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Targeting miR-21 sensitizes Ph+ ALL Sup-b15 cells to imatinib-induced apoptosis through upregulation of PTEN

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ABSTRACT

Philadelphia chromosome positive (Ph+) acute lymphoblastic leukemia (ALL) cells are insensitive to BCR-ABL tyrosine kinase inhibitor imatinib, the underlying mechanisms remain largely unknown. Here, we showed that imatinib treatment induced significant upregulation of miR-21 and downregulation of PTEN in Ph+ ALL cell line Sup-b15. Transient inhibition of miR-21 resulted in increased apoptosis, PTEN upregulation and AKT dephosphorylation, whereas ectopic overexpression of miR-21 further conferred imatinib resistance. Furthermore, knockdown of PTEN protected the cells from imatinib-induced apoptosis achieved by inhibition of miR-21. Additionally, PI3K inhibitors also notably enhanced the effects of imatinib on Sup-b15 cells and primary Ph+ ALL cells similar to miR-21 inhibitor. Therefore, miR-21 contributes to imatinib resistance in Ph+ ALL cells and antagonizing miR-21 demonstrates therapeutic potential by sensitizing the malignancy to imatinib therapy.

1. Introduction

Acute lymphoblastic leukemia (ALL) is a heterogeneous disease with multiple, prognostically relevant genetic aberrations and characterized by the over-production of immature white blood cells [1]. In adults, 20–30% of patients with an aggressive form of ALL express the BCR-ABL oncogene as the result of the Philadelphia-translocation *t*(9;22)(q34;q11) and have a very poor prognosis [2]. The oncoprotein BCR-ABL is a constitutively active tyrosine kinase, which promotes uncontrolled cell proliferation [3]. Imatinib, a potent and selective inhibitor of the tyrosine kinase activity of BCR-ABL, has revolutionized the therapy of another form of Ph+ cancer, chronic myelogenous leukemia (CML), by inducing durable remissions [4]. However, it is much less effective in treating Ph+ ALL, which has not been shown to be related to the BCR-ABL kinase domain mutations, the most common type of imatinib-resistance, the underlying mechanism is largely unknown to date.

MicroRNAs (miRNAs), a new class of 21–23 nucleotides endogenous, non-protein coding RNAs, regulate gene expression by translational repression or gene silencing [5] and participate in many cancer progression [6]. Increasing evidence suggests that many miRNAs are deeply involved in resistance or sensitization to anti-cancer drugs [7] or radiation [8,9]. Among these miRNAs, miR-21 is one of the most commonly and dramatically up-regulated miRNA in many cancers [10] and has been implicated in the inhibition of apoptosis and drug resistance by targeting many tumor suppressor genes related to apoptosis [11]. MiR-21 inhibitor has been shown to enhance chemotherapeutic effectiveness in a variety of solid tumors and hematological malignancies [11].

In the present study, we examined the effect of miR-21 on imatinib-induced apoptosis in Ph+ ALL Sup-b15 cells, which has been shown to be resistant to imatinib-induced apoptosis [12]. We found that imatinib upregulated miR-21 expression and antagonism of miR-21 sensitized Sup-b15 cells to imatinib-induced apoptosis through upregulation of PTEN to inhibit PI3K/AKT signaling.

Abbreviations: ALL, acute lymphoblastic leukaemia; CML, chronic myelogenous leukemia; miRNA, microRNA; qRT-PCR, quantitative real-time PCR.

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2. Materials and methods

2.1. Cell line

The human acute lymphoblastic leukemia cell line Sup-b15 was from The Cell Bank of Type Culture Collection of Chinese Academy of Sciences and grown in RPMI-1640, containing 20% FBS and 100 mg/ml penicillin/streptomycin at 37 °C and 5% CO_2.

2.2. Patient sample

A human peripheral blood sample from Ph+ B-ALL patient with informed consent was obtained from Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University. Mononuclear cells were enriched by Ficoll gradient centrifugation and the B cells were then isolated using a positive magnetic bead selection protocol (Miltenyi Biotech, Germany). The study was performed with the approval of the Medical Ethical Committee of Sun Yat-Sen University, Guangzhou, China.

2.3. Reagents

Imatinib was from Santa Cruz Biotechnology (Santa Cruz, CA); LY294002 and wortmannin were from Cell Signaling Technology (Beverly, MA); AlexaFluor® 488-conjugated goat anti-mouse IgG was from Molecular Probes (Life Technology, USA).

