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Original article

Imatinib regulates the alternative pre-mRNA splicing of Bcl-x in K562 cells

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Background: The alternative splicing of Bcl-x generates the proapoptotic Bcl-xs protein and the antiapoptotic variant Bcl-xl. Previous studies have demonstrated that some chemotherapeutic agents such as emetine, staurosporine, and epigallocatechin gallate (EGCG) in combination with ibuprofen significantly altered the ratio of the Bcl-x variants Bcl-xs/Bcl-xl in various cell lines, suggesting Bcl-x splicing might be affected by the exogenous stimuli.

Objective: We investigated the regulative role of imatinib in the alternative pre-mRNA splicing of Bcl-x in K562 cells and the related mechanism.

Methods: Cell proliferation was measured using WST assay kit. Cell apoptosis was assayed using an Annexin V–FITC Apoptosis Detection Kit. RT-PCR and western blot assay was used to analyze the mRNA and protein level of alternative splicing of exon 2 in the Bcl-x gene respectively.

Results: Imatinib regulated the alternative splicing in the Bcl-x gene in the K562 cells. In addition, we found that hydroxyurea, another agent for the therapy of CML, could enhance the effect of imatinib on the ratio of the Bcl-xl/Bcl-xs. Moreover, the induction of alternative splicing was correlated with protein phosphatase 1 (PP1). Alternatively, pretreatment with calyculin efficiently blocked imatinib-induced alternative splcing in the K562 cells compared with okadaic acid, which showed an important role of PP1 in regulating imatinib-induced splicing.

Conclusion: Imatinib regulates the alternative splicing of Bcl-x in K562 cells, which may be associated with the activation of PP1.

Keywords: Alternative splcing**,** Bcl-x, chronic myelogenous leukemia**,** imatinib, protein phosphatase 1

It has been reported that over 90% of multi-exon pre-mRNA transcripts undergo alternative splcing [1]. And, the majority of the genes involved in apoptosis are alternatively spliced, in many cases alternative splicing produces variants which have opposite functions. Due to its significant contribution to proteome diversity, alternative splcing is becoming increasingly relevant to a variety of human diseases, including cancer [2], and provides a potential therapeutic target [3].

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To our knowledge, Bcl-x is a key regulator in cell apoptosis. Alternative splicing of Bcl-x significantly increased the codings of the two transcripts of Bcl-xl (a long form) and Bcl-xs (a short form) of the Bcl-x protein [4]. It was confirmed that Bcl-xl inhibited cell death, while Bcl-xs suppressed the anti-apoptotic effects of both Bcl-2 and Bcl-xl [4].

Previously, studies have found that 20 major anticancer drugs in clinic could influence the products of Bcl-x splicing variants, and they also could alter a subset of alternative splicing event in some cell lines, suggesting differences of various cell lines in regulating the splicing [5].

Imatinib was found to be a potent and selective inhibitor for abl tyrosine kinases, including bcr-abl [6], and it has been approved efficient in the treatment of

CML since 2002 [7]. After the treatment for five years, the rate of complete cytogenetic response among patients receiving imatinib was 87%, accompanying with an estimated 7% of patients in accelerated phase of CML or blast crisis [8]. *In vitro*, 1 μM imatinib treatment for 24 hours led to apoptosis induction and inhibited the activity of tyrosine kinase in CML cell line K562 [9]. However, mono-targeting of BCR-ABL does not always achieve complete leukemia eradication, and additional strategies enabling complete elimination of leukemic cells need to be developed. In China, because of the high cost of imatinib, hydroxyurea was still routinely used to treat CML in almost all phases, especially in chronic phase (CP) (94.1%; n = 514) [10]. Hydroxyurea is a specific inhibitor of DNA synthesis *in vivo*, and it is an antiviral and antineoplastic agent with S-phase specificity. In the treatmemnt of CML, hydroxyurea achieved a hematological not cytogenetic response [8, 11], So far, hydroxyurea is usually used for initial or palliative cytoreduction [7]. *In vitro*, simultaneous exposures to imatinib and hydroxyurea produced an additive effect in K562 cells [12].

In the present study, we hypothesized that imatinib treatment in K562 cells would regulate alternative mRNA splicing of Bcl-x. And we also investigated the effect of imatinib in combination with hydroxyurea on the splicing in the cells. Furthermore, the related mechanism for the splicing bahavior was also investigated.

