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Title:

Ph-like ALL-related novel fusion kinase ATF7IP-PDGFRB exhibits high sensitivity to tyrosine kinase inhibitors in murine cells

Short title: Ph-like ALL-related novel fusion kinase ATF7IP-PDGFRB

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Abstract

ATF7IP-PDGFRB is a novel *PDGFRB*-related fusion gene identified in B-cell precursor acute lymphoblastic leukemia (BCP-ALL) with a signature similar to that of Ph1 ALL, so-called Ph-like ALL. When we introduced *ATF7IP-PDGFRB*, murine Ba/F3 cells acquired the ability to proliferate in an IL-3-independent manner. On the contrary, the expression of wild-type-*PDGFRB* is not sufficient to acquire IL-3-independent proliferation in Ba/F3 cells. The introduction of *ATF7IP-PDGFRB* also induces a typical gene expression profile for Ph1-ALL in Ba/F3 cells. A series of biochemical and cell biological experiments revealed the constitutive activation of *ATF7IP-PDGFRB* as well as downstream signaling molecules, including AKT and MAPK. Although PI3 kinase inhibitor led to cell death in both *ATF7IP-PDGFRB*-introduced and IL-3-maintained Mock cells, MEK inhibitor selectively led to cell death in *ATF7IP-PDGFRB*-introduced cells. The introduction of tyrosine to phenylalanine mutations at binding sites of adaptor molecules important for the MAPK pathway located in the *PDGFRB* portion abolished *ATF7IP-PDGFRB*-mediated cell transformation, suggesting that MAPK-mediated signals are critical in *ATF7IP-PDGFRB*-mediated cell transformation. Upon treatment with

tyrosine kinase inhibitors (TKIs), ATF7IP-PDGFRB-expressing, but not Mock, Ba/F3 cells underwent rapid apoptosis accompanied by a reduced phosphorylation of MAPK. Importantly, the sensitivity of ATF7IP-PDGFRB-expressing Ba/F3 cells to Imatinib is significantly higher than that of BCR-ABL1-transformed Ba/F3 cells, as assessed by IC50. Taken together, ATF7IP-PDGFRB has a transforming potential via the constitutive activation of MAPK and participates in the pathogenesis of Ph-like ALL. Our observations suggest the therapeutic importance of TKIs and possibly MEK inhibitor for a subset of BCP-ALL harboring PDGFRB-related fusion kinases.

Keywords: tyrosine kinase; fusion gene; PDGFRB; MAP kinase; Ph-like ALL

Word count: 3,671

Introduction

The *PDGFRB* gene encodes platelet-derived growth factor receptor- β (PDGFRB) that belongs to the type III receptor tyrosine kinase family [1, 2] and is found rearranged in a subset of myeloproliferative neoplasms (MPNs) with an incidence of 1.8% [3, 4]. These MPN-related PDGFRB fusion proteins are thought to be constitutively activated by enforced dimerization, inducing the stimulation of molecules downstream of PDGFRB in a ligand-independent manner, leading to cell transformation [5-7]. More than 20 fusion partners, including *ETV6* [8], have been reported on MPN-related *PDGFRB* fusion, whereas they have never been found in lymphoid neoplasms.

In contrast, recent reports have shown that distinct *PDGFRB* fusions specifically occur in B-cell precursor acute lymphoblastic leukemia (BCP-ALL) [9-12]. Among these, *EBF1-PDGFRB*, a fusion gene between *early B-cell factor 1* (*EBF1*) and *PDGFRB*, is the most recurrent one.

BCP-ALL expressing *EBF1-PDGFRB* possesses a *BCR-ABL1*-positive (Ph1) ALL-like gene-expression profile and is significantly correlated with a poor prognosis, regardless of the absence of a *BCR-ABL1* fusion gene, so-called Ph-like ALL [9, 12-14]. It is noteworthy that

EBF1-PDGFRB-positive ALL cases were reported to exhibit high sensitivity to tyrosine kinase inhibitor (TKI) [9-11].

In connection with this, we recently reported another novel BCP-ALL-specific *PDGFRB*-related fusion gene, *ATF7IP-PDGFRB*, that originated from t(5;12)(q33;p13) [15], which also revealed a Ph-like gene expression profile and relapsed 5 months after the cessation of therapy [15, 16]. Although the fusion partner activating transcription factor 7-interacting protein (ATF7IP) is a transcriptional cofactor suggested to function as a transcriptional regulator upon binding with methylated DNA-binding domain protein 1 (MBD1) [17-20], the ATF7IP-PDGFRB fusion protein most likely does not induce the deregulation of ATF7IP-mediated transcriptional regulation, but is involved in the activation of tyrosine kinase signaling by oligomerization [15], similarly to cases of other MPN-related *PDGFRB* fusions [7], being responsible for the development of Ph-like ALL. However, the actual state has yet to be clarified.

