

# Imatinib and bortezomib induce the expression and distribution of anaphase-promoting complex adaptor protein Cdh1 in blast crisis of chronic myeloid leukemia

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**Abstract.** Anaphase promoting complex cofactor Cdh1 plays a critical role in tumor suppression and genomic stability in cancer. However, its role in chronic myeloid leukemia (CML) remains unclear. We treated both wild-type and imatinib-resistant K562 cells with imatinib or nilotinib and bortezomib, respectively. The siRNAs of Cdh1 and Skp2 were designed and transiently transfected with HiPerFect transfection reagent into CML cells. Expression of Cdh1-Skp2-p27 pathway proteins were detected by Western blotting. Cell cycle, cell apoptosis and cellular morphology were detected by flow cytometry and Wright staining. Our study revealed that Cdh1 was expressed at lower levels in imatinib-resistant CML blast crisis (BC) patients than imatinib-sensitive ones. Moreover, imatinib and bortezomib induced cell cycle quiescence or arrest, upregulation and nuclear relocation of Cdh1 in CML cells. Furthermore, nilotinib and bortezomib resulted in upregulation of Cdh1 in imatinib-resistant CML cells. Conversely, Cdh1 silencing resulted in stabilization of Skp2 and Cdc20, subsequently promoting G1-S transition and formation of multinucleated cells. Our study shows that TKIs and bortezomib can regulate the cell cycle and cell apoptosis via regulation of the expression and redistribution of Cdh1 in CML-BC, which sheds light on the orchestration of crosstalk between TKIs and bortezomib in imatinib-resistant CML-BC. Additionally, Cdh1 tends to play an important role

in maintenance of genomic stability, the detailed mechanisms deserve further study.

## Introduction

Chronic myeloid leukemia (CML) is a malignant clonal disorder of hematopoietic stem/progenitor cells, characterized by Philadelphia chromosome resulting from reciprocal translocation between the breakpoint-cluster region (BCR) of chromosome 22 and the Abelson leukemia gene (ABL) on chromosome 9 (1). CML is well-known as the best understood human malignancy and a paradigm for cancer research from bench to bedside (1,2). Tyrosine kinase inhibitor (TKI) targeting BCR-ABL fusion protein has remarkably changed the treatment patterns (2-5). Imatinib, a selective inhibitor of the BCR-ABL tyrosine kinase, produces high response rates in CML patients, it has been established as the standard first-line therapy for patients with chronic-phase CML (2,3). However, treatment options for imatinib-resistant patients are still limited except new TKIs and hematopoietic stem cell transplantation. As reported, it remains like a Maltese Falcon for hematologist (6-8). Bortezomib, a proteasome inhibitor, not only prolonged life span and was shown to be superior to high-dose dexamethasone for relapsed multiple myeloma (MM) patients (9), but also induced cell apoptosis in CML and lymphoma cells (10). However, the efficacy of TKIs and bortezomib on imatinib-resistant CML remains obscure.

The natural history of CML is a stereotypical progression from a relatively benign chronic phase (CP) through accelerated phase (AP), into fatal blast crisis (BC) (1). Comprehensive clinical research, including our previous reports, have verified the relentless march can be postponed or even diverted by TKIs or allogeneic hematopoietic stem cell transplantation in some cases (2-5,10,11). The rest might inevitably approach to the destination of blast crisis. Unfortunately, underlying mechanism of disease progression still remains unclear. Acquisition of additional genetic events such as double Ph, trisomy 8, or molecular abnormalities such as activation of PI3K/Akt, Wnt/ $\beta$ -catenin and loss of C/EBP $\alpha$  function in CML stem cells, are believed to speed up disease progression (8,12-14).

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*Abbreviations:* CML, chronic myeloid leukemia; APC, anaphase promoting complex; TKI, tyrosine kinase inhibitor; BC, blast crisis

*Key words:* chronic myeloid leukemia, Cdh1, tyrosine kinase inhibitor, bortezomib, imatinib-resistant

The anaphase promoting complex/cyclosome (APC) is a ubiquitin protein ligase that, together with Cdc20 or Cdh1, targets cell cycle proteins for degradation and has a critical role in mitotic and cell cycle control (15-17). APC substrates include mitotic cyclins, mitotic kinases and proteins involved in chromosome segregation and replication. In addition, both Cdc20 and Cdh1 subunits are themselves substrates of APC-mediated degradation. Cdh1 specifically promotes protein degradation in late mitosis and G1 (15-17). Previous studies have shown that Cdh1 has a critical role in cell cycle control and tumor suppression. Furthermore, Cdh1-deficient cells accumulate numeric and structural chromosomal aberrations, indicating that Cdh1 contributes to the maintenance of genomic stability (18,19). It is well known that BCR-ABL of CML can induce expression of Skp2, the substrate of Cdh1, through PI3K/AKT pathway to promote p27 degradation and G1-S cell cycle transition (20). The role of Cdh1 in CML, has been marginally addressed including interaction of Cdh1-Skp2-p27 cascade and Cdh1-Cdc20 interaction. This inspired us to explore the expression and location of Cdh1-Skp2 proteins in patients with CML-BC, and the potent relationship between TKIs or bortezomib and Cdh1 in both imatinib-sensitive and -resistant CML-BC cells. Moreover, we investigated the biological effect of Cdh1 as well as the change of Skp2-p27 cascade and the expression of Cdc20 after transiently silencing by siRNA in CML-BC.

