Anlotinib Exerts Potent Antileukemic Activities in B-Cell Acute Lymphoblastic Leukemia Via Attenuation of PI3K/AKT/mTOR Signaling Cascade

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Abstract

**Background:** Adult patients with B-cell acute lymphoblastic leukemia (B-ALL) have unfavorable prognosis. Recurrent disease is the major challenge for treatment success and has limited effective therapeutic strategies, suggesting that alternative therapeutics are unmet medical needs. Anlotinib, a novel multi-target inhibitor, is capable of blunting the proangiogenic activity of VEGFR, PDGFR and FGFR, and has shown strong antitumor effects across distinct solid tumors. No study to date has investigated the antileukemic efficacy of Anlotinib in B-ALL, thus prompting us to initiate this study.

**Methods:** A published B-ALL microarray data was analyzed to identify the novel potential treatment choice using Expression2Kinases program. Cell viability was determined by Cell Counting Kit-8 (CCK-8). Annexin V/PI staining kit and staining kit were used to assess cell apoptosis and cell cycle distribution, respectively. The mechanism of action of Anlotinib was investigated with Quantitative real-time polymerase chain reaction (qRT-PCR) and Western blotting. A B-ALL Patient Derived Xenograft (PDX) animal model was generated and then use to analyze the in vivo antileukemia activity of Anlotinib.

**Results:** In this study, we observed that recurrent B-ALL patients exhibited high proangiogenic activity relative to newly-diagnosed patients, as evidenced by upregulation of VEGF/VEGFR signaling and its downstream targets, including PIK3/AKT/mTOR pathway. In vitro studies demonstrated that Anlotinib was potent to reduce cell viability, induce cell apoptosis and block cell cycle at G2/M phase in B-ALL cells. In a patient-derived-xenograft (PDX) model, Anlotinib dramatically attenuated leukemia burden and improved prognosis as compared to the control group. Mechanistically, blockade of three important proangiogenic mediators, comprising VEGFR2, PDGFR-beta and FGFR3, played a critical role in the cytotoxicity of Anlotinib against B-ALL. Moreover, Anlotinib significantly dampened the activity of PI3K/AKT/mTOR pathway which resides in the convergence of these proangiogenic signals, manifested by dephosphorylation of PI3K, AKT and mTOR.

**Conclusions:** In summary, our results indicate that Anlotinib exerts potent anti-leukemic efficacy in B-ALL preclinical models via perturbation of proangiogenic signalings and inactivation of the common downstream PI3K/AKT/mTOR signaling. This work provides evidence and a rationale for future evaluation of Anlotinib in B-ALL management in clinical settings.

Background

Acute lymphoblastic leukemia (ALL) arises from malignant transformation and uncontrollable proliferation of B- or T-lineage lympholiod precursor cells, with B-cell originate ALL (B-ALL) accounting for around 80% of all newly-diagnosed cases in both pediatric and adult population (1). In children, over 80% of patients with B-ALL can be cured with contemporary pediatric therapeutic regimens (2, 3), while only 30–40% of adult counterparts will attain long-term survival (4). Primary (refractory) and acquired (relapsed) chemoresistance are two main reasons leading to failure to treatment and subsequent unfavorable clinical outcomes (5). New emergent therapeutic approaches, including bispecific T-cell
engager Blinatumomab and CD19-specific chimeric-antigen receptor (CAR)-modified autologous T cells (CAR-T), have considerably improved the prognosis of adults with refractory/relapsed B-ALL (6–8). However, failure to or losing initial response to these novel strategies invariably exist and consequently hamper the success of long-term survival (9). Therefore, it is unmet medical needs to explore alternative high-efficient treatment strategies for the treatment of patients with refractory or relapsed B-ALL (RR B-ALL).

Angiogenesis is a process of fresh blood vessels developing from a pre-existing vascular network. It is under exquisite control of pro- and anti-angiogenic factors in physiological circumstances (10–12). Inconsistently, the dynamic balance between pro- and anti-angiogenic signals is broken down during malignant transformation and progression possibly because of upregulation of diverse pro-angiogenic mediators, thus tilting toward pro-angiogenic switch (13). VEGF, PDGF and FGF are three main proangiogenic components and exert their functions predominantly through binding to their corresponding receptors, VEGFR, FGFR and PDGFR (14, 15). Given the pivotal functions of angiogenesis in diverse cancer initiation and development, including ALL, various anti-angiogenic therapeutics have been developed and some of them have moved forward to clinical evaluation in multiple cancer types (16). Nevertheless, most of angiogenesis- blocking approaches show moderate antitumoral effects, probably attributable to the fact that they are incapable of fully disrupting these different pro-angiogenic signals (15, 17). This provides an opportunity for multi-target antiangiogenic agents as a promising anticancer strategy.

Anlotinib is a newly developed oral receptor tyrosine kinase (RTK) inhibitor that primarily targets VEGFR, FGFR and PDGFR, three critical receptors of pro-angiogenic factors (18, 19). Owing to its encouraging clinical outcomes and manageable safety profiles, Anlotinib has been approved in China as a third-line treatment for patients with advanced or metastatic non-small-cell lung cancer (NSCLC) who experienced disease progression or recurrence (20–22). In addition, numerous phase II/III clinical trials have been launched to evaluate the antitumoral activity of Anlotinib against other malignancies, including gastric cancer, hepatocellular carcinoma and renal carcinoma. Preliminary data from these trials have shown promising clinical benefits with Anlotinib administration (23–26). Despite the excellent antitumoral activity of Anlotinib in multiple forms of solid tumor, the anti-leukemia efficacy of Anlotinib in B-ALL remain to be defined. This prompts us to investigate the cytotoxic effects of Anlotinib on B-ALL and its underlying mechanism of action.

