Autophagic degradation determines the fate of T315I-mutated BCR-ABL protein

Marked clinical improvement has been achieved by the use of BCR-ABL-tyrosine kinase inhibitors; however, mutations in BCR-ABL are among the most common causes of resistance to these inhibitors.

We previously found that AIC-47, a novel autophagy inducer, suppresses the expression of BCR-ABL,[1,2] suggesting that AIC-47 could affect the expression of mutated BCR-ABL. So, in the present study we estimated the effects of AIC-47 on BCR-ABL mutant cells by using BCR-ABL-expressing Baf3 cells[3] and TCCY cells.[4] AIC-47 was found to suppress the phosphorylation and expression of wildtype (WT)-BCR-ABL; however, T315I-BCR-ABL expression was hardly suppressed by AIC-47 (Figure 1A and *Online Supplementary Figure S2A*). In Baf3p210 cells harboring other BCR-ABL mutations (M351T or Y253F), the suppression was observed (*Online Supplementary Figure S2B*), suggesting that the regulation of T315I-BCR-ABL generation and/or degradation was different from that of other types of BCR-ABL. We then investigated the generation and degradation of T315I-BCR-ABL. Our previous studies showed that AIC-47 suppresses transcription of *BCR-ABL*.[1] The level of expression of *BCR-ABL* mRNA was decreased after treatment with AIC-47 in both WT- and T315I-BCR-ABL-harboring cells (*Online Supplementary Figure S3A*). AIC-47 also decreased *BCR-ABL* mRNA levels in primary cells from patients (*Online Supplementary Figure S3B*). At steady state, the decay of *BCR-ABL* mRNA was not significantly different between WT- and T315I-BCR-ABL cells (*Online Supplementary Figure S3C*). Moreover, inhibition of translation of *BCR-ABL* mRNA by cycloheximide or puromycin resulted in a delay in the elimination of T315I-BCR-ABL compared with that of WT-BCR-ABL (Figure 1B), suggesting that degradation contributed to the level of BCR-ABL protein expression. In cells, the majority of intracellular proteins are degraded by the ubiquitin-proteasome pathway and autophagy. We found that the autophagy inhibitor 3-methyladenine (3-MA) suppressed the degradation of both types of BCR-ABL; whereas the proteasome inhibitor MG-132 did not sup-