## Patients and methods

**CML patients.** Ten patients with newly diagnosed CML-BC in the Department of Hematology of Nanfang Hospital from April 2009 to November 2010 were enrolled in this study. Diagnosis was done according to the WHO classification (21) and the clinical characteristics are presented in Table I. Bcr-abl was detected by fluorescence *in situ* hybridization (FISH) analysis which was performed on the protocol of Bcr/abl Dual Color Dual Fusion translocation probe set specific for Bcr-abl fusion genes (Vysis LSI, Abbott, USA). Conventional cytogenetic analysis was performed as reference (22) and chromosomal aberrations were described according to ISCN nomenclature (2009) (23). Mononuclear cells (MNC) of primary CML patients were isolated from bone marrow by Ficoll density gradient centrifugation (Amersham Biosciences, Uppsala, Sweden) for immunoblot analysis. Total RNA was purified from cells using a TRIzol Reagent (Takara, Japan). cDNA was amplified with primeScript RT reagent Kit (Takara) according to the manufacturer's instruction.

**Cell culture.** The wild-type K562 human leukemic cell line (K562-WT) derived from CML-BC patient was obtained from American Type Culture Collection (ATCC) (Rockville, USA). K562-Imatinib resistant (K562-IMR) cells were induced as reference (24) and were maintained with 10  $\mu$ M imatinib to ensure drug resistance. The K562-WT and K562-IMR cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island), penicillin (100 U/ml), and streptomycin (200 U/ml). Primary cells derived from CML-BC patients (no. 8 in Table I) were cultured in RPMI-1640 supplemented with 20% FBS (Gibco), and penicillin (100 U/ml), streptomycin (100 U/ml), at 37°C in a 5% CO<sub>2</sub> humidified air atmosphere.

**Agents.** The tyrosine kinase inhibitors imatinib and nilotinib (Novartis Pharmaceuticals, Basel, Switzerland) were dissolved in dimethyl sulfoxide (DMSO) as a 10 mg/ml stocking solution, the proteasome inhibitor bortezomib (Millennium Pharmaceuticals, Cambridge, UK) was dissolved in 0.9% NaCl solution at a concentration of 10 mg/ml. K562-WT cells were treated with imatinib and bortezomib at a final concentration of 800 and 80 nmol/l separately, subsequently, K562-IMR cells were treated with nilotinib and bortezomib at a final concentration of 40  $\mu$ mol/l and 80 nmol/l separately.

**Immunoblot analysis.** Cells were harvested and lysed in RIPA buffer, all immunoblottings were performed following standard biochemical techniques as previously described (10). Antibodies used were from ABcam (Cambridge, MA; anti-Cdh1), Zymed (South San Francisco, CA; anti-Skp2), BD Biosciences-Pharmingen (San Jose, CA; anti-p27), and Sigma (anti-GAPDH as a protein loading control). Quantification of the immunoblot band intensity was performed with Gelpro32 application software.

**Immunofluorescence staining and image acquisition.** For immunofluorescence analysis, 1x10<sup>5</sup> cells were placed on the slide, fixed in paraformaldehyde, permeabilized in Triton X-100, after incubated with appropriate primary antibodies and secondary antibodies conjugated to fluorochromes, cells were finally counterstained with DAPI (4',6-diamidino-2-phenylindole). Fluorescence images were viewed and acquired using an Olympus laser scanning confocal microscope. Olympus FV1000 Viewer (Ver.2.1b) software was used for processing images, and the overlaying and merging of images was done using Adobe Photoshop CS2 (Adobe).

Primary antibodies used for the immunofluorescence analysis were the following: Cdh1 (Santa Cruz Biotechnology goat polyclonal antibody), Skp2 (Zymed mouse monoclonal antibody). The fluorochrome-conjugated secondary antibodies were FITC (rabbit anti-goat), Texas red (Donkey anti-mouse).

**FACS analysis.** For cell cycle analysis, 4x10<sup>5</sup> cells were harvested. Briefly, cells were resuspended and fixed in ethanol and incubated in the propidium iodide (PI) solution with 5  $\mu$ g/ml of RNase, kept on ice until analyzed using a FACScan apparatus (Becton-Dickinson, FACSCalibur) using the ModFit LT Mac 3.1 sp3 software. For cell apoptosis detection, 4x10<sup>5</sup> cells were stained with Annexin V-FITC and PI, and analyzed by flow cytometry using the CellQuest software.

**siRNAs.** For small interfering RNA (siRNA) experiments, single siRNAs of Cdh1 and Skp2 were used to reduce Cdh1 and Skp2 protein expression, respectively. As previously reported (25,26), 100 nM siCdh1 (5'-UGAGAAGUCUCCAGUCAG-3') and siSkp2 (5'-GCAUGUACAGGUGGCUGUU-3') (Takara) were transiently transfected into subconfluent cells with HiPerFect Transfection Reagent (Qiagen, Crawley, UK) in accordance with the manufacturer's instructions.

**Assessment of cell viability.** As previously described (27), Cell proliferation was determined using Cell Counting kit-8 (CCK-8) (Dojin Laboratories, Japan) containing WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfonyl)-2H-tetrazolium, monosodium salt] which allows sensi-

Table I. Characteristics of Ph-positive patients with CML-BC.

Patient no.	Age/sex	IM dose (g)/time (m)	Resistance/sensitive <sup>b</sup>	Blast type	FISH Bcr/abl(+)	Karyotype	APCcdh1/GAPDH
1 <sup>a</sup>	22/F	0.6/2	R	AML	92%	t(9;22),t(3;3)	0.32
2	37/F	0.6/3	R	ALL	92%	t(9;22),+ph	0.49
3	32/M	0.6/13	R	AML	40%	t(9;22),+8,t(3,17)	0.18
4	24/F	0.4-0.6/24	R	AML	69%	t(9;22),+Ph	0.69
5 <sup>a</sup>	42/M	0.6/4	R	AML	89%	t(9;22),+21,+Ph	0.67
6 <sup>a</sup>	30/M	<sup>c</sup>	-	AML	90%	t(9;22)	1.57
7	42/F	0.4/13	S	AML	76%	t(9;22)	1.47
8	30/F	<sup>c</sup>	-	ALL	96%	t(9;22)	1.45
9	24/M	0.4/6	S	AML	95%	t(9;22)	1.65
10	36/F	0.4/10	S	AML	89%	t(9;22)	1.59

<sup>a</sup>Patients have already died. <sup>b</sup>Resistance or sensitive of imatinib. <sup>c</sup>Refused imatinib treatment.

