and poor survival [Rowe, 2009]. Many reports have demonstrated that activating mutations in C-KIT/TKD are associated with such problems. The suppression of PP2A activity appears to be a common event in a number of human neoplasms including chronic myeloid leukemia (CML), gliomas, and lung cancer. Cristobal et al. [2011] reported that in AML PP2A activity is also impaired. However, little is known about the PP2A activity status in AML subgroups harboring C-KIT/TKD.

In this study, we identify 8 C-KIT/D816V mutations out of 286 Chinese AML patients (2.7%). This mutation is preferentially found in M2 and M4 patient, with the incidence of 6.8% and 4.3%, respectively. The frequencies of this mutation seem to be higher in our study when compared with previous reports [Roskoski, 2005; Zaker et al., 2010]. This discrepancy may due to difference of ethnicity and sample size. On the other hand, our data also suggest this mutation is more inclined to occur in male (3.9%) than female (1.5%), consistent with the previous studies [Luck et al., 2010].

PP2A can be inactivated by constitutively active tyrosine kinase in vitro [Neviani et al., 2005; Roberts et al., 2010]. We showed that PP2A activity was modestly decreased in AML patients harboring C-KIT/WT. However, it was dramatically impaired in patients carrying C-KIT/TKD. These data raise the possibility that PP2A is a target for C-KIT/TKD and implicated the potential efficacy of therapies targeting PP2A in such AML in future.

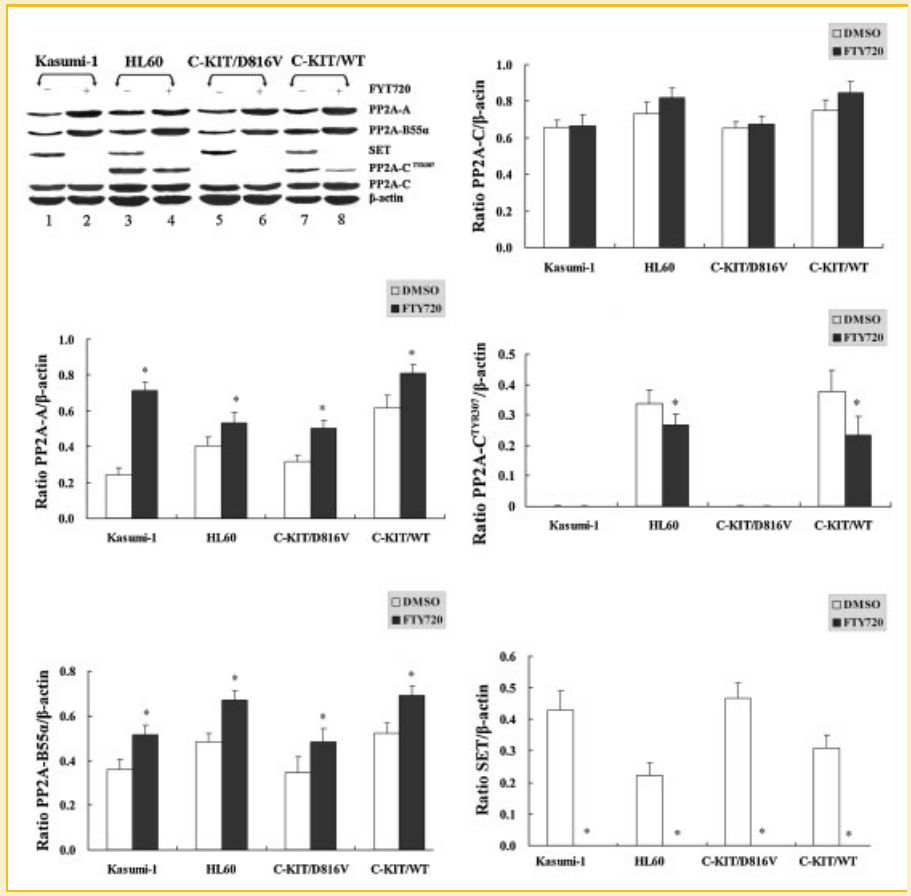

Fig. 6. The effect of FTY720 on the expression of PP2A-A, B55α, C subunit, PP2A-C^{TYR307}, and SET in AML patients harboring C-KIT/D816V and C-KIT/WT, Kasumi-1, and HL60, including a densitometric analysis of each protein. β-Actin was used as a loading control. Blots are a representative of three independent experiments. Asterisks above columns indicate that significance was calculated with respect to normal controls. $^{*}P < 0.05$.