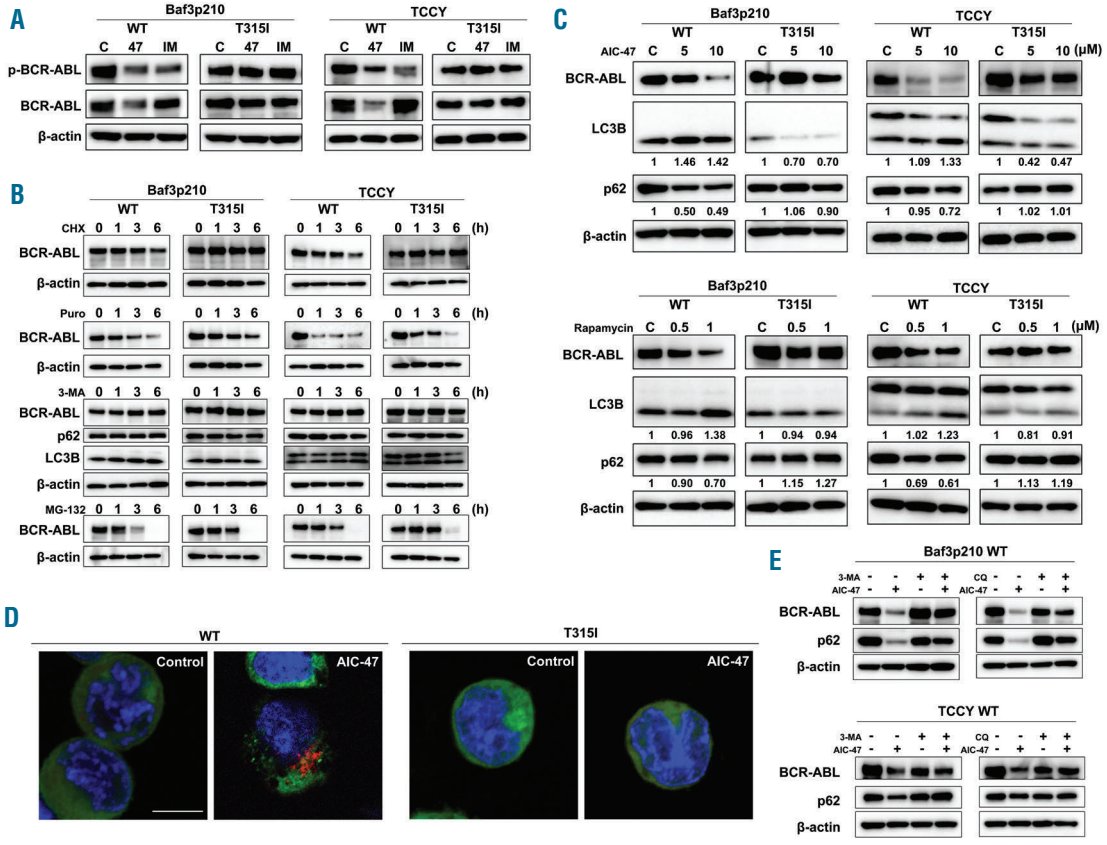

Figure 1. Impaired autophagic degradation increased the stability of T315I-BCR-ABL protein. (A) Effects of AIC-47 or imatinib on phosphorylation and expression of WT- and T315I-BCR-ABL in Baf3p210 and TCCY cells. The cells were treated with dimethylsulfoxide (DMSO; Control: C), AIC-47 (47; 10 μM) or imatinib (IM; 0.25 μM) for 48 h, followed by western blotting analysis. (B) Time-dependent expression of BCR-ABL after the treatments. To inhibit generation of BCR-ABL proteins, Baf3p210 and TCCY cells were treated with cycloheximide (CHX; 2.5 μg/mL) or puromycin (Puro; 1 pg/mL). To inhibit degradation of BCR-ABL proteins, an autophagy inhibitor 3-methyladenine (3-MA; 100 μM) and a proteasome inhibitor MG-132 (10 μM) were used. The levels of expression of BCR-ABL, p62, and LC3B proteins were determined by western blotting analysis. (C) Effect of AIC-47 on autophagy flux and BCR-ABL expression. Expression of BCR-ABL and p62, and conversion of LC3B, in Baf3p210 and TCCY cells treated with AIC-47 (5 or 10 μM) for 24 h were examined by western blotting analysis. The numbers below p62 and LC3B indicate each band density relative to that of the Control (taken as "1"), whose values were determined by densitometry. (D) Distribution of ABL and the autophagy marker LC3 in AIC-47-treated cells. Baf3p210 cells were incubated with AIC-47 (5 μM) for 12 h and then co-immunostained with c-ABL and LC3 antibodies. The cells were viewed with a LSM710 confocal laser scanning microscope. Representative images are shown. ABL is stained green; and the LC3 is stained red. Nuclei appear in blue. (E) Effect of the autophagy inhibitors 3-MA and chloroquine (CQ) on BCR-ABL expression. WT-BCR-ABL-harboring cells were pretreated with 3-MA (100 μM) or CQ (1 μM) for 4 h, and then treated with DMSO or AIC-47 (5 μM) for 24 h. The expression levels of BCR-ABL and p62 were examined by western blotting analysis.

press their degradation (Figure 1B). These findings indicate that autophagic degradation was closely associated with the fate of BCR-ABL protein.

Next, we investigated the induction of autophagy in AIC-47-treated cells. Interestingly, AIC-47 treatment resulted in several findings of autophagy, including: (i) LC3 lipidation, as indicated by the conversion of LC3B I to II; (ii) induction of Atg proteins; and (iii) degradation of p62 in the cells with WT-BCR-ABL, but not in those with T315I-BCR-ABL (Figure 1C and *Online Supplementary Figure S4*). The induction of autophagy occurs preferentially in the G1 and S phases of the cell cycle.[5] The number of WT-BCR-ABL cells in the G0/G1 phase increased after treatment with AIC-47, whereas a change in the profile of the cell-cycle was barely observed in T315I-BCR-ABL cells (*Online Supplementary Figure S1C*). The impaired autophagy in the T315I-mutated cells was also observed in rapamycin-treated cells (Figure 1C and *Online Supplementary Figure S5*). After AIC-47 treatment, LC3 was punctate and co-localized with BCR-ABL in WT-BCR-ABL cells, indicating that BCR-ABL had been degraded in autophagosomes, whereas LC3 puncta were barely observed in T315I-BCR-ABL cells (Figure 1D). To further investigate the AIC-47-induced autophagic degradation of BCR-ABL, we inhibited autophagy flux in WT-BCR-ABL cells by using 3-MA or chloroquine. The pre-treatment with these autophagy inhibitors partly abolished the decreased expression of BCR-ABL induced by AIC-47 (Figure 1E). Based on these results, we speculated that dysregulated autophagic degradation contributed to