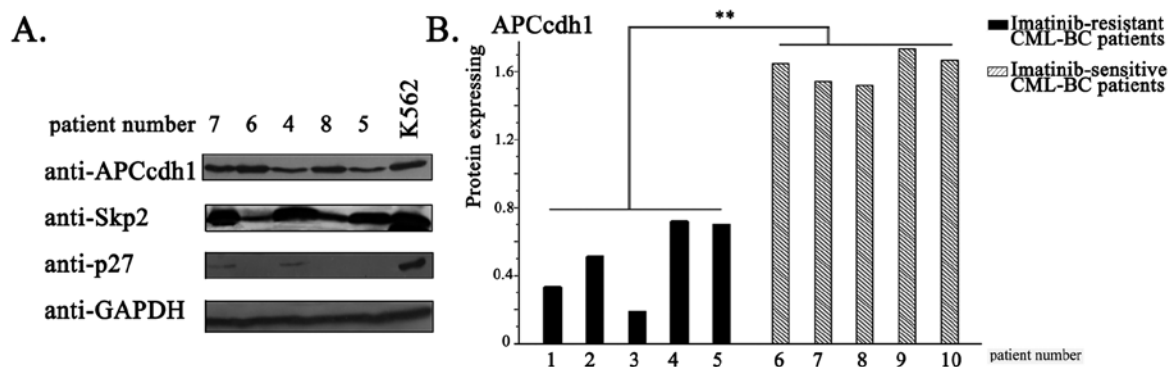


Figure 1. Expression of Cdh1-Skp2-p27 in CML-BC primary cells. (A) The expressions of Cdh1 and Skp2 were detected in CML-BC primary cells by immunoblots using Cdh1, Skp2 and p27 antibodies. K562-WT served as positive control. (B) Densitometry quantification normalized to the level of GAPDH demonstrated the expression of Cdh1 in CML-BC patients; \*\*p<0.01.

tive colorimetric assays for the determination of the number of viable cells, according to the manufacturer's instructions.

**Statistical analysis.** Statistical significance of differences between groups was determined using Independent-sample t-test. p<0.05 was considered to be significant. All data were from 3 independent experiments.

## Results

**Cdh1 had lower expression in imatinib-resistant CML-BC patients.** To explore the role of anaphase-promoting complex cofactor Cdh1 in CML-BC, we first dissected the Cdh1-Skp2-p27 cascade in 10 of CML-BC patients by immunoblot analysis. As shown in Fig. 1, a variety of Cdh1 levels was detected. Skp2 was overexpressed with low-level p27 (Fig. 1A). Furthermore, we respectively analyzed the clinical features of Cdh1-positive samples and divided them into two groups (as shown in Table I). One group was imatinib-resistant including 5 patients, and the other group was sensitive to imatinib, those 5 CML-BC patients resistant to imatinib had additional chromosomal abnormalities. Interestingly, we found that a lower level expression of Cdh1 remarkably correlated with resistance to imatinib and the

secondary chromosome aberrations in CML-BC, and vice versa, higher level of Cdh1 occurred in those sensitive to imatinib and those aberration-free (Fig. 1B) indicating that Cdh1 tends to be related to imatinib-resistance in CML-BC.

**Imatinib/bortezomib induces cell cycle quiescence/arrest and cell apoptosis.** In order to verify the effect of TKI and the proteasome inhibitor in CML-BC cells, we treated the K562-WT cells and CML-BC primary cells with imatinib and bortezomib separately. Treatment with imatinib induced cell cycle quiescence in G0/G1 phase compared with the DMSO-treated group in K562-WT cells indicated by quantification of FACS profiles ( $72.56 \pm 2.30\%$  vs  $37.98 \pm 2.91\%$ , p<0.01) (Fig. 2A) (data are mean  $\pm$  SD n=3). Conversely, bortezomib led to significant increase of the G2/M phase populations compared with the DMSO-treated group ( $73.47 \pm 4.09\%$  vs  $12.86 \pm 0.47\%$ , p<0.01) (Fig. 2B) (data are mean  $\pm$  SD n=3), in line with previous studies (28,29). Treatment of CML-BC primary cells with imatinib or bortezomib also resulted in the same effect on cell cycle progression (Fig. 2A and B).

As expected, we detected an increase of apoptotic cells ( $18.54 \pm 0.88\%$  vs  $3.57 \pm 0.94\%$ , p<0.01;  $56.60 \pm 10.14\%$  vs  $3.57 \pm 0.94\%$ , p<0.05) (data are mean  $\pm$  SD n=3) treated