The precise mechanism for impaired PP2A activity in C-KIT/TKD AML remains unclear. It is believed that the aberrant expression of PP2A subunits and PP2A inhibitor may have important roles. In our study, we found the expression of SET was significantly higher in cells harboring C-KIT/TKD mutation, which could partially explain the decreased PP2A activity in C-KIT/TKD AML cells. Interestingly, Neviani et al. [2005] demonstrated that PP2A was inactivated by the oncogenic tyrosine kinase BCR/ABL through the up-regulation of SET in CML. Since BCR/ABL and C-KIT signaling pathway share some tyrosine phosphorylation targets [Wisniewski et al., 1996], it would be interesting to speculate that PP2A is inactivated by TKD-mutated C-KIT kinase through the up-regulation of SET in AML. However, whether SET is directly regulated by TKD-mutated C-KIT kinase and how C-KIT regulates SET remain to be elucidated. Our data also showed that the reduced expression of PP2A-A, B55α, and C subunit (Fig. 2) may contribute to the decreased PP2A activity as previously described [Eichhorn et al., 2009]. However, PP2A-C^{TYR307} in C-KIT/TKD cells was not detected, that may be due to its initial low expression level in those cells.

Many drugs can efficiently eliminate AML cells with C-KIT/WT but not those harboring C-KIT/TKD [Piccaluga et al., 2007; Verstovsek et al., 2008]. Therefore, developing novel therapies is in urgent need to treat C-KIT/TKD AML. FTY720, currently used as an immunomodulator, has remarkable therapeutic efficacy for patients with imatinib-sensitive and -resistant advanced CML [Neviani et al., 2007; Payne et al., 2007; Liu et al., 2008]. Herein we showed that FTY720 could induce apoptosis in AML cells via increasing PP2A activity. This cytotoxicity was rescued by the pretreatment of OA, a specific inhibitor of PP2A, supporting that FTY720-mediated cytotoxicity was via, at least partially, the activation of PP2A. Furthermore, we showed FTY720 treatment could increase the expression of PP2A-A and B55α subunits and decrease that of PP2A inhibitor SET. Thus, expression changes of these PP2A components induced by FTY720 may contribute to the reactivation of PP2A in AML cells. Of note, while FTY720 did not change PP2A-C expression, it decreased PP2A-C^{TYR307} in AML cells with C-KIT/WT. Dephosphorylation of PP2A-C^{TYR307} may also contribute to PP2A reactivation. However, PP2A-C^{TYR307} was barely detected in Kasumi-1 and primary leukemic cells harboring C-KIT/D816V even before FTY720 exposure, suggesting it may not be the major target for FTY720 to activate PP2A in AML cells carrying C-KIT/TKD (Fig. 6).

Interestingly, the cells harboring C-KIT/TKD are more sensitive to FTY720. This can be explained by different initial PP2A activity in

these cells. Our data suggest that the proliferation inhibition and apoptotic effects of FTY720 are strictly dependent on the activating status of PP2A. In our experiment, AML cells harboring C-KIT/TKD were more sensitive to 10 μM concentrations of FTY720 because they had relatively low PP2A activity (Fig. 1B,C). Our results suggest that the mechanism of FTY720-induced PP2A activation in AML cells may include down-regulation of endogenous PP2A inhibitor SET and dephosporylation of the PP2A-C subunit. The activation of PP2A, together with up-regulation of PP2A-A and B55α subunits jointly contribute to the increased PP2A activity, suggesting that dysfunction of several distinct PP2A complexes contributes to cell transformation.

The studies described herein demonstrate that in C-KIT/TKD AML, PP2A activity is notably decreased, and FTY720 is a potent toxic agent for this C-KIT/TKD AML subgroup. The biologic effect of FTY720 on leukemic cells can be explained by reactivating PP2A, distinguishing it from other therapeutics currently used in this disease. It is encouraging that the toxic effects of FTY720 appear to be most marked in C-KIT/TKD AML patient group where conventional therapeutic approaches are most likely to fail. Altogether, these results highlight the therapeutic effect of reactivating PP2A in C-KIT/TKD AML and strongly support the introduction of the PP2A activator FTY720 in the treatment of C-KIT/TKD refractory AML.

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