We therefore aimed to investigate the functional characteristics of ATF7IP-PDGFRB and its role in the development of ALL in comparison with that of *EBF1-PDGFRB* as well as wild-type (WT)-*PDGFRB*. In this study, we report that ATF7IP-PDGFRB fusion protein is indeed activated

constitutively as tyrosine kinase and functionally involved in the pathogenesis of ALL, showing high sensitivity to TKIs. Notably, our data indicate that mitogen-activated protein kinase (MAPK) plays a critical role in ATF7IP-PDGFRB-mediated leukemic transformation.

Materials and methods

Cells and Reagents

Ba/F3 and WEHI-3 cells (RIKEN Bioresource Center, Tsukuba, Ibaraki, Japan) were maintained as described previously [21]. In the case of Ba/F3 cells, 10% (vol/vol) conditioned medium from the WEHI-3 cell line containing IL-3 was supplemented for maintenance. As we reported previously, WEHI-3- conditioned medium has the same effect as purified IL-3 on the growth and survival of Ba/F3 cells [21].

The MAPK kinase (MEK) inhibitor U0126, PD98059, and phosphatidylinositol 3-kinase (PI3 kinase) inhibitor LY294002 were purchased from Merck Millipore (Darmstadt, Germany). The TKIs imatinib, dasatinib, Nilotinib (LC Laboratories, Woburn, MA, USA), Ponatinib, Rebastinib, and Bafetinib (Selleck, Houston, TX, USA) were used.

Plasmid construction and detection of transduced gene expression

The details of plasmid construction are described in SUPPLEMENTARY INFORMATION. The cDNA of WT-*PDGFRB* was purchased from Promega Corporation (Madison, WI, USA) as the

Flexi® ORF clone pF1KB7023 (kazusa clone cp01083). The coding region was isolated and subcloned into either pGEM-T easy or pGEM-T (Promega).

The use of clinical materials was approved by the institutional review boards and informed consent was obtained from the parents or guardians. A 5' portion of *ATF7IP* cDNA included in the *ATF7IP-PDGFRB* fusion gene was amplified from a clinical specimen of a patient carrying the fusion gene by PCR using KOD plus ver.2 (Toyobo Co., Ltd., Osaka, Japan) and subcloned into pGEM-T easy [21]. To develop full-length ORF of *ATF7IP-PDGFRB* cDNA, the cDNA fragments of *ATF7IP* were subcloned into pGEM-T-WT-*PDGFRB*. Similarly, a 5' portion of *EBF1-PDGFRB* cDNA was amplified and subcloned into pGEM-T easy and the full-length ORF of *EBF1-PDGFRB* cDNA was developed by subcloning of a fragment of *EBF1* into pGEM-T-WT-*PDGFRB*.

To develop a retroviral expression vector, the full-length insert of each cDNA of the fusion gene was digested and subcloned into pRetroX Tight (Clontech Laboratories, Inc., Madison, WI, USA).

The pRetroX Tight-*ATF7IP-PDGFRB* plasmid was used to generate tyrosine to phenylalanine mutants by site-directed mutagenesis using the Transformer™ Site-Directed Mutagenesis kit

(Agilent Technologies, Inc., Palo Alto, CA, USA) according to the manufacturer's protocol. The oligonucleotides used for mutagenesis are listed in Supplementary Table 1.

The expressions of *ATF7IP-PDGFRB*, *EBF1-PDGFRB*, and *WT-PDGFRB* genes were amplified from cDNA prepared from transduced Ba/F3 cells with each gene by PCR (Supplementary Table 1) using rTaq polymerase, as described previously [22]. As an internal control, the primer set for the detection of mouse β -actin (Agilent Technologies, Santa Clara, CA, USA) was also used.

Gene transduction

Chimera gene-inducible cell lines were generated by employing retroviral transduction using the Retro-X™ Tet-On® Advanced Inducible Expression System and Retro-X™ Universal Packaging System (Clontech), basically as described previously [22]. In this system, Tet-on Advanced vector carrying the neomycin resistance gene and pRetroX Tight expression vector carrying the puromycin resistance gene were used. The 6×10^5 Ba/F3 cells that introduced Tet-on Advanced maintained in the presence of neomycin were further infected with retrovirus of pRetroX Tight including

ATF7IP-PDGFRB in a total of 3 mL of medium and exposed for 10 hours. Cells were then washed, divided into two parts, and treated with puromycin for drug selection. One half of the cells sequentially received drug selection. The subsequent stable resistant cells were cloned by repeated limiting dilution 3 times and used in the following experiments. The remaining half was further divided into two parts and treated with or without 1 µg/mL of doxycycline (DOX) to induce the target proteins for 24 hours, and then IL-3-independent cell proliferation was assessed by the cell proliferation assay. The Ba/F3 cells transduced with *EBF1-PDGFRB*, *WT-PDGFRB*, and empty vector, as Mock, were similarly developed. Experiments were repeated at least three times independently and representative data are presented.