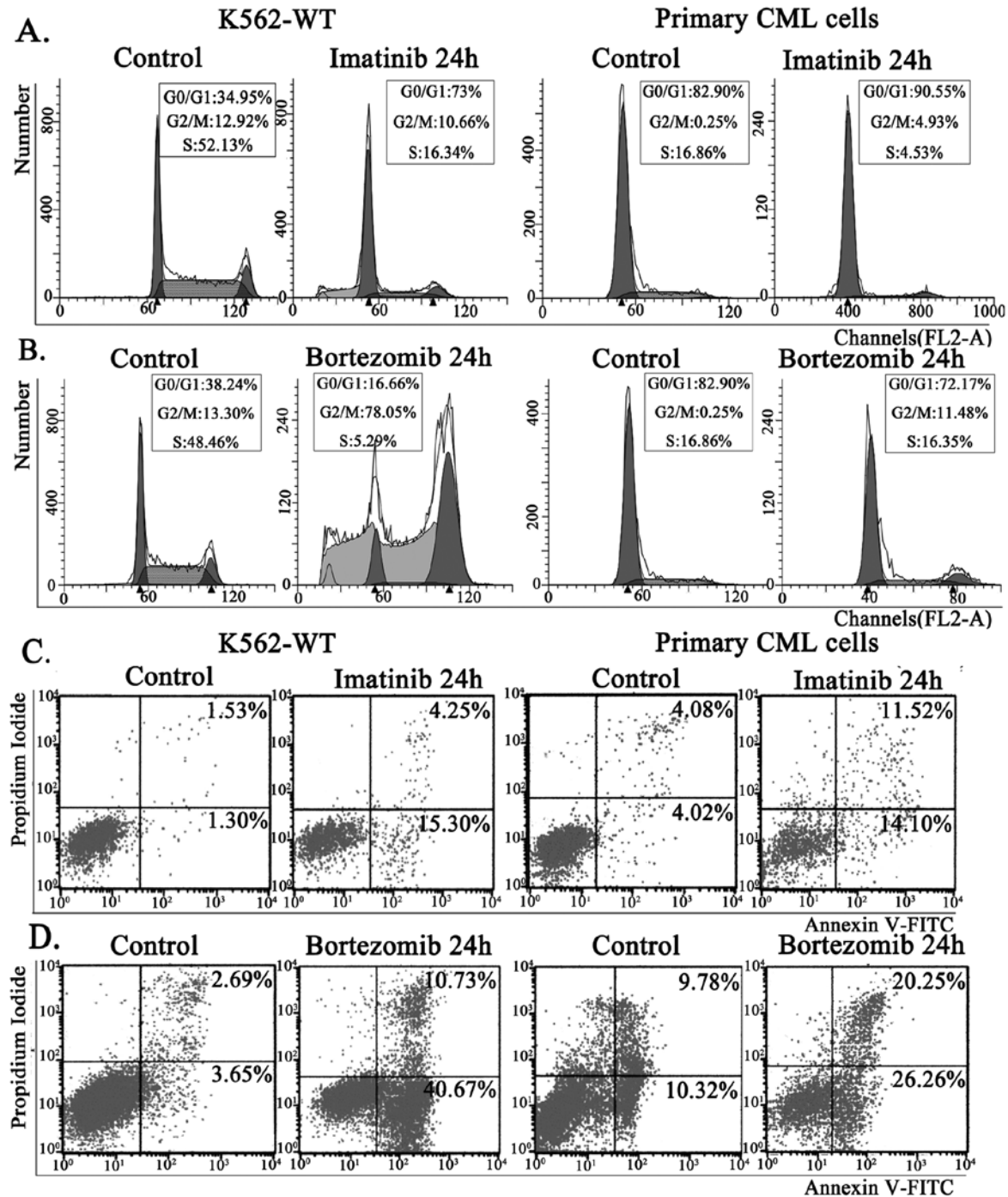


Figure 2. Effects of imatinib and bortezomib on cell cycle and apoptosis. (A) The K562-WT cells were stained with PI, represented by the percentages of two populations (imatinib-treated and DMSO-treated) in the G0/G1, S and G2/M phases of the cell cycle, were examined by FACS. (B) The K562-WT cells were treated with bortezomib and stained with PI for the cell cycle examining by FACS. (C and D) The cells (imatinib-treated and bortezomib-treated) were stained with PI and Annexin V-FITC, and the average percentages distribution of Annexin V-FITC-positive cells, corresponding to late and early apoptotic cells, were determined by FACS. Primary cells of one CML-BC patient (no. 8 in Table I) were treated with imatinib or bortezomib separately, cell cycle and apoptosis were also detected by FACS.

with imatinib or bortezomib in K562-WT cells, compared with DMSO control for 24 h. Similar result was obtained in CML-BC primary cells (Fig. 2C and D).

*Imatinib or bortezomib induce Cdh1 expression in K562 cells.* We wondered which pathway was involved in the change of cell cycle and apoptosis after TKI and proteasome inhibitor treat-

ment, so we treated the imatinib-sensitive cells with imatinib or bortezomib, and imatinib-resistant cells were treated with nilotinib or bortezomib for up to 24 h separately. As illustrated by immunoblot analysis, imatinib or nilotinib led to a G1 quiescence through upregulation of Cdh1, degradation of Skp2 and accumulation of p27 in K562-WT and K562-IMR cells, compared with DMSO-treated control cells (Fig. 3A). The same regulation of