Materials and methods

Main Reagents

Imatinib was purchased from Novartis Co. (Beijing, China). Hydroxyurea, 5, 6-dichloro-1-β -d-ribofuranosyl benzimidazole (DRB), calyculin A, and okadaic acid were purchased from Sigma Co. (Shanghai, China).

Culture and treatment of cells

K562 cell line was derived from CML patient, cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, USA). 2×10^5 K562 cells were inoculated in 35-mm^2 flasks. Drugs were prepared according to manufacturer's instructions. K562 cells were pretreated with 5 nM Calyculin A, or 10nM okadaic acid for 2 hours. DRB with a series of concentrations was added 1 hour before the addition of imatinib. Imatinib and hydroxyurea were added to reach various concentrations. An equal volume of DMSO was added to serve as a control.

WST assay of cell proliferation assay

4-[3-(4-iodopheny)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzene disulfonate (WST, Roche, Indianapolis, IN) is a water soluble tetrazolium salt that is cleaved to formazan only in viable cells. The formazan dye formed is quantified using a microplate reader (Elx808IU Ultra Microplate Reader, Bio-Tek Instruments, Inc., Winooski, VT) and the number of viable cells in wells was directly counted. Cells were seeded at in 96-well plates (5000 cells/well) and treated with imatinib or hydroxyurea at the designated concentrations followed by WST assay according to the manufacturer's instructions (Roche, USA). The inhibition rate was calculated as following fomula: Cell viability (%) = (1-ODtest/ODcontrol) × 100%. Triplicate wells were set for each treatment.

Apoptosis assay

Cell apoptosis was assayed using an Annexin V–FITC Apoptosis Detection Kit I (BD Phar Mingen, CA, USA) in accordance with the manufacturer's instructions. In brief, after treatments cells (2×10^5) were washed twice with cold PBS (0.01 M, pH7.4) and resuspended in 100 μl of 2 × Binding Buffer followed by co-staining with annexin V and propidiumiodide (PI) for 15 minutes. The stained cells were detected and analyzed by a FACScan Flow Cytometer (Becton Dickinson, USA).

RT-PCR assay

Total RNA was extracted from the cultured cells using a Trizol reagent (TIANGEN, Beijing, China) according to the manufacture's instructions. Reverse transcription was performed with 1 μg of total RNA using a Reverse Transcription Kit (Takara Bio., Japan). After incubation for 15 minutes at 37°C, the reactions were terminated by heating at 85°C for 15 seconds. Expression analysis was done using an equal amount cDNA.

To analyze alternative splicing of exon 2 in the Bcl-x gene, the following primers were used for PCR amplification: Bcl-x, Forward: 5′-ATGGCAG CAGTAAAGCAAGCG-3′, Reverse: 5′-TCATT TCCGACTGAAGAGTGA-3′. These reactions were performed for 32 cycles. The annealing temperature was maintained at 55°C; the rest of the conditions included denaturation at 94°C for 30 minutes followed by extension at 72°C for one minute. The

PCR products containing ethidium bromide (EB) were assayed using 1.5% agarose gel electrophoresis. Images of the gels were analyzed by Quantity One 4.6.2 (Bio-Rad, USA) and the ratio of bcl-xs/bcl-xl was calculated.

Western blot assay

After cultured for 24 hours, cells were collected for protein extraction by lysing cells in RIPA buffer containing 50 mM Tris-HCl (pH8.0), 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, and 0.1% SDS supplemented with a protease inhibitor cocktail. Protein concentration was measured by Bradford method. An equal amount of protein was electrophoresed and transferred onto a nitrocellulose membrane by a mini-transblot apparatus (Bio-Rad, American). Briefly, the membranes were incubated overnight with rabbit polyclonal antibody against Bcl-xs/l (S-18, SC-634; Santa Cruz, Heidelberg, Germany; 1:200) and then were washed three times with PBS-0.2% Tween 20 (PBST). After that, the membranes were incubated with anti-rabbit IgG conjugated with horseradish peroxidase (1:3000) at room temperature for 60 minutes and washed again for three times at five minutes each in PBST. The images were visualized by ECL western blot reagent on Hyperfilm ECL.