Cell proliferation assay

The fusion gene-expressing- and Mock Ba/F3 cells were washed twice with medium and dispensed into 96-well plates (2.5×10^4 cells/0.1 mL per well) in the presence or absence of the factors and cultured for the periods indicated in the figures at 37°C. Cell proliferation was analyzed using water-soluble tetrazolium salt (WST) assays (Cell Counting Kit-8, Dojindo, Kumamoto, Japan), as

described previously [23]. All experiments were performed in triplicate, and means and SEM were calculated. Experiments were repeated at least three times independently and representative data are presented.

Flow cytometric analysis and immunocytochemistry

The frequency of apoptotic cells was quantified using the MEBCYTO[®] Apoptosis Kit (Medical and Biological Laboratories, Nagoya, Japan), and then analyzed according to the manufacturer's protocol [21]. Experiments were performed in triplicate, and the means and SEM were calculated. Experiments were repeated three times independently and representative data are presented.

For immunocytochemistry, cells were treated with the PerFix-nc Kit (Beckman Coulter, Inc., Indianapolis, IN, USA) according to the manufacturer's protocol and stained with a combination of rabbit monoclonal anti-PDGFRB antibody (Ab) (Cell Signaling) and Alexa Fluor[®] 546 goat anti-rabbit IgG (Life Technologies, Waltham, MA, USA). The cells were mounted on a slide glass with 4',6-diamidino-2-phenylindole (DAPI) counterstaining using Fluoroshield Mounting Medium

with DAPI (Immunobioscience Corp., Mukilteo, WA, USA) and visualized by DeltaVision Elite (GE Healthcare Life Sciences, Buckinghamshire, UK).

Immunoblotting

Immunoblot analysis was performed as described previously [21, 24] using the Abs listed in Supplementary Table 2, as well as horseradish peroxidase (HRP)-conjugated secondary Abs (Dako, Glostrup, Denmark). Experiments were repeated at least three times independently, and representative data are presented.

Statistical analysis

Data were analyzed using Student's t-test. Values of p less than 0.05 were considered significant.

Results

The transduction of ATF7IP-PDGFRB fusion molecules but not WT-PDGFRB induces the

IL-3-independent proliferation of Ba/F3 cells

Firstly, we examined the effects of ATF7IP-PDGFRB fusion molecule expression on proliferation in Ba/F3 cells by employing a tetracycline-inducible gene expression system. After transduction with *ATF7IP-PDGFRB*-expression vector, some DOX-treated transduced-Ba/F3 cells survived and grew in the absence of conditioned medium containing IL-3, while none of the DOX-untreated transduced Ba/F3 cells could survive without IL-3 (Figure 1A). In contrast, the Mock Ba/F3 cells transduced with empty vector could not survive without IL-3 independently of DOX treatment (Figure 1A). Ba/F3 cells transduced with *EBF1-PDGFRB* also exhibited IL-3-independent proliferation after DOX treatment similarly to *ATF7IP-PDGFRB*, whereas *WT-PDGFRB* did not (data not shown).

After the drug selection and cloning of transduced cells, it was further confirmed that *ATF7IP-PDGFRB*- as well as *EBF1-PDGFRB*-transduced cells showed cell proliferation in the absence of IL-3, whereas Mock cells could only proliferate in the presence of IL-3 (Figure 1B).

The growth rates of transduced Ba/F3 cells with *ATF7IP-PDGFRB* were comparable to parental Ba/F3 cells growing in the presence of IL-3.

In the case of WT-*PDGFRB*, the transduced Ba/F3 cells could not survive without IL-3 even if the ligand PDGFBB was present (Figure 1B). Interestingly, however, the addition of PDGFBB could partially prolong the survival of WT-PDGFRB-transduced Ba/F3 cells in the absence of IL-3, as assessed by WST and Annexin V assays (Figure 1C & D).

As shown in Figure 1E & F, we confirmed appropriate mRNA and protein expressions by RT-PCR and immunoblot analysis, respectively, in each transduced Ba/F3 cell.