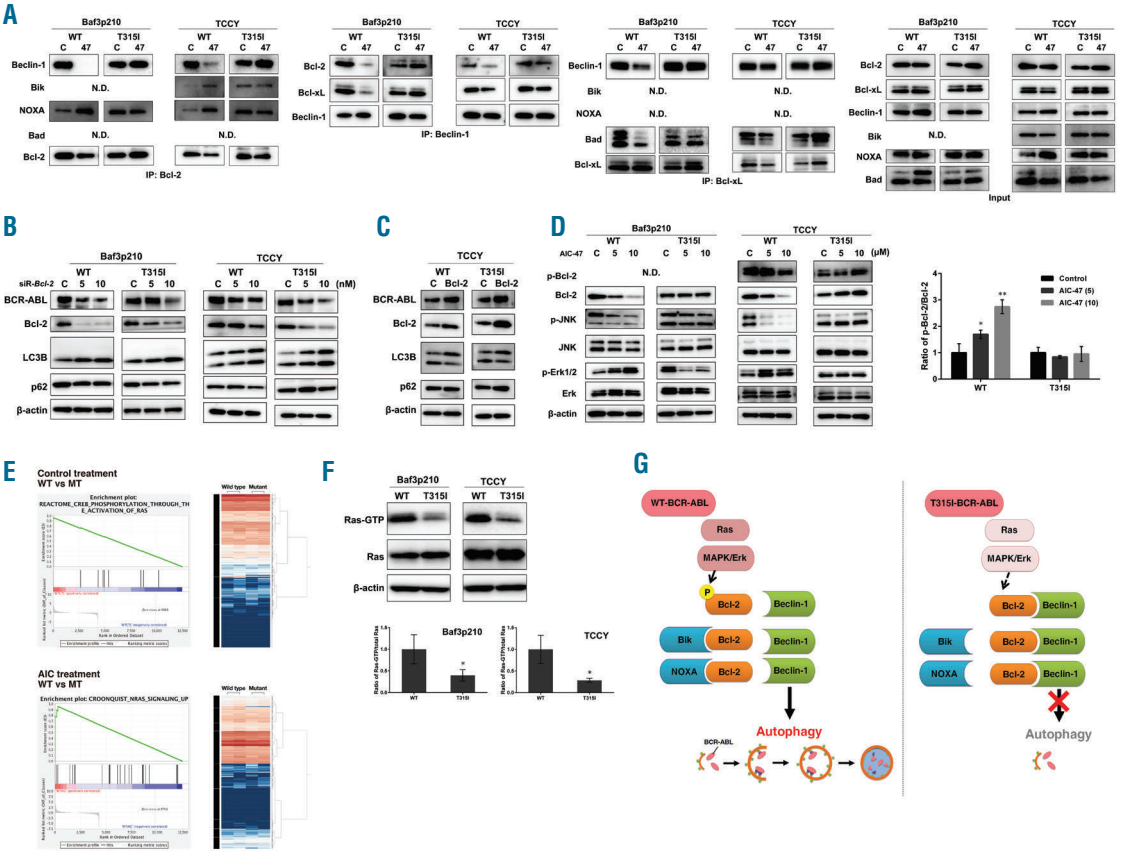

Figure 2. The dissociation of Bcl-2 from Beclin-1 was impaired in T315I-BCR-ABL-harboring cells. (A) Immunoprecipitation of Baf3p210 and TCCY cell lysates. Cells were treated with AIC-47 (5 μM) for 24 h. The expressed Bcl-2, Beclin-1 or Bcl-xL was pulled down by antibodies corresponding to each protein. The binding of each protein was detected by western blotting analysis. Anti-Bik antibody could not detect Bik of mouse origin. (B) Effects of silencing *Bcl-2* on autophagy flux in Baf3p210 and TCCY cells. Cells were transfected with control RNA or siRNA for *Bcl-2* (5 or 10 nM) for 72 h; and then expression levels of BCR-ABL and p62, and the conversion of LC3B, were examined by western blotting analysis. (C) Effects of overexpression of Bcl-2 on autophagy flux in TCCY cells. Cells were transfected with pIRESneo-Bcl-2 vectors for 72 h, and then expression levels of BCR-ABL and p62, and conversion of LC3B, were examined by western blotting analysis. (D) Effects of AIC-47 on the phosphorylation and expression of Bcl-2, JNK, and Erk in Baf3p210 and TCCY cells. The cells were treated with dimethylsulfoxide (DMSO; Control, C), or AIC-47 (5 or 10 μM) for 24 h, followed by western blotting analysis (left panel). Anti-phospho-Bcl-2 antibody could not detect phosphorylated Bcl-2 of mouse origin. The ratio of phosphorylated Bcl-2 was quantified by densitometry scanning and normalized to the expression level of Bcl-2 (right panel). Data are expressed as means ± SD of three different experiments. *P<0.05, ***P<0.001 *versus* Control (Student *t*-test). (E) The heat map shows all genes that were differentially expressed between WT-BCR-ABL cells (WT) and T315I-BCR-ABL cells (MT). Gene set enrichment analysis (GSEA) results of c2 reference gene sets revealed that Ras-related signatures were enriched in steady-state WT-BCR-ABL cells (false discovery rate = 0.138, P<0.001). "NRAS signaling" signatures were also enriched in AIC-47-treated WT-BCR-ABL cells (false discovery rate = 0.039, P<0.001). Horizontal white bars on the left of the heat maps indicate the genes within each gene set. GSEA was performed with GenePattern 2.0 software. (F) Activation of Ras in Baf3p210 and TCCY cells at steady state. GTP-bound Ras was estimated using a Ras assay kit. The level of Ras-GTP was quantified by densitometry scanning and normalized to the total level of Ras. (G) Schematic diagram of the difference in autophagic degradation between WT- and T315I-BCR-ABL. In T315I-BCR-ABL-harboring cells, the activation of Ras/MAPK signaling is less than that in WT-BCR-ABL-harboring cells, which reduces the phosphorylation of Bcl-2. The dissociation of Bcl-2 from Beclin-1 is thus impaired in T315I-BCR-ABL cells, resulting in impaired autophagic degradation of T315I-BCR-ABL.

the increased stability of T315I-BCR-ABL and might be one of the mechanisms of resistance to tyrosine kinase inhibitors.

In the autophagy network, Bcl-2 interacts with Beclin-1 and contributes to the inhibition of autophagy flux.