Data presentation and statistical analysis

The data were expressed as means□standard deviation (SD). One-way analysis of variance (ANOVA) was used to compare the differences among groups by SPSS 11.0 software (SPSS inc. Chicago, USA). p <0.05 was considered statistically significant.

Results

Effect of imatinib or hydroxyurea on K562 cells viability

We first determined the anti-proliferation effect of imatinib or hydroxyurea alone on K562 cells. Cell proliferation inhibitory rate was analyzed by WST assay. It demonstrated that imatinib or hydroxyurea exposure induced the apoptosis of K562 cells in a time-dependent manner. In other experiments, a combined treatment of imatinib (1 μM) in combination with hydroxyurea (15 mM) significantly suppressed cells proliferation compared with imatinib or hydroxyurea treatment alone, suggesting an inhibitory effect of imatinib in combination with hydroxyurea on the proliferation of K562 cells as shown in **Figure 1**.

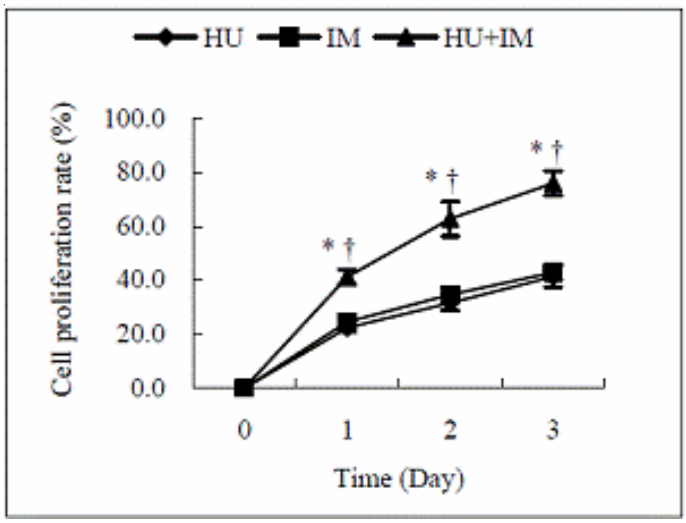

Figure 1. Effect of drugs on the proliferation of K562 cells ($\bar{x}$±s, n=3). WST assay was used to assess the cell proliferation according to the following formula: Cell proliferation inhibitory rate (%) = 1-OD_{test}/$OD_{control}$ × 100%. *p <0.01 vs HU; □p <0.01 vs IM. HU: Hydroxyurea (10 mmM), IM: Imatinib (1 μM).

Effect of imatinib or hydroxyurea on the apoptosis of K562 cells

To assess the effect of imatinib or hydroxyurea on apoptosis of K562 cells, we treated K562 with imatinib or hydroxyurea alone or imatinib combined with hydroxyurea and then analyzed cell apoptosis percentage. Similar to the anti-proliferative effects, the K562 cells rapidly underwent apoptosis in the presence of imatinib or hydroxyurea. Interestingly, the combined treatment significantly increased cell apoptosis by approximately 2-fold (**Figure 2**).

Imatinib regulates the alternative splicing of bcl-x pre-mRNA dose-dependently in K562 cells

For the importance of Bcl-2 in the regulation of cell apoptosis, we assayed whether imatinib could alter the ratio of the Bcl-xs/Bcl-xl splicing variants in K562 cells. After treated with 1 □M of imatinib for 24 hours, the cells were collected at a centrifuge of 1000 × *g* and harvest. Then the mRNA levels of endogenous Bcl-xl and Bcl-xs were determined by RT-PCR assay. As we have expected, Bcl-xl was a predominant form and it was found in untreated cells (**Figure 3A**). Similarly, imatinib significantly induced the protein expression of Bcl-xs and the Bcl-xl protein level was significantly decreased (**Figure 3C**).

To further validate our findings, we treated K562 cells overnight with various concentrations of imatinb. Meanwhile, dimethyl sulfoxide (DMSO) was selected as a control. The results revealed that the ratio of Bcl-xs/Bcl-xl was gradually increased after the treatment of 0.5-5 μM of imatinib (**Figure 3B**). The imatinib with highest concentration could increased the ratio by 4-fold, whereas DMSO slightly affected the ratio by less than 1-fold at its highest concentration.