Immunocytochemistry revealed the cytoplasmic localization of ATF7IP-PDGFRB fusion proteins (Figure 1G). The above data indicate that the expression of ATF7IP-PDGFRB as well as EBF1-PDGFRB chimeric proteins, but not WT-PDGFRB, induces IL-3-independent proliferation in Ba/F3 cells.

Gene expression induced by ATF7IP-PDGFRB in Ba/F3 cells

Since TEL-PDGFRB and BCR-ABL have been reported to regulate overlapping and distinct gene transcription profiles [25], we subsequently examined the effects of ATF7IP-PDGFRB fusion molecules on gene expression in Ba/F3 cells by employing an oligonucleotide microarray. As shown in Supplementary Figure 1A and Supplementary Table 3-8, Ba/F3 cells expressing ATF7IP-PDGFRB also revealed gene transcription profiles overlapping and distinct with those expressing p190 BCR-ABL1. However, only a part of the overlapping genes between ATF7IP-PDGFRB and p190 BCR-ABL1, including *Cish*, *Phlda1*, and *Spred1*, are common with those genes overlapping between TEL-PDGFRB and BCR-ABL1 [25] (Supplementary Table 3).

To test whether or not the expression of ATF7IP-PDGFRB induces the Ph1-ALL-related gene expression profile, we employed gene set enrichment analysis (GSEA), as described previously [15]. When the top 600 genes exhibiting higher expressions were examined with GSEA, ATF7IP-PDGFRB- as well as p190 BCR-ABL1- expressing Ba/F3 cells were revealed to have a gene expression profile that was enriched in Ph1-ALL (Supplementary Figure 1B).

Intracellular signaling mediated by ATF7IP-PDGFRB fusion molecules in Ba/F3 cells

Next, we assessed the molecular basis of the IL-3-independent cell proliferation due to the expression of *ATF7IP-PDGFRB* fusion molecules. Since PDGFRB is a receptor tyrosine kinase, we examined the state of tyrosine phosphorylation of cellular proteins by employing immunoblot analysis. As shown in Figure 2A, intracellular proteins were tyrosine-phosphorylated in *ATF7IP-PDGFRB*- as well as *EBF1-PDGFRB*-expressed Ba/F3 cells in comparison with Mock cells. In the case of WT-PDGFRB, the increase in tyrosine phosphorylation of intracellular proteins was induced after stimulation with PDGFBB. We also confirmed that the *ATF7IP-PDGFRB* as well as *EBF1-PDGFRB* and PDGFBB-stimulated WT-PDGFRB were included in the above-mentioned tyrosine phosphorylated intracellular proteins by detecting the tyrosine phosphorylation of specific residues located in PDGFRB kinase, including Tyr771 (Figure 2B), Tyr751, Tyr1009, and Tyr1021 (Supplementary Figure 2).

Next, we examined the phosphorylation of molecules located downstream of PDGFRB. Upon phosphorylation, the Tyr751 and Tyr771 residues on PDGFRB were reported to allow the binding and activation of PI3 kinase/Shc/Nck β , and RasGap/Shc, respectively [26-29]. Consistent with Tyr751 and Tyr771 phosphorylation on PDGFRB, AKT, MAPK (Erk1/2), p38 MAPK, and JNK

(Figure 2C), downstream molecules of PI3 kinase, Nck β , and Ras signaling pathways, respectively, were phosphorylated in ATF7IP-PDGFRB or EBF1-PDGFRB-transduced Ba/F3 cells as well as PDGFBB-stimulated WT-PDGFRB-introduced Ba/F3 cells. Consistent with Tyr1009 and 1021 phosphorylation on PDGFRB that allows activation of PLC- γ [30], PLC- γ was phosphorylated in Ba/F3-transduced with PDGFRB-related chimeras. Interestingly, the phosphorylation of the same residue on PLC- γ on WT-PDGFRB-expressing cells was not significant, even after stimulation with PDGFBB (Figure 2C).

Importance of MAPK and AKT in IL-3-independent proliferation of Ba/F3 cells mediated by

ATF7IP-PDGFRB

Since ATF7IP-PDGFRB has been shown to phosphorylate AKT and MAPKs, we examined the effect of inhibitors of PI3 kinase and MEK on the IL-3-independent proliferation of ATF7IP-PDGFRB-expressing Ba/F3 cells. As presented in Figure 3A, the AKT inhibitor LY294002 induced cell death in both ATF7IP-PDGFRB-expressing Ba/F3 and Mock cells. Interestingly, however, the MEK inhibitor PD98059 induced cell death only in

ATF7IP-PDGFRB-expressing Ba/F3 cells, and not in Mock cells (Figure 3A). We also examined the effect of another MEK inhibitor, U0126, and essentially similar results were observed (Figure 3A). When IL-3 was simultaneously added, MEK-inhibitor-induced cell death in ATF7IP-PDGFRB-expressing Ba/F3 cells was avoided partially (Figure 3B). We further confirmed that treatment with PD98059 and LY294002 markedly reduced the phosphorylation of AKT and MAPK, respectively, as assessed by immunoblotting (Figure 3C). The data indicate the importance of the activation of both AKT and MAPK in the ATF7IP-PDGFRB-mediated IL-3-independent proliferation of Ba/F3 cells.