Methods And Materials

Microarray data acquisition

Gene array data were screened using the Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo) database. Human B-ALL expression profile datasets GSE60926 was download from GEO. The GSE60926 dataset contains 22 B-ALL bone marrow samples at diagnosis, 20
bone marrow samples at relapse, and 8 cerebrospinal fluid (CSF) samples at relapse. The dataset was analyzed using the GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array platform.

**Differentially expressed genes (DEGs) identification, downstream pathway enrichment, and upstream kinase enrichment analysis.**

The raw data of GSE60926 in the CEL file was effectively processed using the Affy package pair in R, using correction, normalization and log2 conversion (27). Absent probes were filtered. The duplicate probes were merged by the maximum value and processed for probe annotation using “hgu133plus2.db” R package. The DEGs in B-ALL relapse samples compared with the samples at diagnosis were determined using limma package (28). DEGs were screened with a false discovery rate (FDR) corrected P<0.05 and |log fold-change (FC)|>1. Functional enrichment for downstream pathways was performed by the web-based genomic annotation tool: DAVID (https://david.ncifcrf.gov/) (29). DEGs were subjected to molecular function and pathway studies by Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. DEGs were also used to predict upstream regulatory kinase by web-based Expression2Kinases software (30). Sankey diagrams were generated by ggalluvial R package.

**Cell Lines and Reagents**

Nalm6 and SupB15 cells, two B-ALL cell lines, were kept in our lab and routinely cultured in RPMI 1640 medium (Invitrogen, Life Technologies, Carlsbad, California, USA) supplemented with 10% fetal bovine serum and 100 units/ml penicillin and 100 mg/ml streptomyacin at 37°C in a humidified 5% CO2 incubator. Anlotinib, a kind gift provided by Zhengda Tianqing Pharmaceutical Group Co., Ltd, was dissolved with dimethyl sulfoxide (Sigma, St Louis, MO, USA) into a stock concentration of 100 mM and diluted with culture medium into designated concentrations.

**Cell Viability Assay**

Cell viability of Nalm6 and SupB15 cells treated with Anlotinib was determined by a Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) assay. In brief, B-ALL Cells (5×10^4 cells/well) suspended in 100 μl growth medium were seeded in 96-well plates and immediately treated with designated doses of Anlotinib for 24h and 48 h. At the end of the treatment, CCK-8 reagents (10 μl/well) were added and continued to incubate for an additional 2 h, after which the absorbances were detected at 450 nm by microplate reader (ELx800; BioTek Instruments Inc., Winooski, Vermont, USA). Cell viability was calculated with the following formula: cell viability rate (%) = (absorbance of experimental group – absorbance of blank well) / (absorbance of control group- absorbance of blank well) × 100%. IC50 value of each cell line was calculated based on the results of cell viability. The data was obtained from three independent experiments in triplicates, and presented as mean ± SD.

**Apoptosis and Cell Cycle Analysis**
Nalm6 and SupB15 cells were exposed to different concentrations of Anlotinib (0, 1, 2, 4, 8 µM) for 24 h or 48 h. Anlotinib-treated cells were then stained with Annexin V/PI staining kit (eBioscience, San Diego, California, USA) per the manufacturer’s instructions. The stained cells were analyzed by flow cytometry (FACS Caliber; BD Biosciences, San Jose, California, USA). Apoptotic cells were defined as Annexin-V positive cells.

For cell cycle assessment, $2 \times 10^5$ of either Nalm6 or SupB15 cells were exposed to Anlotinib (0, 2, 4, 8 µM) for 24 h. Subsequently, Anlotinib-exposed cells were stained with PI/RNase staining buffer and then referred to flow cytometer analysis (BD Biosciences, San Jose, California, USA), following the manufacturer’s instructions.

Both assays were performed in independent triplicates for three times.

A B-ALL Patient Derived Xenograft (PDX) animal model

NOD-Prkdc−/-IL2rg−/- mice (male, NPI, IDMO ltd., Beijing, China) were housed under specific-pathogen-free (SPF) environment in accordance with the animal care guidelines of Xiamen University Animal Care and Use Committees. In this study, each mouse was intravenously engrafted with $1 \times 10^6$ of B-ALL cells from a primary PDX B-ALL mouse tissue within 24 hours of receiving 1 Gy of irradiation. Once detecting human CD45 staining (clone HI30, Biolegend) in peripheral blood ≥ 1%, a total of 22 PDX mice were randomized to control or Anlotinib group (5 mg/kg, 5-day on and 2-day off) for three-week treatment. At the end of treatment, 5 PDX mice from each group were euthanized and used to evaluate the antileukemia efficacy of Anlotinib in a B-ALL preclinical model. The remaining 6 mice of both groups were utilized to determine the survival curve. During the treatment course, body weight of each mouse was monitored daily to assess the toxicity profiles of Anlotinib for the treatment of B-ALL in preclinical model.