[6,7] Treatment with Z36, another inducer of autophagy, which dissociates Bcl-2/Bcl-xL and Beclin-1,[8] resulted in induction of LC3 lipidation in WT- and T315I-BCR-ABL cells (*Online Supplementary Figure S6*). The expression level of BCR-ABL protein decreased after treatment with Z36 in both WT- and T315I-BCR-ABL cells (*Online Supplementary Figure S6*). These results suggest that the interaction between Bcl-2/Bcl-xL and Beclin-1 is associated with the impaired autophagy in T315I-BCR-ABL cells. Thus, we examined the interaction of Bcl-2/Bcl-xL with Beclin-1 in AIC-47-treated cells. After AIC-47 treatment, Bcl-2 clearly dissociated from Beclin-1 in WT-BCR-ABL cells; whereas binding remained in the T315I-BCR-ABL cells (Figure 2A). Bcl-xL slightly dissociated from Beclin-1 (Figure 2A). There are several different means to regulate the dissociation of Bcl-2 from Beclin-1, including competitive displacement of the Beclin-1 BH3 domain by other Bcl-2 family proteins, or by MAPK (JNK and Erk)-mediated phosphorylation of Bcl-2.[6,7] The interaction with BH3-only proteins Bik or NOXA and Bcl-2 was increased after treatment with AIC-47 in WT-BCR-ABL cells (Figure 2A). Binding with Bcl-2 and Bad was not observed (Figure 2A), suggesting that Bik and NOXA bound to the BH3-binding groove of Bcl-2 and disrupted the interaction between Beclin-1 and Bcl-2. Bcl-xL bound Bad; however, the interaction was slightly decreased (Figure 2A). An interaction between Bik or NOXA and Bcl-xL was not observed (Figure 2A). Based on these data, we speculated that Bcl-2, rather than Bcl-xL, contributed predominantly to the induction of AIC-47-mediated autophagy. In primary chronic myeloid leukemia cells, dissociation of Bcl-2 and Beclin-1 was also observed in WT-BCR-ABL cells, whereas binding remained in T315I-BCR-ABL cells (*Online Supplementary Figure S7A*). The knockdown of Bcl-2 induced autophagy and decreased BCR-ABL expression even in T315I-BCR-ABL cells (Figure 2B). Overexpression of Bcl-2 by using a pIRESneo-Bcl-2 vector[9] reversed the expression of BCR-ABL (Figure 2C). A Bcl-2 inhibitor, ABT-737, induced dissociation of Bcl-2/Beclin-1 and autophagic degradation of both WT- and T315I-BCR-ABL; these effects were enhanced in combination with AIC-47 (*Online Supplementary Figure S7B*). These results suggested that Bcl-2 was one of the essential molecules for autophagic degradation of BCR-ABL. We also investigated the MAPK-mediated phosphorylation of Bcl-2. AIC-47 decreased Bcl-2 expression; whereas the ratio of phosphorylated Bcl-2 was increased in WT-BCR-ABL cells (Figure 2D). After treatment with AIC-47, the level of phosphorylation of JNK was decreased in WT-BCR-ABL cells; however, the level of phosphorylation of Erk was increased in these cells (Figure 2D). A MEK inhibitor decreased the phosphorylation of Bcl-2 and disrupted the AIC-47-mediated autophagy flux (*Online Supplementary Figure S8*). These results suggested that Erk-mediated phosphorylation of Bcl-2 also contributed to the induction of autophagy by AIC-47 and that inactivation of MAPK/Erk signaling was one of the causes of impaired autophagy in T315I-BCR-ABL cells. We speculated that some upstream kinases might contribute to the difference in phosphorylation levels. We, therefore, used gene set enrichment analysis, which revealed that Ras-related signaling was significantly upregulated in WT-BCR-ABL cells (Figure 2E). Previous experiments demonstrated that Ras/MAPK signaling promotes autophagic cell death through the NOXA/Beclin-1 pathway.[10] Ras functions as a molecular switch by cycling between an active GTP-bound form and an inactive GDP-bound form. Interestingly, the amount of GTP-bound Ras was less in T315I-BCR-ABL cells than in WT-BCR-ABL cells (Figure 2F), indicating that the activation of Ras was associated with the difference in activation of MAPK/Erk signaling. However, further investigation is needed.