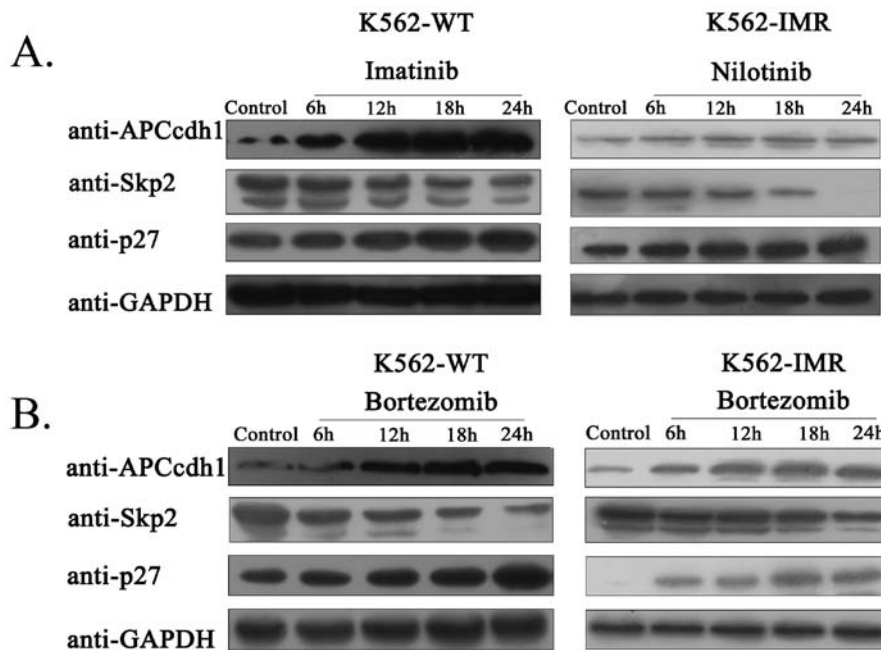


Figure 3. Effects of imatinib or nilotinib and bortezomib on Cdh1-Skp2-p27 pathway in the K562-WT and K562-IMR cells. (A) The K562-WT and K562-IMR cells were treated with imatinib and nilotinib separately at the concentrations indicated for 24 h. (B) Additionally, the K562-WT and K562-IMR cells were treated with bortezomib at the concentrations indicated for 24 h. Protein lysates were all analyzed by immunoblot with the antibodies of Cdh1-Skp2-p27 signaling pathway regulators.

Cdh1-Skp2-p27 pathway was detected after being treated with bortezomib in both K562-WT and K562-IMR cells (Fig. 3B).

*Imatinib or bortezomib induce nuclear distribution of Cdh1.* Previous studies reported that Cdh1 expression was low in imatinib-resistant CML-BC cells. To explore the subcellular localization and interaction in space between Cdh1 and Skp2, immunofluorescence microscopic analysis was performed in CML-BC cell lines and primary cells. It illustrated that Cdh1 and Skp2 were co-localized more abundantly in the cytoplasm of imatinib-sensitive cells, but co-distributed to the nuclei in imatinib-resistant cells (Fig. 4A and B).

We hence studied the subcellular localization of Cdh1 by immunofluorescence analysis in imatinib- or bortezomib-treated K562-WT cells. Cdh1 showed a mainly cytoplasmic localization in DMSO-treated control cells. In contrast, we found a rapid redistribution of Cdh1 from cytoplasm to nucleus where Cdh1 is known to be active, after the treatment with imatinib or bortezomib (Fig. 4C).

*Cdh1 silencing resulted in promotion of G1-S transition resulting from dysregulation of Cdh1-Skp2-p27 pathway and accumulation of Cdc20.* Gene silencing by siRNA targeting Cdh1 was carried out to observe the effect on cell cycle of K562 cells. Down-regulation by Cdh1-siRNA result in promotion of proliferation, the proliferation inhibition ratio was ~24%. Furthermore, cell cycle assay by FACS revealed that the proportion of G0/G1 cells was significantly less in siCdh1 cells compared with control in K562 cells ( $26.82 \pm 3.99\%$  vs  $39.16 \pm 5.49\%$ ,  $p < 0.01$ ) (Fig. 5A) (data are mean  $\pm$  SD  $n=3$ ). In contrast, siSkp2 resulted in remarkable increase of G0/G1 phase cells in comparison with negative control in K562-WT cells ( $49.74 \pm 7.13\%$  vs  $39.16 \pm 5.49\%$ ,

$p < 0.05$ ) (Fig. 5B) (data are the mean  $\pm$  SD  $n=3$ ). Similar result was obtained in CML-BC primary cells (Fig. 5).

In order to further elucidate the mechanism of Cdh1 regulating cell cycle and protecting genomic stability in CML-BC, we detected Cdh1-Skp2-p27 cascade and the substrates facilitating genomic instability Cdc20. We showed that transiently silencing of Cdh1 significantly accumulated Skp2, which in turn degraded the downstream signaling molecule p27 (Fig. 6A). Attenuation of Skp2 with siRNA increased p27 expression (Fig. 6B), which are consistent with previous reports in gastrointestinal stromal tumor cells and breast cancer (30,31). Furthermore, we revealed that Cdh1 silencing led to accumulation of Cdc20, the substrate of Cdh1, which might greatly contribute to cell cycle change and facilitating genomic instability (Fig. 6A).

*Cdh1 silencing led to morphology abnormalities.* As shown in Table I, those 5 CML-BC patients resistant to imatinib were with additional chromosomal abnormalities. Of particular interest, Western blot analysis revealed that a lower level expression of Cdh1 not only remarkably correlated with imatinib-resistance, but also those with the secondary chromosome aberrations (Fig. 1), which indicated that Cdh1 tends to be related to genetic stability in CML-BC. General morphology analysis was applied to further determine the biological effect of Cdh1 silencing in K562 and primary cells. We found that siCdh1 induces petal-like morphology cells in K562 (Fig. 6C). Moreover, we observed the marked morphological change of the formation of binucleated or multinucleated cells (19%) after siCdh1 transfection in primary CML-BC cells whose blast type was ALL (Fig. 6D), which might be an indication of defects in cytokinesis and mitotic hyperactivity. These new-shaped cells were negative for both peroxidase (POX) and alkaline phosphatase