2.4. Cell proliferation assay

Cell proliferation was determined by CellTiter-Blue® assay according to the manufacturer's protocol (Promega, Madison, WI). Absorption was measured at 570 nm using a Synergy ELISA reader (Biotek, Winooski, USA).

2.5. AgomiR-21 and antagomiR-21 Transfection

The human miR-21 agomiR and antagomiR were purchased from RiboBio (Guangzhou, China) and the transfection procedure was performed using Lipofectamine RNAiMAX (Life Technology, USA) according to the manufacturer's protocol. The transfection efficiency was detected by flow cytometry using Cy3-labeled control miRNA. This reagent exhibits at least 95% transfection efficiency in Sup-b15cells (Supplementary Fig. S1).

2.6. Transfection of short-interfering RNA (siRNA) of PTEN

The siRNA sequence targeted human PTEN (GeneBank accession No. NM_000314) transcript was: 5′-GTA TAG AGC GTG CAG ATA A. A scrambled sequence (sense-5′-CGU ACU GUC GAC ACU GAA ACG GAC A; antisense-5′-UAU CCG UUU CAG UGU CGA CAG UAC GTG) named si-NC, which was non-homologous to any human DNA sequence, was used as negative control. The transfection procedure of PTEN siRNA as described previously [13].

2.7. Quantitative real-time PCR (qRT-PCR) analysis of miRNA-21, PTEN and PDCD4 expression

Bulge-loop™ miRNA qRT-PCR Primer Set (one RT primer and a pair of qPCR primers for each set) specific for mature miR-21 was designed by RiboBio (Guangzhou, China). Briefly, total RNA was isolated with the RNeasy mini kit (Qiagen, Hilden, Germany) and 100 ng of total RNA was used to synthesize a specific cDNA of miR-21 using the First-Strand cDNA Synthesis Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. For detection of PTEN and PDCD4 expression, RNA was extracted and reverse transcribed as previously described [13]. The sequences of gene-specific PCR primers were: 5′-ACC GGC AGC ATC AAA TGT TT and 5′-AGT TCC ACC CCT TCC ATC TG for PTEN; 5′-TGA GAT TTA AGG GCT GGG CA and 5′-ACC ATC TCG ACT CAC TGC AA for PDCD4; 5′-CTC TGC TCC TCC TGTTCG AC and 5′-ACG ACC AAA TCC GTT GAC TC for glyceraldehydes-3-phosphate dehydrogenase (GAPDH). Then, qRT-PCR was performed on the LightCycler®480 detection system (Roche, Swiss) using the SYBR Green Real-Time PCR Master Mix Kit (Applied Biosystems) according to the manufacturer's instructions. Expression of miR-21 was normalized to U6 small noncoding RNA (U6-snRNA) and expression of PTEN and PDCD4 was normalized to GAPDH.

2.8. Apoptosis assays

Apoptosis analysis was performed using the Alexa Fluor® 647-conjugated Annexin V and PI as recommended by the manufacturer's instructions (Biolegend, San Diego, CA, USA). The stained cells were detected on a FACSCalibur flow cytometer (BD, San Jose, CA, USA) and analyzed by CellQuest Pro software program.

2.9. Western blot

Protein extracts were resolved through 10% SDS–PAGE, transferred to PVDF membranes, probed with mouse monoclonal antibodies against human PTEN or pAKT (Y473) (Millipore, Billerica, MA, USA), or rabbit polyclonal antibodies against human PDCD4 and AKT (Cell Signaling Technology, Beverly, MA), and then with horseradish peroxidase-conjugated goat anti-rabbit or mouse IgG secondary antibody (DAKO, Carpinteria, CA), and then visualized by chemiluminescence (Pierce, Rockford, IL).

2.10. Intracellular staining for pAKT protein

The cells were fixed with 4% formaldehyde for 10 min at 37 °C, permeabilized with ice-cold 90% methanol for 30 min on ice and blocked by incubation in 0.5% BSA/PBS for 10 min at room temperature. The permeabilized cells were incubated with pAKT antibody or isotype control antibody at room temperature for 1 h. Primary antibody was visualized by incubating the cells with AlexaFluor® 488-conjugated goat anti-mouse IgG for 30 min. pAKT protein level was measured by flow cytometry.