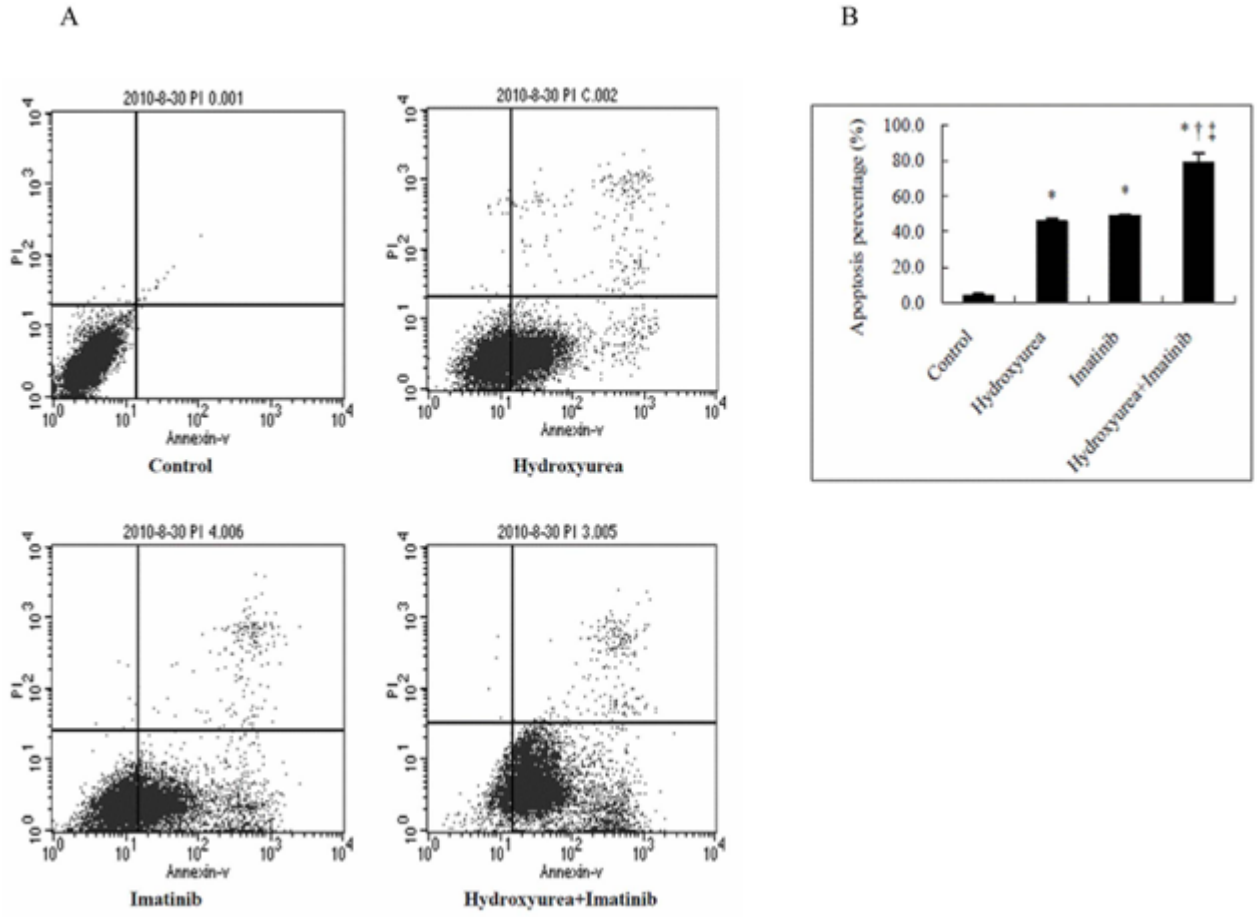

Figure 2. Effect of chemotherapeutic agents on the apoptosis of K562 cells ($\bar{x}$±s, n=3). K562 cells were treated with imatinib (1 μM) or hydroxyurea (10 mmM) for 24 hours. Thereafter, they were stained with both annexin V and propidium iodide. Subsequently, the cells apoptosis percentage was analyzed by double fluorescence assay. $^{*}p$ <0.01 vs Control; □p <0.01 vs Hydroxyurea; □p <0.01 vs Imatinib.