The increased stability of T315I-BCR-ABL protein was expected to result in a cell growth advantage. Rapamycin (0.5 nM) significantly inhibited cell growth in WT-BCR-ABL cells, whereas inhibition was barely observed in T315I-BCR-ABL cells (*Online Supplementary Figure S9*). The dissociation of Bcl-2/Beclin-1 by treatment with Z36 or silencing of Bcl-2 induced growth inhibition in both WT- and T315I-BCR-ABL cells (*Online Supplementary Figure S9*), but this effect was more marked in the T315I-BCR-ABL cells. These results partly supported our contention that the autophagic degradation of BCR-ABL might be related to cell growth. However, this study has limitations to elucidate the association between autophagy and cell growth completely. AIC-47 could have multiple targets other than autophagic signaling and exhibit anti-leukemic effects even in T315I-BCR-ABL cells. Autophagy is intimately connected with apoptosis, as the same regulators, such as Bcl-2, can control both autophagy and apoptosis.[11] Rapamycin (1 nM) could also inhibit cell growth by induction of apoptosis (*Online Supplementary Figure S9*). Further studies are needed to reveal the association between autophagic degradation of BCR-ABL protein and cell growth.

Autophagy is essential for the maintenance of cellular homeostasis in response to various stress conditions. Depending on the circumstances, autophagy can either promote or inhibit cell survival. Several groups reported that autophagy regulates leukemogenesis[12] and that pharmacological inhibition of autophagy enhances the effects of tyrosine kinase inhibitors.[13] There is opposing evidence with respect to the tumor-inhibiting effects of autophagy, which showed that BCR-ABL acts as a suppressor of autophagy.[14] The anti-leukemic effects of arsenic trioxide are also caused by autophagic degradation of WT-BCR-ABL.[15] Our data indicate that the dissociation of Bcl-2 from Beclin-1 was impaired in T315I-BCR-ABL cells, resulting in the impaired autophagic degradation that contributed to the extension of the half-life of T315I-BCR-ABL (Figure 2G). Although inhibition of autophagy is effective at enhancing the sensitivity of tyrosine kinase inhibitors to BCR-ABL, it could also contribute to increasing the stability of BCR-ABL. Our findings suggest that there is still room for argument about autophagy-targeting cancer therapy.

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Acknowledgments: we thank Dr. Brian J. Druker and Dr. Yuko Sato for giving us the cell lines used.

Funding: this work was supported by grant-in-aid for a research fellow from the Japan Society for the Promotion of Science (JSPS; 16J08131 to HS), grant-in-aid for Scientific Research from the JSPS

(JP18K15273 to HS), and grant-in-aid for scientific research from the Ministry of Education, Science, Sports, and Culture of Japan (25293218 to TN).

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doi:10.3324/haematol.2018.194431

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.

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