2.11. Statistical analysis

Values represent the mean ± S.D. from at least three independent experiments. P values were obtained from t-tests with paired or unpaired samples, with significance set at $P < 0.05$.

3. Results

3.1. MiR-21 is closely related with imatinib resistance in Sup-b15 cells

Previous study has shown that Ph+ ALL cell line Sup-b15 is inherently resistant to imatinib [12], we firstly confirmed this report according to the results of proliferation (Supplementary Fig. S2A) and apoptosis assay (Supplementary Fig. S2B). To investigate whether miR-21 participates in imatinib resistance in Sup-b15 cells, the effects of miR-21 inhibition or overexpression on imatinib-induced apoptosis were determined. We found that imatinib induced small apoptosis in antagomiR-NC-transfected cells, whereas inhibition of miR-21 by antagomiR-21 notably increased imatinib-induced apoptosis (Fig. 1A). In contrast, ectopic expression of miR-21 by agomiR-21 transfection further conferred imatinib resistance (Fig. 1B). Interestingly, overexpression of

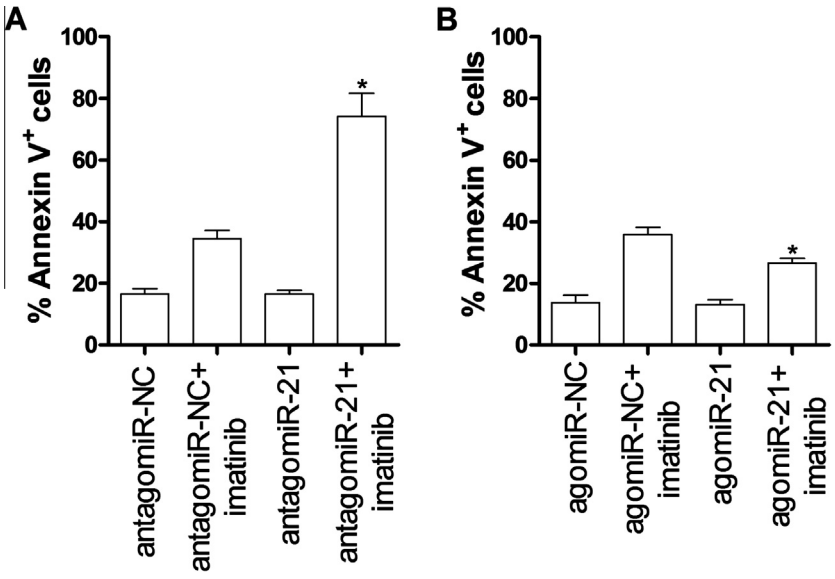

Fig. 1. Effects of antagomiR-21 and agomiR-21 on imatinib-induced apoptosis. (A) and (B) Sup-b15 cells were transfected with antagomiR-NC (100 nM), antagomiR-21 (100 nM), agomiR-NC (100 nM) or agomiR-21 (100 nM) for 48 h, followed by treatment with or without 1.0 μM imatinib for another 48 h and the apoptosis was analyzed by Annexin V/PI double labeling. All these data are shown as mean ± S.D. collected from three independent experiments. $^{*}P < 0.05$ versus non-target control group.

miR-21 significantly inhibited imatinib-induced apoptosis in Ph+ imatinib-sensitive CML cell lines K562 and Ku812 (Supplementary Fig. S3A and B). qRT-PCR analysis showed that imatinib increased miR-21 expression in imatinib-resistant Sup-b15 cells (Supplementary Fig. S4A), but decreased miR-21 expression in imatinib-sensitive K562 and Ku812 cells (Supplementary Fig. S4B and C). These results suggest that miR-21 plays an important role in suppressing imatinib-induced apoptosis in Sup-b15 cells.

3.2. MiR-21 regulates PTEN expression in Sup-b15 cells

We next sought to identify the target molecule(s) responsible for the miR-21-induced imatinib resistance. PTEN and PDCD4 have been shown to be the potential target molecules of miR-21 and related to apoptosis [14,15], we thus focused on the effects of miR-21 inhibition or overexpression upon the expression of PTEN and PDCD4. qRT-PCR analysis showed that transfection of antagomiR-21 or agomiR-21 resulted in significant decrease and increase in cellular levels of miR-21 in Sup-b15 cells treated with or without imatinib (Fig. 2A), respectively. Imatinib treatment increased in miR-21 expression (Fig. 2A) in antagomiR-NC-transfected or agomiR-NC transfected cells accompanied by decreased in mRNA and protein expression of PTEN compared with their counterparts (Fig. 2B and D). MiR-21 inhibition or overexpression cells exposed to imatinib showed further upregulation and downregulation of PTEN than imatinib treatment alone (Fig. 2B and D), respectively. The expression of PDCD4 was essentially unchanged by the manipulation of miR-21 cellular levels (Fig. 2B and C). These results suggest that PTEN is the target of miR-21 in Sup-b15 cells.