Identification of the ATF7IP-PDGFRB tyrosine residues critical for IL-3-independent proliferation in Ba/F3 cells

To determine the tyrosine residues critical for IL-3-independent proliferation in Ba/F3 cells, we generated a series of ATF7IP-PDGFRB mutants containing tyrosine to phenylalanine point mutations designed to abrogate the binding of specific downstream signaling molecules to the PDGFRB portion (Figure 4A). The Ba/F3 cell lines expressing each of the tyrosine to

phenylalanine mutants were assessed for their ability to proliferate in the absence of IL-3. As presented in Figure 4B, Ba/F3 cells expressing the phenylalanine mutant of Tyr1009 and Tyr1021 residues (Y1009F and Y1021F) on PDGFRB conferred IL-3-independent proliferation in Ba/F3 cells. However, Ba/F3 cells expressing Y716F-, Y751F-, Y775F-mutant as well as kinase-inactive arginine mutant of Lys634 (K634R) could not survive without IL-3. In contrast, Ba/F3 cells expressing Y740F- and Y771F-mutant exhibit very slower proliferation in the absence of IL-3. As we mentioned above, the Tyr716, Tyr740, Tyr751, Tyr771, and Tyr775 residues allow the binding and activation of Grb2, PI3 kinase/Shc/Nck β , RasGAP/Shc, and Grb2/Grb7, located upstream of MAPK and the AKT pathway, while Tyr1009 and Tyr1021 residues allow the binding and activation of PLC- γ . Therefore, the data indicate that the specific tyrosine residues which allow the binding of upstream molecules of MAPK and the AKT pathway are critical for IL-3-independent proliferation in Ba/F3 cells mediated by ATF7IP-PDGFRB.

Effect of TKIs on survival of Ba/F3 cells expressing ATF7IP-PDGFRB fusion molecules

We subsequently examined the effect of TKIs on the IL-3-independent proliferation of Ba/F3 cells expressing ATF7IP-PDGFRB fusions. When ATF7IP-PDGFRB-expressing Ba/F3 cells were exposed to imatinib and dasatinib, cell proliferation was significantly inhibited in a concentration-dependent manner, and the viable cell number was reduced to under 10% after 48-hour incubation with imatinib at a concentration of 25 nM and dasatinib at a concentration of 2.5 nM (Figure 5A). Similarly, EBF1-PDGFRB-expressing Ba/F3 cells also revealed high sensitivity against imatinib and dasatinib (Figure 5A). The calculated IC₅₀ values of imatinib against ATF7IP- and EBF1-PDGFRB-expressing Ba/F3 cells at 24 hours of incubation were 7.8 and 14.1 nM, respectively, being much lower than the 2.5 μ M of previously established [21] BCR-ABL-expressing Ba/F3 cells (data not shown). In contrast, the calculated IC₅₀ values of dasatinib against ATF7IP- and EBF1-PDGFRB-expressing Ba/F3 cells at 24 hours of incubation were 1.0 and 5.0 nM, respectively, not being significantly different from the 5.0 nM of BCR-ABL-expressing Ba/F3 cells (data not shown).

We also examined the effect of other next-generation TKIs, including nilotinib, bafetinib, rebastinib, and ponatinib, and observed an effective reduction in numbers of viable Ba/F3 cells

expressing ATF7IP-PDGFRB or EBF-PDGFRB by each TKI (Figure 5B). We further confirmed that the phosphorylation of AKT and MAPK, as well as PDGFRB, was indeed reduced in PDGFRB-fusion-expressing Ba/F3 cells after treatment with TKIs, as assessed by immunoblot analysis (Figure 5C). The data indicate that TKIs effectively suppress cell survival in Ba/F3 cells mediated by ATF7IP-PDGFRB by inhibiting tyrosine kinase activity as well as the consequent activation of AKT and MAPK.