Western blotting analysis and Quantitative real-time polymerase chain reaction (qRT-PCR)

Nalm6 and SupB15 cells ($5 \times 10^5$/ml) were cultured with Anlotinib (0, 2, 4, 8 µM) for 24 h. At the end of treatment, cells were harvested and lysed in RIPA buffer (Thermo Scientific, USA) supplemented with protease inhibitor (Roche Diagnostics, Mannheim, Germany) and phosphatase inhibitor (Roche Diagnostics, Mannheim, Germany). Protein concentrations were quantified and normalized by a BCA protein Assay (Pierce, Thermo Scientific, USA). Equal protein concentrations of each treatment group (20 µg/lane) were electrophoresed on 8 to 12% gels and then transferred to PVDF membrane (Millipore, Billerica, MA, USA). The membrane was probed with primary antibodies and HRP-conjugated secondary antibodies (all from Cell Signaling Technology, Danvers, MA, USA). Ultimately, an ECL Western Blotting Detection Kit (GE Healthcare, Chicago, USA) was used to detect the protein signals that were visualized by the Amersham Imager 600 (AI600, GE Healthcare, Chicago, USA).

Total RNA was extracted from cultured cells using TRIzol reagent (Invitrogen, USA). It was reverse transcribed into cDNA using the PrimeScript RT kit (Takara, Japan) according to the manufacturer's
instructions. The cycling conditions were 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 20 s. Each reaction was repeated three times. The primer sequences for RT-PCR are shown in Table S1.

**Statistical analysis**

Statistical analyses were performed with IBM SPSS 19.0 software (SPSS, Chicago, IL) and GraphPad Prism 8 (GraphPad Software Inc, San Diego, CA, USA). The results for continuous variables were presented as mean ± standard deviation values. Student's t tests or Wilcoxon signed-rank tests were used for between-group comparisons. Categorical data were analyzed using chi-squared tests or Fisher's exact tests. P values < 0.05 (two-tailed) were considered to indicate statistically significant results. All triplicate results were quantifications of independent experiments.

**Results**

**In silico analysis predicts Anlotinib as a potential anti-leukemic therapeutic regimen in patients with B-ALL**

To discover new fundamental pathways and cellular biomarkers that might be crucial for B-ALL, we harbored a published B-ALL microarray data (GSE60926) with 40 B-ALL bone marrow samples at diagnosis (n = 20) or at relapse (n = 20) from Gene Expression Omnibus (GEO) database. Raw data was directly downloaded from the GEO database. Quality control and background normalization were performed with the “affy” R package (27). After excluding the absent probes, 39177 and 39008 normalized probes were separately observed in newly-diagnosed and relapsed B-ALL samples. Next, we merged the duplicate probes by the maximum value and processed the remaining probes for probe annotation by “hgu133plus2.db” R package. “Limma” R package was employed to determine differential expression genes (DEGs) between the newly-diagnosed and relapsed groups. To the end, the two groups exhibited a different expression pattern, manifested by a clustered expression heatmap which was generated by the DEGs with a cutoff value of |fold change (FC)| > 1 (Fig. 1A). Importantly, we identified 27 downregulated genes and 64 upregulated genes (Fig. 1B).

To uncover novel potential pathways regulated by these DEGs for B-ALL, we performed Gene Ontology (GO) enrichment analysis with KEGG pathway database (31). Consequently, several significant affected pathways, including VEGF signaling cascade, were selected and shown in a bar graph (Fig. 1C). VEGF signaling pathway, an angiogenesis-associated pathway, was also enriched by significant DEGs. PI3K/Akt signaling pathway which had been implicated in both the pathogenesis and the progression of leukemia was also highlighted (32). Knowing that upstream receptor tyrosine kinase can cause the change of the downstream gene expression profile (33, 34). We applied “Expression2Kinases” to identify upstream kinase of the DEGs (30). Upstream kinases like AKT1, CDK1, FGFR1, RET, and so on were combined with enriched pathways using Sankey diagram to emphasize the potential regulatory kinase in the relapse B-ALL (Fig. 1D). Protein kinase inhibitors were gaining more and more attention in leukemia therapies (35) and also relapsed B-ALL (36). Next, we combined some China's Food and Drug
Administration (CFDA) approved TKIs, their potential target kinase, downstream regulatory pathways and their corresponding relationship in the Sankey diagram (Fig. 1E). Venn diagram showed the overlapped kinases (Fig. 1F) and downstream enriched pathways (Fig. 1G). RET and ALK were shown in the overlapped kinases diagram (Fig. 1F), and PI3K-AKT signaling pathway, VEGF signaling pathway, MAPK signaling pathway, and cell cycle pathway were shown in the overlapped pathways diagram (Fig. 1G). Anlotinib was proven to target RET kinase and downstream pathways like PI3K-AKT signaling pathway and VEGF signaling pathway (Fig. 1E) (18, 37). Taken together, our bioinformatic analyses point out that anlotinib might be a potential therapeutic regimen in B-ALL treatment.

**Anlotinib attenuates cell viability, blocks cell cycle at G2/M phase and induces apoptosis in B-ALL cells**

In this study, Nalm6 and SupB15 cells were used as B-ALL *in vitro* models. To investigate the antileukemic effect of Anlotinib on B-ALL, the two cell lines were cultured with or without Anlotinib (1, 2, 4, 8 µM) for 24 and 48 hours, respectively. Cell viability was determined by a CCK8 assay and cell apoptosis was evaluated with an annexin V/PI dual-staining assay. As compared with the untreated group, Anlotinib treatment significantly decreased the viability of B-ALL cells in dose- and time-dependent manners (Fig. 2A-B). The IC50 values of Nalm6 and SupB15 cells after 24 hours of Anlotinib treatment, were 3.224 ± 0.875 µM and 3.803 ± 0.409 µM, respectively. For 48-hour treatment, the IC50 values of Nalm6 and SupB15 cells were 2.468 ± 0.378 µM and 2.459 ± 0.443 µM, respectively (Table 1). As expected, exposure Nalm6 and SupB15 cells to Anlotinib remarkably increased the proportion of cell apoptosis, including both early and late apoptosis in dose- and time-dependent fashions (Fig. 2C-F).