3.3. Apoptosis induction of miR-21-knockdown Sup-b15 cells by imatinib requires PTEN

To determine the role of PTEN in mediating the effects of miR-21 inhibition on imatinib-induced apoptosis, we evaluated whether siRNA-mediated PTEN knockdown could mitigate the effects of miR-21 inhibition on imatinib-induced apoptosis. Our results showed that inhibition of miR-21 markedly promoted imatinib-induced apoptosis and decreased AKT phosphorylation in siRNA-NC-transfected cells, whereas depletion of PTEN significantly reduced imatinib-induced apoptosis and increased phosphorylation level of AKT in antagomiR-21-transfected cells (Fig. 3A and B). These data indicate that PTEN is required for imatinib-induced apoptosis in miR-21-knockdown Sup-b15 cells.

3.4. PI3K inhibitors cooperate with imatinib to induce apoptosis

As PTEN is a major negative regulator of AKT signaling, our above results suggest that inactivation of PI3K/AKT signaling pathway is essential for imatinib-induced apoptosis through miR-21 inhibition. We thus determined whether PI3K specific inhibitors (LY294002 and wortmannin) also sensitize Sup-b15 cells to imatinib-induced apoptosis. We found that wortmannin alone induced a small apoptosis of Sup-b15 cells, but LY294002 treatment led to more proliferation inhibition and apoptosis induction than imatinib (Fig. 4A and B). Similar to miR-21 inhibition, inhibition of PI3K combined with imatinib resulted in significant apoptosis than either agent did alone. Moreover, imatinib or LY294002 treatment alone slightly inhibited phosphorylation of AKT, respectively, whereas combination of LY294002 with imatinib resulted in notable reduction of AKT phosphorylation (Fig. 4C). Most important, inhibition of PI3K by LY294002 also resulted in marked inhibition of proliferation, enhancement of the induction of apoptosis and significant reduction of AKT phosphorylation by imatinib in primary Ph+ ALL cells (Fig. 4D, E and F). Taken together, these results indicate that PI3K/AKT signaling pathway contributes to imatinib resistance in Ph+ ALL cells.

4. Discussion

Although imatinib monotherapy is effective in treating Ph+ CML, the response of Ph+ ALL patients is not nearly as good, leading to shorter remissions and more rapid emergence of imatinib resistance [2]. Imatinib resistance has been attributed to BCR-ABL-dependent and -independent mechanisms. BCR-ABL-dependent mechanisms include amplification of the BCR-ABL gene and mutations within ABL that disrupt binding to imatinib [16]. BCR-ABL-independent mechanisms include chromosomal abnormalities in addition to the Ph chromosome abnormalities, disruptions in drug uptake and efflux [17], and activation of alternative