Discussion

In this study, we revealed that the expression of ATF7IP-PDGFRB induces IL-3-independent proliferation in Ba/F3 cells, indicating the transforming potential of ATF7IP-PDGFRB, similarly to cases of EBF1-PDGFRB and other PDGFRB-related MPN-type fusion-kinases [9, 5-7]. Our data also show that ATF7IP-PDGFRB is constitutively phosphorylated on tyrosine residues and activates downstream molecules, including AKT, PLC- γ , and MAPKs. Furthermore, we observed that the expression of ATF7IP-PDGFRB induces the Ph1-ALL-related gene expression profile (Supplementary Figure 1B). Based on the above observations, we concluded that *ATF7IP-PDGFRB* is a driver gene for the development of lymphoid neoplasms and it participate in the pathogenesis of Ph-like ALL.

In contrast, our data also revealed that WT-PDGFRB introduced into Ba/F3 cells was not sufficient for IL-3-independent survival, while stimulation with PDGFBB partially protected Ba/F3 cells from the apoptotic cell death induced by the withdrawal of IL-3. Therefore, constitutive activation as tyrosine kinase mediated by fusion protein conformation should be necessary for cell transformation.

We further observed that the activation of AKT and MAPK was important for the IL-3-independent survival of Ba/F3 cells. The phenylalanine mutations of tyrosine residues 716, 740, 751, 771, and 775 that allow the binding and activation of upstream molecules of the AKT and MAPK pathway abolished the IL-3-independent proliferation of Ba/F3 cells mediated by ATF7IP-PDGFRB. However, Tyr1009 and Tyr1021, which allows the binding of PLC γ 1, did not affect the ability of ATF7IP-PDGFRB to induce IL-3-independent proliferation, indicating the distinct magnitude of each Tyr residue in the molecular mechanism of leukemic transformation mediated by ATF7IP-PDGFRB.

We showed that PI3 kinase inhibitor leads to cell death in both ATF7IP-PDGFRB-introduced cells and Mock cells maintained by IL-3, indicating that signals mediated by AKT are essential for cell survival in both cases. In contrast, MEK inhibitors led to cell death in ATF7IP-PDGFRB-introduced cells but not Mock cells, indicating the difference in cell survival signals induced by ATF7IP-PDGFRB and IL-3 receptor. Although the precise mechanism is not known at this time, it was suggested that MAPK-mediated signals are more critical in ATF7IP-PDGFRB-mediated cell transformation. One possible explanation is that the IL-3

receptor could simultaneously activate a surrogate pathway for cell survival that can substitute for the MAPK cascade, whereas ATF7IP-PDGFRB could only activate the MAPK cascade, and no other substitution pathway. Although the detailed mechanism has not yet been clarified, the importance of the MAPK cascade in malignant transformation has been well-described [31, 32]. Further clarification of the downstream molecular event for MAPK should provide new insight into PDGFRB kinase-mediated cell transformation. In addition, it will also be useful to develop a new therapeutic strategy targeting the MAPK pathway [33] against hematologic malignancies caused by PDGFRB translocation.

Interestingly, Tomasson and his coworker reported that the introduction of tyrosine to phenylalanine mutations had no effect on PDGFRB kinase activity in the context of the TEL-PDGFRB fusion and Ba/F3 cell lines expressing each of these mutants which conferred IL-3-independent growth to Ba/F3 cells [34] and, thus, their observation on TEL-PDGFRB fusion is totally distinct from our observation on ATF7IP-PDGFRB fusion. Although the precise reason for the difference is not known at this time, it is possible that the distinct fusion partners differentially affect the downstream signaling of the PDGFRB kinases.

As stated above, ATF7IP-PDGFRB and EBF1-PDGFRB have so far been found only in ALL patients but other PDGFRB fusion genes have been found only in myeloid neoplasms, suggesting selectivity between fusion partners of PDGFRB and the lineage of the developing neoplasms, whereas the precise mechanism for the selectivity has yet to be clarified. Clarification of the mechanism of ATF7IP-PDGFRB selectivity for ALL development is awaited.

Importantly, our data also demonstrate that ATF7IP-PDGFRB-expressing Ba/F3 cells show high sensitivity for TKIs, especially imatinib. The estimated IC50 of imatinib is much lower than that of BCR-ABL1. This observation is consistent with the clinical observations of others who reported the effectiveness of imatinib for patients with PDGFRB-rearranged leukemia [3, 10, 11]. TKIs can effectively inhibit kinase activity of PDGFRB and reduce the activation of AKT and MAPK, required for cell survival. The high sensitivity of rearranged PDGFRB kinases to TKIs is important in the therapeutic consideration of TKI usage for patients with PDGFRB-rearranged ALL.