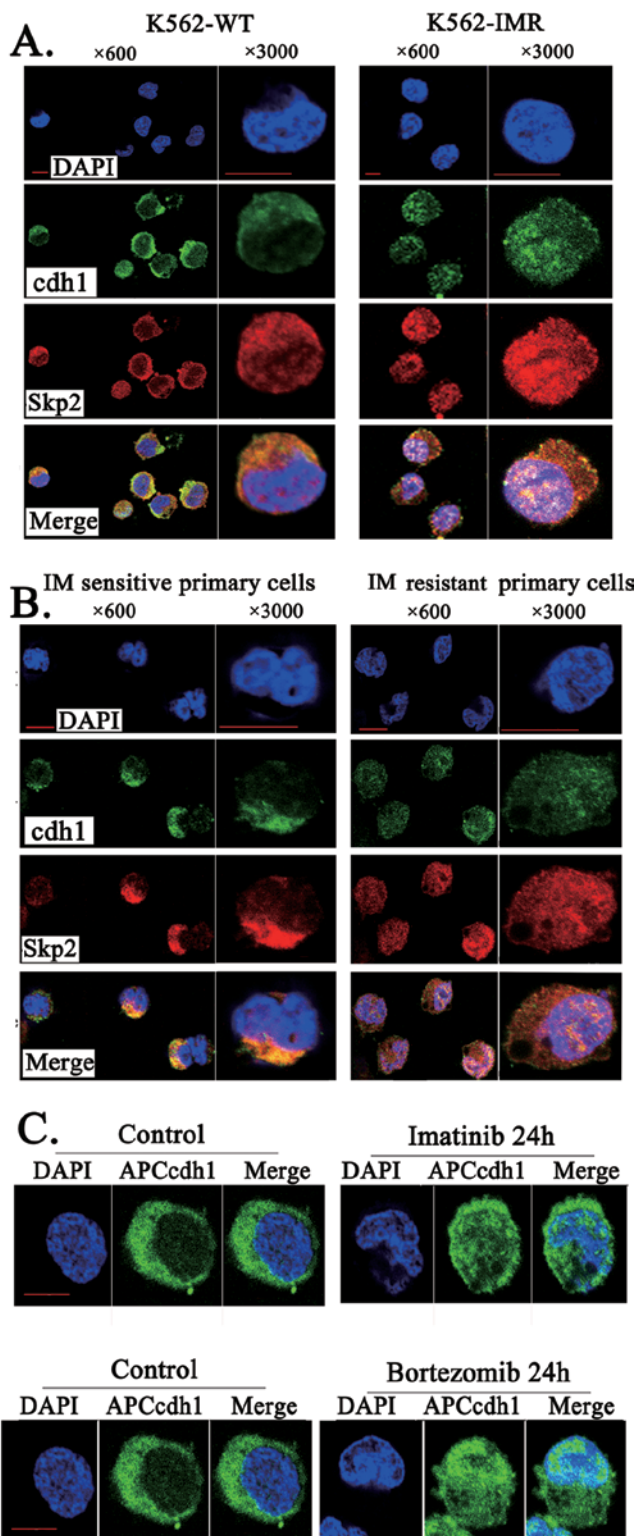


Figure 4. Dynamic distribution of subcellular location of Cdh1. (A and B) Colocalization of Cdh1 and Skp2 in K562-WT cells, K562-IMR cells, IM sensitive-CML primary cells and IM resistant-CML primary cells. The DAPI staining shows the nuclear localization. FITC and Texas Red fluorescence show localization of Cdh1 and Skp2 separately. The double-labeled immunofluorescence shows immunoreactivity for both Cdh1 and Skp2. (C) K562-WT cells were treated with imatinib or bortezomib for up to 24 h as indicated. Immunofluorescence analysis illustrated the rapid redistribution of Cdh1. FITC fluorescence shows localization of Cdh1 (scale bars, 10  $\mu$ m).

tion of Cdh1 induced accumulation of mitotic errors rather than cellular differentiation.

## Discussion

In this study, we explored the interpretation of underlying mechanism of imatinib and bortezomib via regulating the expression and redistribution of Cdh1 and subsequent pathway in CML-BC. We also investigated the effect of imatinib, nilotinib and bortezomib on Cdh1 regulation, subsequent regulation on Cdh1-Skp2-p27 cascade and cell cycle progression. We studied the change of biological and morphology in Cdh1-silencing CML-BC cells. Our results provide evidence that TKIs and bortezomib have a novel anti-leukemic mechanism suppressing the cell cycle and induction of cell apoptosis in CML-BC by inducing the expression and nuclear redistribution of Cdh1 and the degradation of its substrate such as Skp2 and Cdc20.

Following the initial success of imatinib as frontline therapy for CML, nilotinib and dasatinib have been developed with increased potency and the ability to inhibit IM-resistant BCR-ABL kinase. They are designed chiefly to inhibit directly ATP binding sites of BCR-ABL kinase with minor difference in target spectrum. BCR-ABL kinase exhibits transcriptional activation of Skp2 expression and promotes the cell cycle via PI3K/AKT pathway in CML cells (20,32). A recent study demonstrated that in addition to activating the PI3K/AKT pathway, Bcr-Abl may phosphorylate Emi1 to enhance its stability which in turn inhibits Cdh1 and attenuate Skp2 protein ubiquitination (33). The mechanism of bortezomib in CML-BC cells was unclear. In the present study, we demonstrated both TKIs and bortezomib exerted an effect on the control of the cell cycle progression and apoptosis, by inducing Cdh1 expression and nuclear redistribution. Imatinib or bortezomib treatment was able to induce cell cycle quiescence or arrest and apoptosis in most of CML-BC cells, in which cells not undergoing apoptosis are removed from the proliferative pool by entering quiescence through modulation of Cdh1-Skp2-p27 axis. Quiescence after imatinib treatment might be one of the reasons for result of the STIM study (34), in which CML patients could remain in complete molecular remission after discontinuation of the TKI treatment.