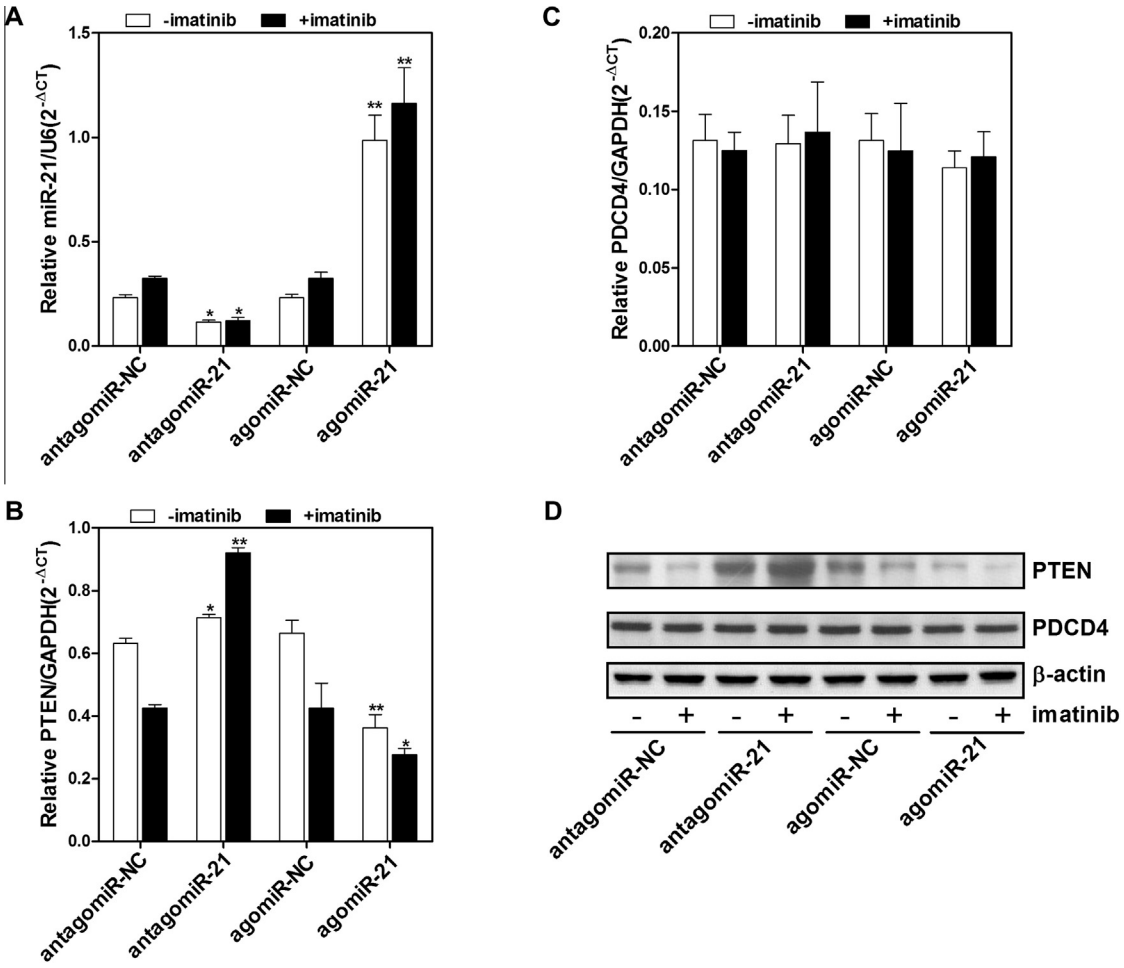

Fig. 2. MiR-21 regulates PTEN expression in Sup-b15 cells. (A)–(C) Sup-b15 cells were transfected with antagomiR-21 (100 nM), agomiR21 (100 nM), antagomiR-NC (100 nM) or agomiR-NC (100 nM) for 48 h, and then treated with imatinib (1.0 μM) for 48 h. The level of miR-21 (A) was assayed by qRT-PCR. In parallel, the mRNA (B) and protein (C) level of PTEN and PDCD4 were measured by qRT-PCR and Western blot, respectively. All these data are shown as mean ± S.D. collected from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ versus non-target control group.