In conclusion, ATF7IP-PDGFRB has a transforming potential due to the constitutive activation of AKT and MAPK, participating in the pathogenesis of Ph-like ALL. Although further investigation is needed to assess the precise mechanisms, further clarification of the molecular basis

of the importance of the MAPK pathway in the fusion PDGFRB kinase-mediated cell transformation should provide a new therapeutic strategy against hematologic malignancies caused by PDGFRB translocation. In addition, our experimental observation that ATF7IP-PDGFRB has significant sensitivity to TKIs in comparison with BCR-ABL1 is consistent with the clinical findings, indicating the therapeutic usefulness of TKIs for a subset of BCP-ALL harboring PDGFRB-related fusion kinases.

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Authorship

TI and NK conceived and coordinated the study, performed and analyzed all of the experiments, and wrote the paper. AK and KT designed and constructed vectors for expression of mutant proteins performed and analyzed the experiments shown in Figures 3 and 4. KI provided technical

assistance and analyzed the experiments shown in Supplementary Figure 1 and Supplementary Tables. HU, OT, KK, HO and KO provided technical assistance, analyzed the experiments, and contributed to the preparation of the figures. JF and TS conceived the study and drafting the article. All authors approved the final version of the manuscripts.

Competing interests

The authors declare that they have no conflicts of interest with the contents of this article.

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Legends for Figures

Figure 1. IL-3-independent cell proliferation induced by *ATF7IP-PDGFRB* but not wild-type

(WT)-*PDGFRB*. **(A)** Ba/F3 cells were transduced with *ATF7IP-PDGFRB*-expressing retroviral vector or empty vector (Mock) by employing a tetracycline-inducible system. After 10-hour exposure to retrovirus followed by 24-hour treatment with or without doxycycline (Dox), cells were washed and IL-3 was withdrawn. After 60-hour incubation, the numbers of living cells were estimated by water-soluble tetrazolium salt (WST) assays. The mean $\pm$ SEM of triplicates of each treatment is indicated by a bar graph. Data shown are representative of three independent experiments. **(B)** Cloned Ba/F3 cells transduced with *ATF7IP-PDGFRB*, *EBF1-PDGFRB*, and wild-type *PDGFRB* (WT) were cultured in the absence of IL-3 for the indicated periods and examined with the WST assay. In the case of WT transduced cells, two conditions: with or without (w/o) 100 ng/mL of PDGFBB, were tested. As a control, Mock Ba/F3 cells transduced with empty vector with or without (w/o) IL-3 were simultaneously examined. OD values are shown as the mean $\pm$ SEM of triplicates. Data shown are representative of three independent experiments. **(C)** The results of WT with or w/o

PDGFBB and Mock w/o IL-3 presented in B were extracted, magnified on the Y-axis, and presented. (D) The same sample preparations of WT-*PDGFRB*-transduced cells as in B were examined by the Annexin-V-binding assay. As controls, Mock cells treated with (+) or without (-) the indicated combinations of PDGFBB and IL-3 were also similarly examined. Experiments were performed in triplicate and the mean $\pm$ SEM of positivity (%) are presented with a typical cytogram. Data shown are representative of three independent experiments. X-axis, fluorescence intensity; Y-axis, relative cell number. (E) Total RNA was extracted from 1×10^7 of Ba/F3 cells transduced with *ATF7IP-PDGFRB* (AT), *EBF1-PDGFRB* (EB), WT-*PDGFRB* (WT), and empty vector (Mock, MO), and the expressions of each gene and *β -actin*, as an internal control, were examined by PCR. The ϕ /*Hae*III DNA markers were applied to the same gel (Ma). (F) The expression of each type of PDGFRB was examined by immunoblot analysis. In the case of ATF7IP-PDGFRB, the fusion proteins were detected by both anti- (α -)ATF7IP antibody and anti- (α -)PDGFRB antibody, respectively. As an internal control, *β -actin* was also blotted. Data shown are representative of three independent

experiments. (G) The expression of ATF7IP-PDGFRB in Ba/F3 cells was detected by immunocytochemistry. Scale bar, 100 μ m.

Figure 2. Increased tyrosine phosphorylation mediated by the expression of ATF7IP-PDGFRB.