Next, we assessed the cell cycle distribution of both Nalm6 and SupB15 cells treated with or without Anlotinib (2, 4, 8 µM). Herein, treatment with Anlotinib clearly increased the percentage of Nalm6 cells at G2/M phase, which was accompanied by decreased percentage of G1/G0 phase (Fig. 2E). Accordingly, the similar results of cell cycle distribution were also observed in the SupB15 cells treated with or without Anlotinib (Fig. 2F). Altogether, these findings demonstrate that Anlotinib exerts potent anti-leukemic activity in B-ALL cell lines.

**Anlotinib abrogates leukemic cell growth in a patient-derived xenograft (PDX) B-ALL mouse model**

To further consolidate the robust anti-B-ALL efficacy of Anlotinib *in vivo* model, we generated a PDX model by intravenously engrafting 1 x 10^6 of B-ALL cells from a primary PDX B-ALL mouse tissue into NOD-scid IL2Rγ null (NSG) mice (Fig. 3A). Upon detecting human CD45 percentage > 1% in the peripheral blood, B-ALL PDX mice were randomized into either vehicle (n = 11) or Anlotinib treatment group (n = 11). Anlotinib (5 mg/kg/d) was orally administered from Monday to Friday (5 days on, 2 days off) for 3 consecutive weeks (Fig. 3A). Body weight of each mouse was measured for 5 days per week during the entire treatment course. As compared with the untreated group, Anlotinib administration appeared to moderately increase mice body weight (Fig. 3B), indicating that Anlotinib is well tolerated in the treatment of B-ALL preclinical model. In addition, no other treatment relevant safety profiles had been noticed in the Anlotinib group versus the vehicle group. These observations were in concert with the facts that Anlotinib
was active against multiple malignant diseases and had acceptable adverse effects in a series of clinical trials.

At the end of the experiment, 5 of 11 PDX mice from each group were randomly selected and then euthanized to evaluate the antileukemic activity of Anlotinib. The remaining mice in each group continued to be housed and utilized to analyze survival curve. As a result, administration B-ALL PDX mice with Anlotinib significantly reduced the size and weight of spleen when compared to the Anlotinib-untreated group (Fig. 3C-D). More notably, treatment with Anlotinib remarkably attenuated the leukemia burden in both bone marrow and spleen, as evidenced by substantial decreases of human CD45 and CD19 positive cells (Fig. 3E-H). In contrast to the control group, Anlotinib treatment strikingly prolonged the survival period of B-ALL PDX mice (Fig. 3I). Taken together, the in vivo data reveal that administration with Anlotinib shows robustly antitumoral effects on B-ALL models with neglectable safety profiles.

Anlotinib decreases the levels of key proangiogenic factor receptors and perturbs the activity of the PI3K/AKT/mTOR signaling pathway in B-ALL cells

Anlotinib is designed to predominantly target VEGFR, PDGFR and FGFR, which are receptors of VEGF, PDGF and FGF and play important roles in angiogenesis. Not surprisingly, in this study we found Anlotinib also significantly diminished the mRNA expression levels of VEGFR2, PDGFR-beta and FGFR3 in B-ALL cells (Fig. 4A-B).

Our bioinformatic results revealed that perturbation of PI3K/AKT signaling cascade might contribute to the cytotoxicity of Anlotinib against B-ALL (Fig. 1G). This observation drove us to examine whether it still held true in our cellular models. As expected, exposure B-ALL cells to Anlotinib downregulated the enrichment of the phosphorylation of several crucial components of this pathway, including PI3K, AKT and mTOR (Fig. 4C) (18). Altogether, these results suggest that Anlotinib exhibits its effective antileukemia activity in B-ALL cells, primarily through attenuation of three key proangiogenic mediators and decrease of the function of the PI3K/AKT signaling pathway (Fig. 4D).

Discussion

In this study, we showed that patients with RR B-ALL had distinct gene expression signature as compared to those with newly-diagnosed B-ALL. This signature included significant enhancement of pro-angiogenic VEGF/VEGFR signaling and its downstream PI3K/AKT pathway in RR B-ALL. The observation indicates that proangiogenic signals might be important for the development of RR B-ALL and could be a potential therapeutic target to treat this malignant entity. VEGF, a well-established pro-angiogenic factor, plays a crucial role in angiogenesis through binding to VEGFRs which are composed of three members: VEGFR-1, VEGFR-2, and VEGFR-3, with VEGFR-2 being the main receptor to transmit the proangiogenic signal (14, 38). Blockade of VEGF/VEGFR signaling has been extensively investigated over the past decades, and some of these investigations have shown that attenuation of VEGF/VEGFR associated angiogenesis resulted in modest or significant antitumor effectiveness (39–41). Therefore, several anti-VEGF/VEGFR strategies have been approved for the treatment of numerous solid tumors, encompassing metastatic
colorectal cancer, hepatocellular carcinoma, non-small-cell lung cancer (NSCLC), and renal cell carcinoma, etc (42, 43). However, resistance to inhibition of VEGF/VEGFR pathway invariably occurs and correlates with poor outcomes (44). Upregulation of alternative proangiogenic signals, incorporating PDGF/PDGFR and FGF/FGFR pathway, partially contribute to failure to anti-VEGF/VEGFR regimen (45–47). This suggests that simultaneously targeting VEGF/VEGFR, PDGF/PDGFR, and FGF/FGFR pathways might become more potential potent treatment option than solely inhibiting VEGF/VEGFR signaling.