We also found both imatinib and bortezomib induced nuclear redistribution of Cdh1, which might be the underlying mechanism of Cdh1-mediated anti-leukemia effect in CML-BC. Our results revealed that similar to IM-sensitive CML-BC cells, Cdh1 was induced by imatinib to up-regulate and re-distribute from cytoplasm to nuclei to degrade its substrate, such as Skp2 and Cdc20. In contrast, lower level Cdh1 was located in the nuclei of IM-resistant CML-BC cells, which might result from failure on inhibiting Skp2 expression by imatinib and compensatory recruitment of Cdh1 for degradation of Skp2. TKIs, including imatinib, nilotinib and dasatinib, directly suppressed BCR-ABL kinase activity, and proteasome inhibitor could also degrade BCR-ABL fusion protein directly (10). Moreover, expression and dynamic distribution of Cdh1, induced by imatinib and bortezomib, provides a novel interpretation of underlying mechanism to inhibit BCR-ABL downstream cascade via Cdh1-Skp2-p27 axis. In terms of TKI-sensitivity in CML-BC, it might make sense to evaluate the different abundance of Cdh1 expression in the nuclei.

(AKP) (Fig. 6D), which affect cellular differentiation to mature myelocyte. These morphological changes illustrated that inhibi-



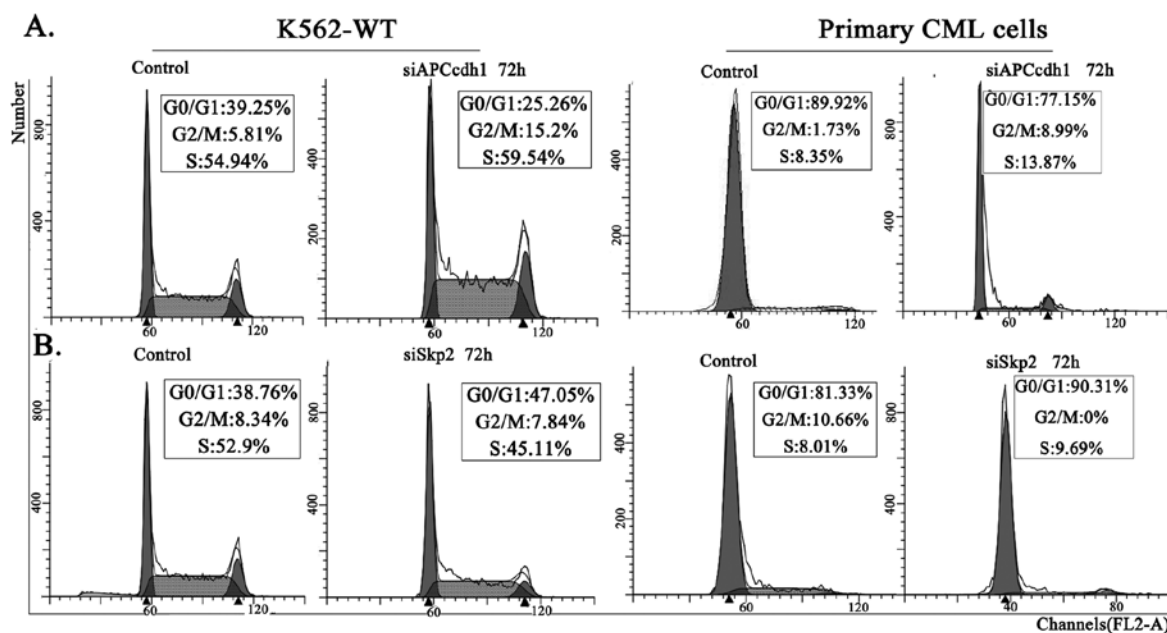


Figure 5. Effects of siCdh1 or siSkp2 on cell cycle in K562-WT and CML-BC primary cells. K562-WT cells and primary cells from a CML-BC patient were infected with the negative control siRNA and siCdh1 (A) or siSkp2 (B), and cultured under condition as previously described for 72 h. The cell cycle profile was examined by FACS. The data in the figure were obtained from one experiment.