(A) Cell lysates were prepared from transduced Ba/F3 cells, and protein tyrosine phosphorylation was detected by immunoblotting using anti- (α -)phosphotyrosine antibody 4G10. (B) The same sample preparations as in A were similarly examined using phospho-specific anti- (α -)pY771-PDGFRB antibodies. As an internal control, β -actin was also blotted. (C) Enhanced phosphorylation of signaling molecules located downstream of PDGFRB. The same sample preparations as in A were similarly examined using phospho-specific antibodies indicated in the figure. As an internal control, entire proteins of each molecule were also detected by non-phospho-specific Abs. ATF7IP-PDGFRB, AT; EBF1-PDGFRB, EB; WT-PDGFRB, WT; with ligand (L) stimulation (100 ng/mL of PDGFBB for 5 minutes, unless otherwise indicated), +; without ligand stimulation, (-); Mock/empty vector, MO. Data shown are representative of at least three independent experiments. The

immunoblot experiment with α -pErk1/2 was performed using membranes divided into two parts because of the excessive exposure.

Figure 3. Effect of inhibitors against PI3 kinase and MEK on the proliferation of Ba/F3

transduced with ATF7IP-PDGFRB. (A) ATF7IP-PDGFRB-expressing Ba/F3 cells in the absence of IL-3 and Mock Ba/F3 cells in the presence of IL-3 were treated with or without either the PI3 kinase inhibitor LY294002 or MEK inhibitor PD98059 and U0126 at the indicated concentrations for 24 hours. Subsequent numbers of living cells were estimated by the WST assay, as in Figure 1B. Data obtained from inhibitor-treated cells are expressed as the ratio of the OD value against that obtained from untreated cells (Relative cell viability) and the means $\pm$ SEMs of triplicates. Data shown are representative of three independent experiments. Statistical significance was observed (PD98059 50 and 100 μ M, $p < 0.01$; U0126 40 and 50 μ M, $p < 0.05$). (B) ATF7IP-PDGFRB-expressing Ba/F3 cells were treated with PD98059 in the presence or absence of IL-3 and examined as in A. Data shown are representative of three independent experiments. Statistical significance was observed (50

μM , $p<0.05$; $100 \mu\text{M}$, $p<0.01$). (C) Cell lysates were prepared from the Ba/F3 cells treated with $50 \mu\text{M}$ of LY294002 or PD98059 for 12 hours. Immunoblot analysis was performed as in Figure 2. Without inhibitors, None; with LY294002, LY; with PD98059, PD. Data shown are representative of at least three independent experiments.

Figure 4. The role of tyrosine residues on the PDGFRB portion in ATF7IP-PDGFRB-mediated transformation. (A) Schematic presentation of tyrosine to phenylalanine mutation of ATF7IP-PDGFRB is shown. Each tyrosine residue related to the downstream signal transduction of PDGFRB was replaced with phenylalanine by site-directed mutagenesis as indicated. The K634R mutant was also included as a control for nontransforming ATF7IP-PDGFB mutant. The K634R mutant contains a lysine to arginine substitution in the catalytic domain of the PDGFB portion of the fusion that abrogates tyrosine kinase activity. (B) The proliferation of Ba/F3 cells transduced with each ATF7IP-PDGFRB-mutant in the absence of IL-3 was examined by the WST assay, as indicated in Figure 1B. As a control, Mock Ba/F3 cells transduced with empty vector with or without IL-3 were simultaneously

examined. OD values are shown as the mean $\pm$ SEM of triplicates. Data shown are representative of six independent experiments.

Figure 5. Effect of tyrosine kinase inhibitors on the proliferation of Ba/F3 transduced with PDGFRBs. ATF7IP- and EBF1-PDGFRB-expressing cells as well as Mock Ba/F3 cells maintained with IL-3 were treated with or without the tyrosine kinase inhibitors (**A**), imatinib or dasatinib and (**B**), other next-generation TKIs at the indicated concentrations for 48 hours. Subsequent numbers of living cells were estimated by the WST assay, as in Figure 1B. Data obtained from inhibitor-treated cells are expressed as the ratio of the OD value against that obtained from untreated cells (Relative cell viability) and the means $\pm$ SEMs of triplicates. Data shown are representative of at least three independent experiments. (**C**) Cell lysates were prepared from the same sample preparations treated with (+) or without (-) 100 nM of imatinib for 12 hours as in A. Immunoblot analysis was performed as in Figure 2. Data shown are representative of at least three independent experiments.

Figures
Figure 1

Figure 2

Figure 3

Figure 4

A

B

* Mock with IL-3, *ATF7IP-PDGFRB*, Y1009F, Y1021F vs. Y740F, Y771F, $p < 0.01$
Mock with IL-3, *ATF7IP-PDGFRB*, Y1009F, Y1021F
vs. Mock w/o IL-3, K634, Y716F, Y751F, Y775F, $p < 0.01$
Y740F, Y771F vs. Mock w/o IL-3, K634, Y716F, Y751F, Y775F, $p < 0.01$ *

Figure 5