Anlotinib is a newly developed oral multi-target RTK inhibitor that was designed to abrogate angiogenesis via inhibition of VEGFR, PDGFR and FGFR (18, 19). Previous studies demonstrated that Anlotinib were potent to decrease cell viability and promote cell apoptosis in dozens of solid tumor preclinical models, including NSCLC and colorectal cancer (20–22). Most importantly, Anlotinib showed encouraging clinical data in phase II/II clinical trials involving patients with recurrent NSCLC, manifested by significantly increased overall response rate and prolonged median overall survival (23–26). Owing to these promising results, this drug has received an approval from CFDA to treat recurrent NSCLC (22). Our prior work revealed that this agent was cytotoxic to MLL-rearranged acute myeloid leukemia via suppression of SETD1A/AKT-mediated DNA damage response (48). Nevertheless, there has limited experience with Anlotinib treatment in ALL. In the present study, the in vitro preclinical models illustrated that Anlotinib markedly diminished cell viability, triggered apoptotic cell death and enriched cell cycle in G2/M phase in B-ALL cell lines. Furthermore, oral administration with Anlotinib for 3 weeks considerably abrogated leukemia burden in bone marrow and spleen, and improved prognosis in a PDX model engrafted with patient-derived B-ALL cells. Consistent with favorable adverse effects of Anlotinib reported in various other types of cancers, administration of Anlotinib in B-ALL in vivo model also showed minimal side effects. All these observed findings inform that clinical treatment with Anlotinib in patients with RR B-ALL is probably feasible with regard to its attractive preclinical data and tolerable adverse effects.

In concert with prior published series, we observed that Anlotinib exerted its cytotoxicity against B-ALL preclinical model potentially through interference with proangiogenic functional targets, including VEGFR2, PDGFR-beta and FGFR3, in a dose-dependent manner. PI3K/AKT/mTOR signaling cascade is a classical pro-survival pathway and plays a critical role in promoting cell proliferation and survival in normal circumstances. Aberrant activation of PI3K and its downstream AKT/mTOR signaling is often observed in a wide range of malignant diseases, including B-ALL (49–51). Aberration of PI3K/AKT/mTOR pathway is closely associated with chemotherapeutic resistance and inferior prognosis in pediatric and adult B-ALL patients (52, 53). Accordingly, inhibition of the activity of PI3K/AKT/mTOR pathway cascade has been widely evaluated in B-ALL cellular models, most of which have been proven to be active against B-ALL cells, thus prompting several small molecular inhibitors targeting the PI3K-associated cascade entering clinical evaluation (54). Of interest, the PI3K/AKT/mTOR cascade resides in the convergence of the three important proangiogenic signaling pathways (55–58), suggesting that downregulation of proangiogenic signals with Anlotinib treatment might result in perturbation of the function of PI3K/AKT/mTOR pathway. Not surprisingly, in the present study, exposure B-ALL cells to Anlotinib indeed decreased the phosphorylation levels of PI3K and also dephosphorylated the levels of AKT and mTOR.
Conclusions

Collectively, this study found that RR B-ALL showed high proangiogenic activity and activation of PI3K/AKT/mTOR pathway, a pivotal downstream of angiogenesis. Anlotinib was effective to kill B-ALL cells \textit{in vitro} and \textit{in vivo}. Mechanistically, the anti-leukemia effects of Anlotinib on B-ALL correlated with downregulation of VEGFR2, PDGFR-beta and FGFR3, three important proangiogenic mediators. Furthermore, attenuation of the activity of PI3K/AKT/mTOR pathway was one of the potential mechanisms of action of Anlotinib to kill B-ALL cells. Overall, our preclinical results provide a promising rational therapeutic option using Anlotinib for the treatment of B-ALL but need further confirmatory investigations in clinical trial.

Abbreviations

ALL: Acute lymphoblastic leukemia; TKI: Tyrosine Kinase Inhibitor; CAR-T: Chimeric-antigen receptor (CAR)-modified autologous T cells; DEGs: Differential expresssive genes; CCK-8: Cell Counting Kit-8; PDX: Patient Derived Xenograft.

Declarations

Consent for publication

Not applicable.

Availability of data and materials

The datasets supporting the conclusions of this study are included in the figures and table.

Competing interests

All authors declare no conflict of interest.

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Ethical statements

All animal studies were performed following protocols approved by Xiamen University Animal Care and Use Committees (XMULAC20170065).
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Not applicable.

Authors’ contributions

Conception and design were performed by QLC, QL, GWL, MMD, and BX. Research performance was performed by QLC, QL, and YLJ. Provision of study thought, materials, and technology were performed by QLC, QL, YLJ, QWC, GSC, GWL, MMD, and BX. Collection and assembly of data were done by QLC, QL, YLJ, QWC, GSC, GWL, MMD, and BX. Data analysis and interpretation were performed by QLC, QL, MMD, GWL, and BX. Manuscript writing was performed by QL, MMD, and BX. Final approval of manuscript was performed by all authors who read and approved the final manuscript.