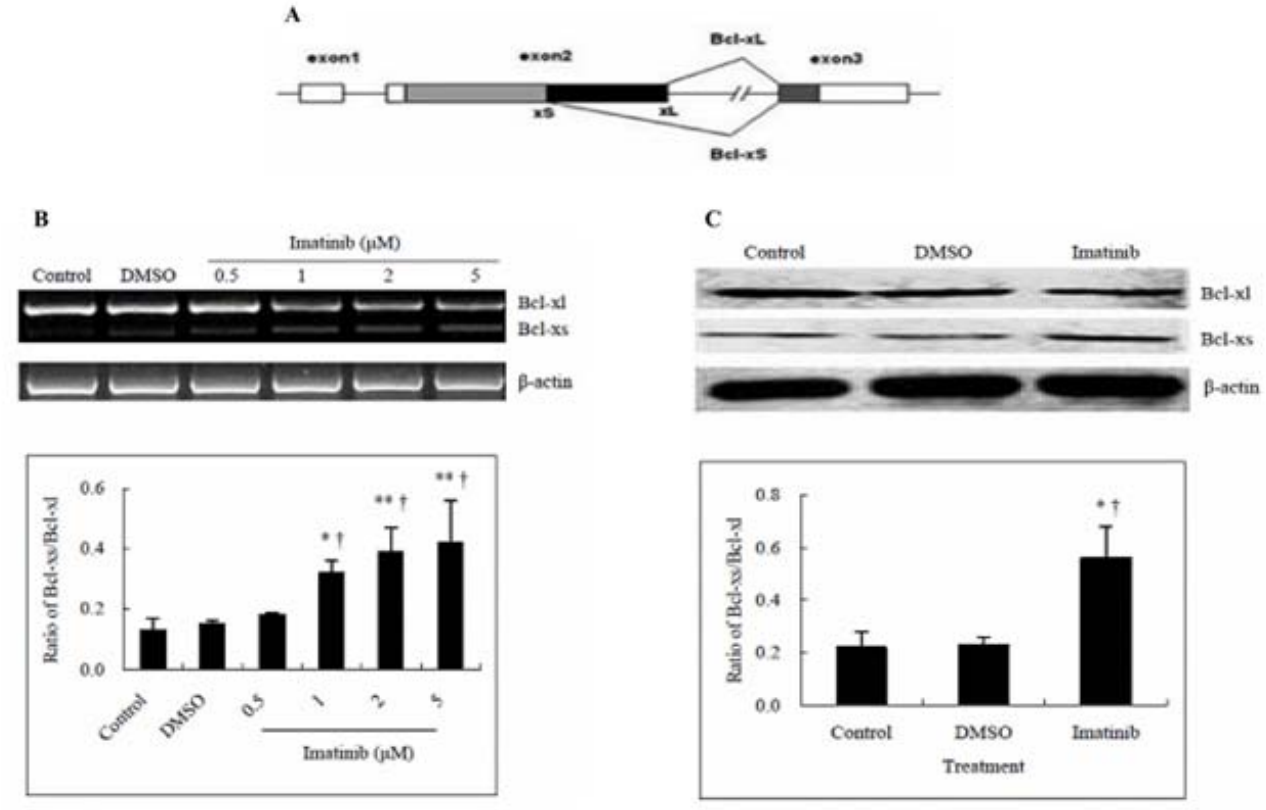

Figure 3. Imatinib regulates the Bcl-x splcing in K562 cells ($\bar{x}$±s, n = 3). K562 cells were treated with imatinib for 24 hours. Total RNA was extracted and then RT-PCR was used to analyze the alternative splcing of Bcl-x. **A:** Alternative splicing of exon 2 in the Bcl-x gene produces larger Bcl-xl and smaller Bcl-xs. **B:** Bcl-xl and Bcl-xs levels are correlated with the concentration of imatinib. **C:** Effect of imatinib on the protein expressions of Bcl-xs and Bcl-xl. K562 cells were treated with 1 □M imatinib for 24 hours. The total protein was extracted and quantified. Western blot was used to assay the Bcl-xs and Bcl-xl protein levels. The molecular weight of Bcl-xs and Bcl-xl was 18 kD and 30 kD respectively. $^{*}p$ <0.05, $^{**}p$ <0.01 vs Control; □p <0.01 vs DMSO.