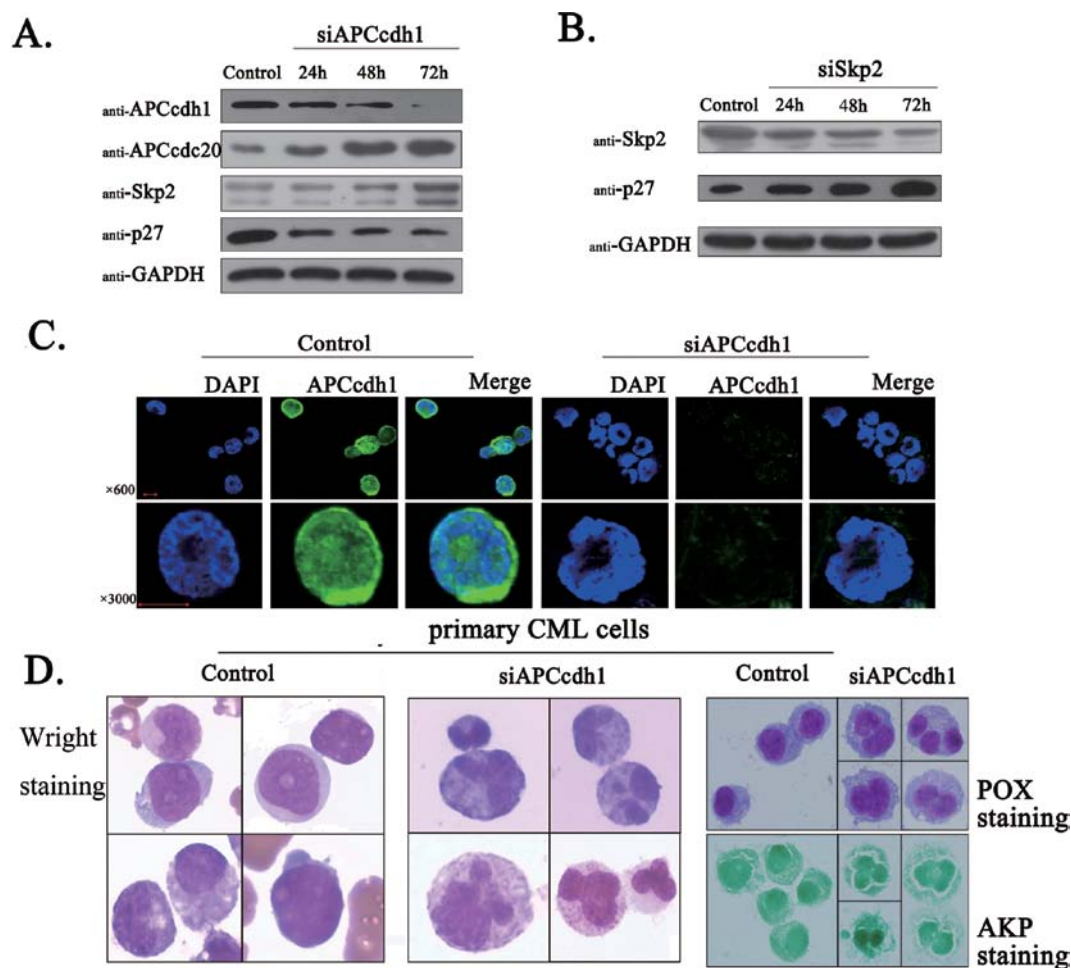


Figure 6. Effects of Cdh1-silencing on Cdh1-Skp2-p27 pathway and Cdc20 and cellular morphology. Cdh1 and Skp2 were specifically suppressed by siCdh1 and siSkp2. (A) Engineering of siCdh1 for up to 72h in K562-WT cells as indicated, immunoblots were probed for Cdh1, Skp2, p27 and Cdc20. (B) After transiently transfected with siSkp2, immunoblots were probed for Skp2 and p27. GAPDH stain is shown to demonstrate equal loading. (C) The petal-like morphology of cells were observed after transfected with siCdh1. Fluorescent staining showed as previously indicated. (D) Wright staining showed binucleated or multinucleated cells after siCdh1 transfection in CML-BC primary cells whose blast type was ALL. Cells transfected with the negative control siRNA (D, left) and siCdh1 cells (D, middle) were shown. Both the POX staining and AKP staining (D, right) were performed to confirm the cellular differentiation to mature myelocytes.

We demonstrated that bortezomib still exerted a regulation effect on expression and relocation of Cdh1 even in IM-resistant CML-BC cells, which provided evidence for synergy combination of TKI and proteasome inhibitor for overcoming IM-resistant. We have reported that it has a synergistic effect between proteasome inhibitors and imatinib in CML via suppression on BCR-ABL, E2F1 and  $\beta$ -catenin, suggesting that combined use of tyrosine kinase inhibitor and proteasome inhibitor might be helpful for optimizing CML treatment (10). In IM-resistant CML-BC, ABL domain mutation and the dysregulation of hOCT1 protein could facilitate BCR-ABL kinase escape from TKIs, and result in promotion of the cell cycle and cell proliferation. Treatment with either imatinib and bortezomib resulted in recruitment of Cdh1 to the nuclei in CML-BC, degradation of BCR-ABL-related activation of Skp2 and subsequent cascade. Regulation on the expression and nuclear redistribution might be a novel potent target for synergy of TKIs and bortezomib in both imatinib-sensitive or -resistant CML-BC cells.

Our results revealed Cdh1 tends to be related to genomic stability in CML-BC. Cdh1 is required for preventing unscheduled proliferation and protecting primary mammalian cells or immortalized cells from genomic instability. Cdh1-deficient cells showed a large variety of chromosomal aberrations (18). On the contrary, Cdc20, the substrate of Cdh1 contributed to genetic aberration. Adult T-cell lymphoma/leukemia is often aneuploid with complex chromosomal abnormalities in genetic, multi-nucleated or convoluted cells in morphology, which are related with overexpression of Cdc20 (35-37). Firstly, we found lower level of Cdh1 in those with additional genetic abnormalities (Fig. 1B). Additionally, our study demonstrated Cdh1-silencing led to promotion of G1-S transition resulting from dysregulation of the Cdh1-Skp2-p27 pathway (Figs. 5 and 6). Furthermore, Cdh1 silencing resulted in morphology abnormalities with bi-nucleated or multi-nucleated cells in CML-BC (Fig. 6). We next showed Cdh1-silencing led to accumulation of Cdc20, which contributed to G1-S transition and genomic instability (Fig. 6A). Based on these results, we propose that Cdh1 may protect genomic stability in CML-BC through the control of the cell cycle at G0/G1 phase from unscheduled proliferation and degradation of the substrates facilitating genomic instability, such as Skp2 and Cdc20.

In conclusion, we found that TKI and bortezomib could induce cell cycle arrest and cell apoptosis via regulating the expression and redistribution of Cdh1 in CML-BC, and our study shed new light on synergy combination of TKIs and bortezomib in imatinib-resistant CML-BC cells. The role of Cdh1 in maintenance of genomic stability and detailed mechanism of Cdh1 involved in the progression of CML, are worth further exploration.

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