References

1. Terwilliger T, Abdul-Hay M. Acute lymphoblastic leukemia: a comprehensive review and 2017 update. Blood Cancer J. 2017;7(6):e577.

2. Smith MA, Seibel NL, Altekruse SF, Ries LA, Melbert DL, O’Leary M, et al. Outcomes for children and adolescents with cancer: challenges for the twenty-first century. J Clin Oncol. 2010;28(15):2625-34.

3. Bhojwani D, Pui CH. Relapsed childhood acute lymphoblastic leukaemia. Lancet Oncol. 2013;14(6):e205-17.

4. Geyer MB, Hsu M, Devlin SM, Tallman MS, Douer D, Park JH. Overall survival among older US adults with ALL remains low despite modest improvement since 1980: SEER analysis. Blood. 2017;129(13):1878-81.

5. Hunger SP, Mullighan CG. Redefining ALL classification: toward detecting high-risk ALL and implementing precision medicine. Blood. 2015;125(26):3977-87.

6. Loffler A, Kufer P, Lutterbuse R, Zettl F, Daniel PT, Schwenkenbecher JM, et al. A recombinant bispecific single-chain antibody, CD19 x CD3, induces rapid and high lymphoma-directed cytotoxicity by unstimulated T lymphocytes. Blood. 2000;95(6):2098-103.

7. Fousek K, Watanabe J, Joseph SK, George A, An X, Byrd TT, et al. CAR T-cells that target acute B-lineage leukemia irrespective of CD19 expression. Leukemia. 2021;35(1):75-89.

8. Kantarjian H, Stein A, Gokbuget N, Fielding AK, Schuh AC, Ribera JM, et al. Blinatumomab versus Chemotherapy for Advanced Acute Lymphoblastic Leukemia. N Engl J Med. 2017;376(9):836-47.

9. Wudhikarn K, Flynn JR, Riviere I, Gonen M, Wang X, Senechal B, et al. Interventions and Outcomes of Adult Patients with B-ALL Progressing After CD19 Chimeric Antigen Receptor T Cell Therapy. Blood. 2021.
10. Kerbel RS. Tumor angiogenesis. N Engl J Med. 2008;358(19):2039-49.

11. De Palma M, Biziato D, Petrova TV. Microenvironmental regulation of tumour angiogenesis. Nat Rev Cancer. 2017;17(8):457-74.

12. Passaro D, Di Tullio A, Abarrategi A, Rouault-Pierre K, Foster K, Ariza-McNaughton L, et al. Increased Vascular Permeability in the Bone Marrow Microenvironment Contributes to Disease Progression and Drug Response in Acute Myeloid Leukemia. Cancer Cell. 2017;32(3):324-41 e6.

13. Kazerounian S, Lawler J. Integration of pro- and anti-angiogenic signals by endothelial cells. J Cell Commun Signal. 2018;12(1):171-9.

14. Simons M, Gordon E, Claesson-Welsh L. Mechanisms and regulation of endothelial VEGF receptor signalling. Nat Rev Mol Cell Biol. 2016;17(10):611-25.

15. Qin S, Li A, Yi M, Yu S, Zhang M, Wu K. Recent advances on anti-angiogenesis receptor tyrosine kinase inhibitors in cancer therapy. J Hematol Oncol. 2019;12(1):27.

16. Jayson GC, Kerbel R, Ellis LM, Harris AL. Antiangiogenic therapy in oncology: current status and future directions. Lancet. 2016;388(10043):518-29.

17. Annese T, Tamma R, Ruggieri S, Ribatti D. Angiogenesis in Pancreatic Cancer: Pre-Clinical and Clinical Studies. Cancers (Basel). 2019;11(3).

18. Gao Y, Liu P, Shi R. Anlotinib as a molecular targeted therapy for tumors. Oncol Lett. 2020;20(2):1001-14.

19. Shen G, Zheng F, Ren D, Du F, Dong Q, Wang Z, et al. Anlotinib: a novel multi-targeting tyrosine kinase inhibitor in clinical development. J Hematol Oncol. 2018;11(1):120.

20. Zhang K, Ma X, Gao H, Wang H, Qin H, Yang S, et al. Efficacy and Safety of Anlotinib in Advanced Non-Small Cell Lung Cancer: A Real-World Study. Cancer Manag Res. 2020;12:3409-17.

21. Han B, Li K, Zhao Y, Li B, Cheng Y, Zhou J, et al. Anlotinib as a third-line therapy in patients with refractory advanced non-small-cell lung cancer: a multicentre, randomised phase II trial (ALTER0302). Br J Cancer. 2018;118(5):654-61.

22. Syed YY. Anlotinib: First Global Approval. Drugs. 2018;78(10):1057-62.

23. Zhou AP, Bai Y, Song Y, Luo H, Ren XB, Wang X, et al. Anlotinib Versus Sunitinib as First-Line Treatment for Metastatic Renal Cell Carcinoma: A Randomized Phase II Clinical Trial. Oncologist. 2019;24(8):e702-e8.

24. Chi Y, Fang Z, Hong X, Yao Y, Sun P, Wang G, et al. Safety and Efficacy of Anlotinib, a Multikinase Angiogenesis Inhibitor, in Patients with Refractory Metastatic Soft-Tissue Sarcoma. Clin Cancer Res.
25. Sun Y, Niu W, Du F, Du C, Li S, Wang J, et al. Safety, pharmacokinetics, and antitumor properties of anlotinib, an oral multi-target tyrosine kinase inhibitor, in patients with advanced refractory solid tumors. J Hematol Oncol. 2016;9(1):105.