Although the switch in the ratio between Bcl-xs/Bcl-xl suggested that imatinib might infuence Bcl-x alternative splicing, it is possible that this drug differentially affected the stability of the Bcl-x variants' mRNAs. In view of this, we in advance pretreated K562 cells with the transcriptional elongation inhibitor DRB for 1 hour before the treatment of imatinib. However, increasing DRB gradually antagonized the shift towards Bcl-xs, suggesting the imatinib-induced Bcl-xs/Bcl-xl required active transcription as can be seen in **Figure 4**. The results suggested that the imatinib -induced shift was not completely caused by differential stability of Bcl-x mRNA.

Hydroxyurea enhances the imatinib-induced splicing effect

To clarify the effect of a combined treatment of imatinib and hydroxyurea on the alternative splicing in K562 cells, we treated K562 cells with the two indicated drugs for 24 hours and assayed the mRNA levels of the Bcl-x products. The results revealed that combined treatment led to a stronger increase in the ratio of Bcl-xs/Bcl-xl compared with a moderate increase of the ratio after imatinib or hydroxyurea treatment alone as shown in **Figure 5**.

Imatinib-induced alterantive splicing is inhibited by calyculin A, but not by okadaic acid

To further investigate whether protein phosphatase play a role in regulating the alternative splicing of Bcl-x, we pretreated K562 cells with 5 nM of calyculin A, an inhibitor of both PP1 and PP2A-type protein phosphatases for 2 hours. Calyculin A completely blocked the imatinib effects on alternative splicing (**Figure 6**). Meanwhile, we also pretreated K562 cells

with 10 nM of okadaic acid, a selective PP2A inhibitor for 2 hours to identify whether PP1 or PP2A was the imatinib-responsive protein phosphatase in regulating Bcl-x alternative splicing. The result indicated that okadaic acid had no effect on Bcl-x alternative splicing. Taken together, PP1 mediated the regulatory effects of imatinib in the alternative splicing of Bcl-x.

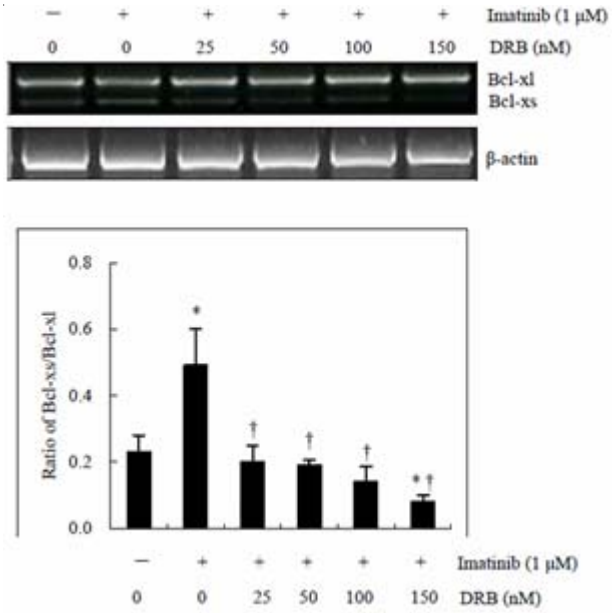

Figure 4. The imatinib-induced alternative splicing requires active transcription ($\bar{x}$±s, n = 3). The K562 cells were treated with various concentrations of DRB 1 hour before addition of 1 μM of imatinib, then the total RNA was extracted and mRNA levels of Bcl-xl and Bcl-xs were analyzed by RT-PCR. And, the ratio of Bcl-xs/Bcl-xl was calculated. There was a decrease in the Bcl-xs/Bcl-xl ratio along with the increasing concentration of DRB. $^{*}p$ <0.01 vs Control; $^{\dagger}p$ <0.01 vs Imatinib.