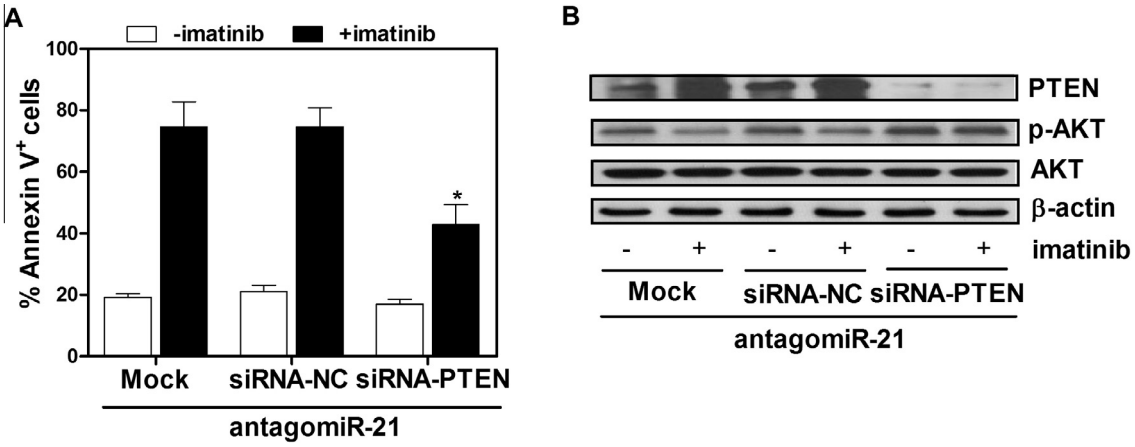

Fig. 3. Knockdown of PTEN attenuates anticancer activity of imatinib. Sup-b15 cells were transfected with PTEN siRNA (si-PTEN, 100 nM) or control siRNA (si-NC, 100 nM) with or without antagomiR-21 (100 nM) for 48 h, and then treated with imatinib (1.0 μM) for 48 h. Apoptosis was detected by Annexin V/PI double staining (A). In parallel, the protein level of PTEN, p-AKT and AKT was analyzed by Western blot (B). All these data are shown as mean ± S.D. collected from three independent experiments. $^{*}P < 0.05$ versus non-target control group.

signaling pathways that cause cell proliferation or promote cell survival [18,19]. In the present study, we suggest a new molecular mechanism of Ph+ ALL cells resistance to imatinib therapy, in which miR-21 is upregulated in response to imatinib treatment and consequently confers resistance to imatinib-induced apoptosis in Sup-b15 cells by decreasing PTEN expression. Interestingly, the