26. Wang J, Wu DX, Meng L, Ji G. Anlotinib combined with SOX regimen (S1 (tegafur, gimeracil and oteracil potassium capsules) + oxaliplatin) in treating stage IV gastric cancer: study protocol for a single-armed and single-centred clinical trial. BMJ Open. 2020;10(6):e034685.

27. Gautier L, Cope L, Bolstad BM, Irizarry RA. affy--analysis of Affymetrix GeneChip data at the probe level. Bioinformatics. 2004;20(3):307-15.

28. Diboun I, Wernisch L, Orengo CA, Koltzenburg M. Microarray analysis after RNA amplification can detect pronounced differences in gene expression using limma. BMC Genomics. 2006;7:252.

29. Dennis G, Jr., Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, et al. DAVID: Database for Annotation, Visualization, and Integrated Discovery. Genome Biol. 2003;4(5):P3.

30. Chen EY, Xu H, Gordonov S, Lim MP, Perkins MH, Ma'ayan A. Expression2Kinases: mRNA profiling linked to multiple upstream regulatory layers. Bioinformatics. 2012;28(1):105-11.

31. Kanehisa M, Sato Y, Kawashima M, Furumichi M, Tanabe M. KEGG as a reference resource for gene and protein annotation. Nucleic Acids Res. 2016;44(D1):D457-62.

32. Sujobert P, Bardet V, Cornillet-Lefebvre P, Hayflick JS, Prie N, Verdier F, et al. Essential role for the p110delta isoform in phosphoinositide 3-kinase activation and cell proliferation in acute myeloid leukemia. Blood. 2005;106(3):1063-6.

33. Potratz J, Tillmanns A, Berning P, Korschning E, Schaefer C, Lechtape B, et al. Receptor tyrosine kinase gene expression profiles of Ewing sarcomas reveal ROR1 as a potential therapeutic target in metastatic disease. Mol Oncol. 2016;10(5):677-92.

34. Du Z, Lovly CM. Mechanisms of receptor tyrosine kinase activation in cancer. Mol Cancer. 2018;17(1):58.

35. Ling Y, Xie Q, Zhang Z, Zhang H. Protein kinase inhibitors for acute leukemia. Biomark Res. 2018;6:8.

36. Chougule RA, Shah K, Moharram SA, Vallon-Christersson J, Kazi JU. Glucocorticoid-resistant B cell acute lymphoblastic leukemia displays receptor tyrosine kinase activation. NPJ Genom Med. 2019;4:7.

37. Song F, Hu B, Cheng JW, Sun YF, Zhou KQ, Wang PX, et al. Anlotinib suppresses tumor progression via blocking the VEGFR2/PI3K/AKT cascade in intrahepatic cholangiocarcinoma. Cell Death Dis. 2020;11(7):573.
38. Cardones AR, Banez LL. VEGF inhibitors in cancer therapy. Curr Pharm Des. 2006;12(3):387-94.

39. Zirlik K, Duyster J. Anti-Angiogenics: Current Situation and Future Perspectives. Oncol Res Treat. 2018;41(4):166-71.

40. Ferrara N, Hillan KJ, Gerber HP, Novotny W. Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. Nat Rev Drug Discov. 2004;3(5):391-400.

41. Deng M, Zha J, Jiang Z, Jia X, Shi Y, Li P, et al. Apatinib exhibits anti-leukemia activity in preclinical models of acute lymphoblastic leukemia. J Transl Med. 2018;16(1):47.

42. Mawalla B, Yuan X, Luo X, Chalya PL. Treatment outcome of anti-angiogenesis through VEGF-pathway in the management of gastric cancer: a systematic review of phase II and III clinical trials. BMC Res Notes. 2018;11(1):21.

43. Bruix J, Takayama T, Mazzaferro V, Chau GY, Yang J, Kudo M, et al. Adjuvant sorafenib for hepatocellular carcinoma after resection or ablation (STORM): a phase 3, randomised, double-blind, placebo-controlled trial. Lancet Oncol. 2015;16(13):1344-54.

44. Itatani Y, Kawada K, Yamamoto T, Sakai Y. Resistance to Anti-Angiogenic Therapy in Cancer-Alterations to Anti-VEGF Pathway. Int J Mol Sci. 2018;19(4).

45. Kono SA, Heasley LE, Doebele RC, Camidge DR. Adding to the mix: fibroblast growth factor and platelet-derived growth factor receptor pathways as targets in non-small cell lung cancer. Curr Cancer Drug Targets. 2012;12(2):107-23.

46. Hosaka K, Yang Y, Nakamura M, Andersson P, Yang X, Zhang Y, et al. Dual roles of endothelial FGF-2-FGFR1-PDGF-BB and perivascular FGF-2-FGFR2-PDGFRbeta signaling pathways in tumor vascular remodeling. Cell Discov. 2018;4:3.

47. Bergers G, Hanahan D. Modes of resistance to anti-angiogenic therapy. Nat Rev Cancer. 2008;8(8):592-603.

48. Chen J, Feng J, Fang Z, Ye J, Chen Q, Chen Q, et al. Anlotinib suppresses MLL-rearranged acute myeloid leukemia cell growth by inhibiting SETD1A/AKT-mediated DNA damage response. Am J Transl Res. 2021;13(3):1494-504.