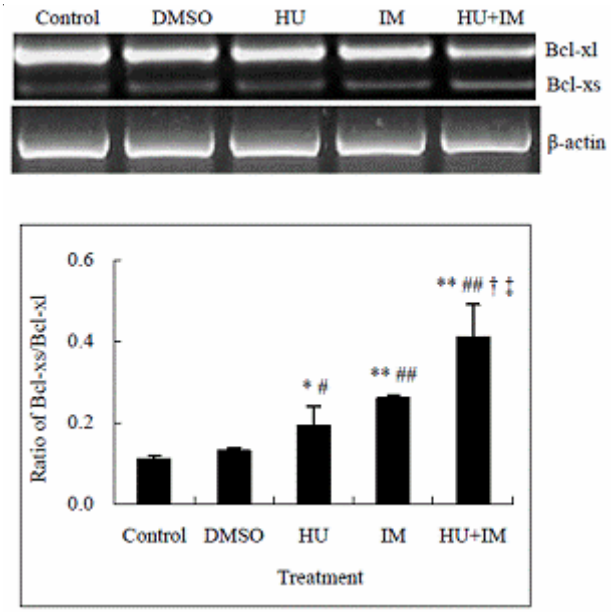

Figure 5. Hydroxyurea enhances the splcing efficacy of imatinib ($\bar{x}$±s, n = 3). The K562 cells were treated with imatinib (1 μM) or hydroxyurea (10 mM) for 24 hours. After that the total RNA was extracted and the mRNA levels of Bcl-xl and Bcl-xs were analyzed by RT-PCR. And , the ratio of Bcl-xs/Bcl-xl was calculated. HU or IM alone modestly changed the ratio of Bcl-xs/Bcl-xl, while imatinib in combination with hydroxyurea significantly increased the ratio of Bcl-xs/Bcl-xl in K562 cells. $^{*}p$ <0.05, $^{**}p$ <0.01 vs Control; $^{\#}p$ <0.05, $^{\#\#}p$ <0.01 vs DMSO; $^{\dagger}p$ <0.01 vs HU; $^{\ddagger}p$ <0.01 vs IM. IM: Imatinib, HU: Hydroxyurea.

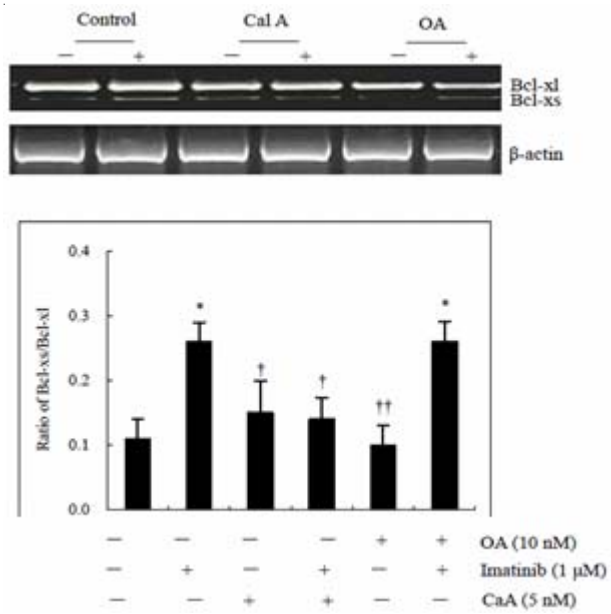

Figure 6. Imatinib-induced alterantive splicing is inhibited by calyculin A, but not by okadaic acid ($\bar{x}$±s, n=3). The K562 cells were treated with 5 nM calyculin A or 10 nM okadaic acid for 2 hours followed by 1 μM imatinib treatment for 24 hours. Total RNA was extracted and RT-PCR was employed to analyze imatinib-induced Bcl-x alternative splcing. PP1 not PP2A mediates the imatinib-induced the alternative splcing of Bcl-x. $^{*}p$ <0.01 vs Control; $^{\square}p$ <0.05, $^{\square\square}P$<0.01 vs Imatinib. Cal: Calyculin A, OA: Okadaic acid.

Discussion

Alterations in apoptosis are usually present in cancer and are a characteristics of nearly all types of cancers [13]. Likewise, aberrant profiles of alternative splicing frequently occur in cancer, but the precise contributions of these alterations to malignancy and metastasis remain poorly understood [14]. The regulation of the Bcl-x gene expression is of direct relevance to the apoptotic protein such as Bcl-x that has been documented in cell apoptosis. Moreover, the differential regulation of apoptosis by Bcl-x variants via alternative splicing is as important as the Bcl-xs overexpression-induced apoptosis, whereas the overexpression of Bcl-xl inhibited apoptosis. Although the expression of specific splice forms possibly influence cancer progression and the successfulness of anticancer regimens, little is known about effects of drugs on alternative splicing. Previously, it has been reported that some chemotherapeutic agents like staurosporine, and EGCG in combination with ibuprofen altered the ratio of the Bcl-xs/Bcl-xl in various cell lines, suggesting a regulatory role of exogenous stimuli in Bcl-x splicing. Based on these, we proposed a speculation that imatinib might affect the alternative splicing of Bcl-x [15, 16].