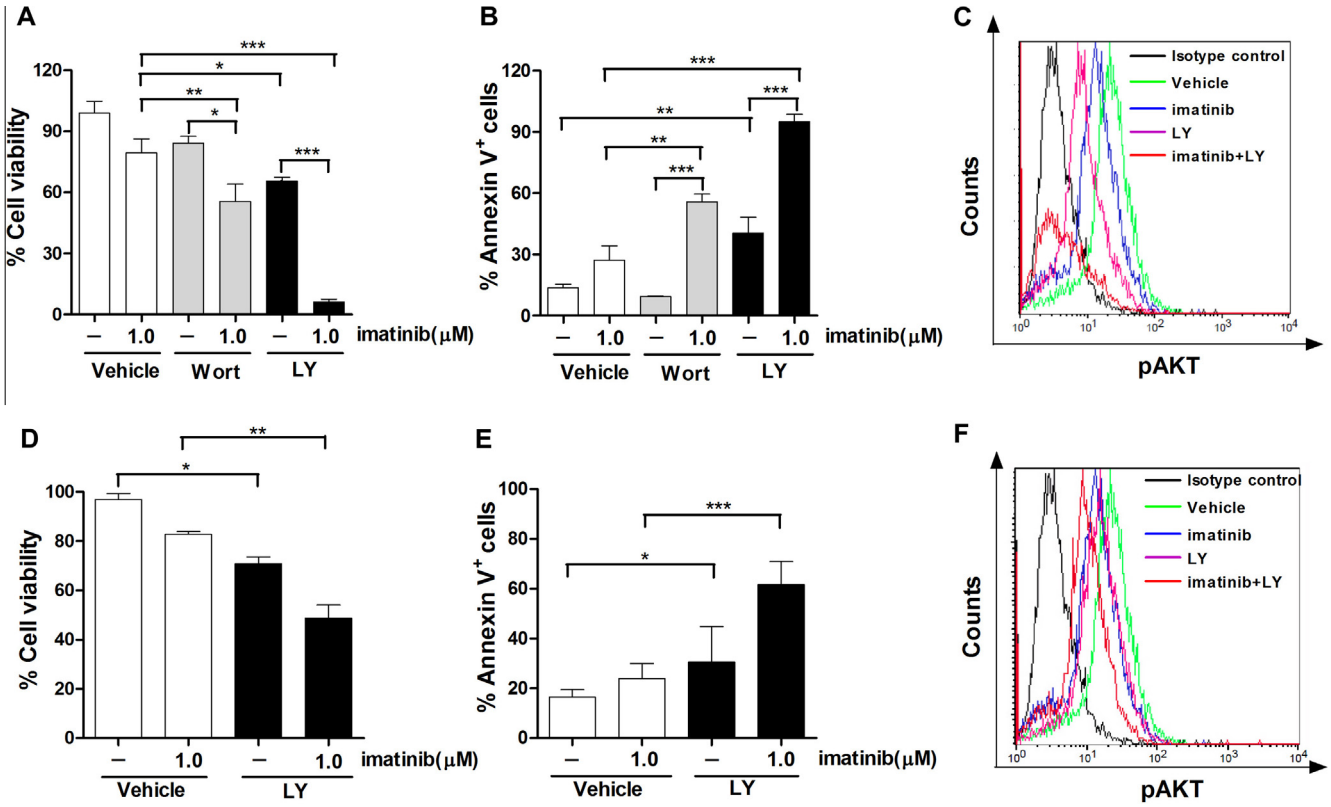

Fig. 4. PI3K inhibitors cooperate with imatinib to inhibit cell proliferation, induce apoptosis and decrease AKT phosphorylation. Sup-b15 (A–C) and primary Ph+ ALL cells (D–E) were pretreated with LY294002 (50 μM) or wortmannin (1 μM) for 2 h, followed by incubation with 1.0 μM imatinib for another 48 h in the presence of LY294002 and wortmannin, and then subjected to detection of cell proliferation (A and D), apoptosis (B and E) and pAKT level (C and F). All these data are shown as mean ± S.D. collected from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. LY, LY294002; Wort, wortmannin.

cytokine IFN had also been found to induce miR-21 expression in human cancer cells, and inhibition of miR-21 resensitized resistant cancer cells to IFN-induced apoptosis [20]. In multiple myeloma cells, IL-6 promoted the survival of these cells through the induction of miR-21 expression [21]. These data strongly suggest that induction of miR-21 by various stimuli also contributes to cancer cells resistance to apoptosis besides miR-21 overexpression in cancer cells was associated with chemoresistance.

It is well established that miR-21 functions via inhibition of a variety of mRNA targets including PTEN [22], CDC25A [23], PDCD4 [24], RHOB [25], Spry1 [26], BTG2 [27] and TIMP3 [28] validated in various cancer cell types. For example, miR-21 via repression of PDCD4 affects the PI3K/AKT pathway in breast cancer cells [29], whereas miR-21 in hepatocellular carcinoma cells regulates the PI3K antagonist PTEN [22]. Here, we found that imatinib treatment resulted in upregulation of miR-21 and downregulation of PTEN but not PDCD4, suggesting that PTEN might be the downstream target of miR-21 in Sup-b15 cells. Therefore, the effect of antagomiR-21 or agomiR-21 on PTEN expression was determined after imatinib treatment. Our results showed that combined miR-21 inhibitor and imatinib treatment significantly upregulated PTEN expression compared to miR-21 inhibitor or imatinib alone, while overexpression of miR-21 resulted in further decrease of PTEN in imatinib-treated cells. Moreover, inhibition of miR-21 markedly decreased AKT phosphorylation in siRNA-NC-transfected cells, whereas depletion of PTEN significantly increased phosphorylation level of AKT in antagomiR-21-transfected cells. Taken together, these results suggest that miR-21 knockdown-mediated inactivation of PI3K/AKT pathway is through PTEN in Ph+ ALL cells.

Aberrant PI3K/AKT signaling has been linked to oncogenesis and therapy resistance in various malignancies and the PI3K/AKT pathway is thus believed to be an attractive target for cancer therapy. Quentmeier H et al. reported that the constitutive and BCR-ABL1-independent activation of the PI3K/AKT pathway was a common feature of all Ph+ imatinib-resistant ALL and CML cell lines [12], inhibition of PI3K/mTOR activities with dual PI3K/mTOR inhibitor enhanced nilotinib efficiency in Sup-b15 cells [19]. Here, we also demonstrated that inhibition of PI3K by PI3K inhibitors could significantly enhance imatinib-induced apoptosis in Sup-b15 cells. Furthermore, the combination of imatinib and miR-21 inhibitor led to a similar result. These data suggest that inhibition of PI3K/AKT pathway via combination of miR-21 inhibitor and imatinib is related with increased apoptosis in Sup-b15 cells. In addition, we also found that PI3K inhibitor LY294002 treatment alone induced more apoptosis of Sup-b15 cells than imatinib. These results strongly support the view that targeting components of PI3K survival pathways, alone or in combination with TKIs, represents an attractive potential therapeutic approach for targeting Ph+ ALL [30].

In summary, our present study suggests that miR-21 is required for survival of Ph+ ALL cells after imatinib treatment, and the combination of miR-21 inhibitor and imatinib is a promising strategy for Ph+ ALL therapy.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.10.107.

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