49. Janku F, Yap TA, Meric-Bernstam F. Targeting the PI3K pathway in cancer: are we making headway? Nat Rev Clin Oncol. 2018;15(5):273-91.

50. Slomovitz BM, Coleman RL. The PI3K/AKT/mTOR pathway as a therapeutic target in endometrial cancer. Clin Cancer Res. 2012;18(21):5856-64.
51. Ediriweera MK, Tennekoon KH, Samarakoon SR. Role of the PI3K/AKT/mTOR signaling pathway in ovarian cancer: Biological and therapeutic significance. Semin Cancer Biol. 2019;59:147-60.

52. Neri LM, Cani A, Martelli AM, Simioni C, Junghanss C, Tabellini G, et al. Targeting the PI3K/Akt/mTOR signaling pathway in B-precursor acute lymphoblastic leukemia and its therapeutic potential. Leukemia. 2014;28(4):739-48.

53. Badura S, Tesanovic T, Pfeifer H, Wystub S, Nijmeijer BA, Liebermann M, et al. Differential effects of selective inhibitors targeting the PI3K/AKT/mTOR pathway in acute lymphoblastic leukemia. PLoS One. 2013;8(11):e80070.

54. Fransecky L, Mochmann LH, Baldus CD. Outlook on PI3K/AKT/mTOR inhibition in acute leukemia. Mol Cell Ther. 2015;3:2.

55. Deng M, Zha J, Zhao H, Jia X, Shi Y, Li Z, et al. Apatinib exhibits cytotoxicity toward leukemia cells by targeting VEGFR2-mediated prosurvival signaling and angiogenesis. Exp Cell Res. 2020;390(1):111934.

56. Karar J, Maity A. PI3K/AKT/mTOR Pathway in Angiogenesis. Front Mol Neurosci. 2011;4:51.

57. Starska K, Forma E, Lewy-Trenda I, Stasikowska-Kanicka O, Skora M, Brys M. Fibroblast growth factor receptor 1 and 3 expression is associated with regulatory PI3K/AKT kinase activity, as well as invasion and prognosis, in human laryngeal cancer. Cell Oncol (Dordr). 2018;41(3):253-68.

58. Zhang H, Bajraszewski N, Wu E, Wang H, Moseman AP, Dabora SL, et al. PDGFRs are critical for PI3K/Akt activation and negatively regulated by mTOR. J Clin Invest. 2007;117(3):730-8.

Tables

Table 1. The IC50 values of Nalm6 and Sup15 cells treated with Anlotinib for 24 and 48 hours

| Cell lines | IC50 ± S.D (μmol/L) |
|------------|---------------------|
|            | 24 hours            | 48 hours            |
| Nalm6      | 3.224 ± 0.875       | 2.468 ± 0.378       |
| Sup15      | 3.803 ± 0.409       | 2.459 ± 0.443       |

Supplemental Tables

Table S1 is not available with this version.

Figures
Figure 1

In silico analysis predict Anlotinib as a potential anti-leukemic therapeutic choice. (A) & (B) Clustered heatmap and volcano plot were generated by the normalized and annotated significant differential expressed genes (corrected P<0.05 and |log fold-change (FC)|>1). (C) GO analysis of DEGs whose expression was altered in relapsed B-ALL cases. (D) Potential upstream regulatory kinase and downstream enriched pathways network was constructed by DEGs. (E) TKI-Kinase-Pathway network was constructed and shown. (F) & (G) Potential upstream kinase and potential downstream pathways were suggested by the overlapped section.
Figure 2

The cytotoxic effects of Anlotinib on B-ALL cells. (A) & (B) Exposure either Nalm6 or SupB15 cells to designated concentrations of Anlotinib for 24 and 48h, cell viability was determined with a CCK-8 kit. (C) & (D) Flow cytometric analysis of cell cycle distribution of B-ALL cells treated with distinct concentrations of Anlotinib for 24h. (E-F) Annexin V/PI dual-staining assay was used to analyze cell apoptotic proportion of Nalm6 and SupB15 cells treated with or without Anlotinib for 24 and 48 h. (G-H) The representative flow cytometric plots of B-ALL cells exposed to indicated doses of Anlotinib for 24 and 48h. Each experiment was independently performed three times in triplicate. *P < 0.05, **P < 0.01, ***P < 0.001.
Figure 3

Anlotinib reduces leukemic burden and prolongs survival in a patient-derived xenograft (PDX) B-ALL mouse model. (A) The scheme for the timeline of the experiments using a PDX mouse model. (B) Monitoring of body weight once daily in both vehicle and Anlotinib groups during the entire treatment course. Each point represents mean ± S.D for body weight in each group. (C) & (D) The size and weight of spleen were measured in each group. (E-H) Flow-cytometric analysis of the percentage of human CD45+ and human CD19+ leukemic cells in spleen and femur bone marrow. (I) Assessment of Kaplan–Meier survival curve in control and Anlotinib groups.
Anlotinib decreases the levels of key proangiogenic mediators and blunts the activity of PI3K/AKT/mTOR pathway in B-ALL cells. Nalm6 and SupB15 cells were treated with or without Anlotinib for 24 h. (A) & (B) Real-time quantitative PCR analysis of mRNA levels of VEGFR2, PDGFR-beta and FGFR3. (C) Western blot analysis of the expression of p-PI3K, p-AKT, AKT, mTOR and p-mTOR. (D) Schematic depiction of the underlying mechanism of Anlotinib cytotoxicity against B-ALL cells.