Further, there were evidences indicating that alternative splicing was regulated by endogenous factors like hormones, ceramide, and growth factors such as insulin and insulin-like growth factor1 that have been shown to regulate the alternative splicing of insulin receptor, protein kinase C□, indicating the alternative splicing is likely coupled to signaling pathway [17-19]. Recently, more and more studies have showed that some signaling pathways including AKT kinase, phosphatidylinositol 3-kinase, and Jun N-terminal protein kinase are involved in the splicing control [20-22]. In the present study, we have confirmed the anti-proliferative and pro-apoptotic effects of imatinib on CML cell line K562, which was consistent to the study of Carroll [9].

Bcr-abl fusion protein played an antiapoptotic effect in CML cells, and it was increased the antiapoptotic variants Bcl-xl by stimulating STAT5 [23]. And, imatinib could induce the apoptotic of K562 cells via blocking Bcl-xl antiapoptotic pathway [23]. Our results indicated that imatinib stimulated the upregulation of proapoptic Bcl-xs accompanying with a concomitant downregulation of anti-apoptotic Bcl-xl in K562 cells. The involvement of imatinib-induced splicing is not totally surprising because suppression

of tyrosine kinase was associated with the apoptosis induction, which at least in some cases is accompanied by a reduction in the expression of Bcl-xl [23]. Importantly, imatinib regulating the alternative splicing of Bcl-x is one of the underlying mechanisms of the pro-apoptotic effect of imatinib. However, the mechanism for the imatinib-induced splicing requires further investigation.

As observed, apparently a combination of imatinib and hydroxyurea significantly enhanced the ratio of Bcl-xs/Bcl-xl significantly compared with imatinib or hydroxyurea treatment alone. Hydroxyurea is a specific inhibitor of DNA synthesis and dose not affect RNA and protein synthesis. In the present study we found that hydroxyurea enhanced the effect of imatinib-induced alternative splicing of Bcl-x in CML cell line. Simultaneous treatments to imatinib and hydroxyurea produced an additive effect, which might have some therapeutic advantages in clinic [12]. Because the combined therapy may reduce agent to a minimum dose compared with individual administration. Obviously, a combination of imatinib and hydroxyurea is more efficient and well tolerant for the treatment of recurrent GBM [24], and the efficiency was also confirmed in a phase II study [25]. Additional, it was found that imatinib significantly increased the susceptibility of K562 cells to cisplatin by simultaneous inhibition of p53 trans-activation and reduction of Bcl-xl [26]. Thus, a combined treatment for CML is more efficient than the individual treatment.

Dephosphorylation of SR splicing factors by PP1 was involved in the regulation of alternative splicing of Bcl-x in ceramide-treated lung carcinoma cells [17]. Thus, we speculated that imatinib-induced alternative splicing is relevant to the activation of PP1. In this study, the results demonstrated that the PP1 and PP2A inhibitor, calyculin A completely blocked imatinib-induced alternative splicing compared with okadaic acid, a specific inhibitor of PP2A, suggesting PP1 mediated the regulation of imatinib in the alternative splicing of Bcl-x.

PP1 activity is not only present in the cytosol, but in the nucleus [27] where it is likely to regulate nuclear processes including pre-mRNA splicing [28]. The dephosphoreylation of a family of RNA splice factors, SR proteins, has been well decumented in the activation of PP1 [29]. Dephosphorylation of SR protein by PP1 induced alternative 5' splice site selection *in vitro* [30] while Bcl-x alternative splicing was determined by alternative selection of 5' splice site within Bcl-x exon 2.

In conclusion, imatinib could regulate the alternative splicing of Bcl-x in K562 cells, which may be associated with the activation of PP1. However, further study is needed to investigate the role of tyrosine kinase in this event. In addition, whether imatinib selectively affect the stability of Bcl-xl or Bcl-xs mRNA is also required investigation.

Acknowledgements

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