CD34 is a target for covalent EGFR inhibitors to eliminate stem/progenitor cells in acute and chronic myeloid leukemia

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Abstract

The effect of epidermal growth factor receptor (EGFR) inhibitors on acute myeloid leukemia (AML) was discovered over one decade ago. However, clinical trials of EGFR inhibitors in AML have yielded controversial outcomes. Leukemia cells lack EGFR expression, and the mechanism by which EGFR inhibitors affect leukemia cell growth is unknown, obscuring the precise subset of AML patients that might be targeted by these compounds. Since myeloid leukemia arises from malignant stem/progenitors, here we evaluated the effect of EGFR inhibitors on primary leukemia stem/progenitors that expressed the stem cell marker CD34 which were sorted from leukemia patients. EGFR inhibitors induced significant apoptosis of primary CD34\(^+\) but not CD34\(^-\) cells derived from AML and chronic myeloid leukemia (CML) patients both in vitro and in patient-derived xenotransplantation model. Using two EGFR inhibitors osimertinib and afatinib, we demonstrated binding and covalent adducts of the inhibitors with the cysteine(C) 199 residue of the CD34 protein, which downregulated phosphorylation of tyrosine 329(Y329) of CD34, leading to the dissociation of CD34 from tyrosine kinase Src and thereafter the inhibition of STAT3 phosphorylation. Most importantly, administration of osimertinib yielded clinical responses in two CD34-high AML patients identified by quantitative proteomics with reduced levels of Y329 phosphorylation of CD34 after treatment. Collectively, these findings delineate a novel molecular pathway whereby EGFR inhibitors kill leukemia and reveal that the CD34 antigen is a targetable signaling molecule that mediates cell survival signals via connecting to Src-STAT3 pathway.

Introduction

Acute myeloid leukemia (AML) is a lethal hematological malignancy, with a 5-year overall survival rate of less than 30% with chemotherapy\(^1\),\(^2\),\(^3\),\(^4\). Although 60–70% of AML patients enter complete remission after induction regimen (combination of cytarabine and anthracycline), most of them relapse within 3 years due to the outgrowth of chemotherapy-resistant leukemic stem/progenitor cells\(^5\),\(^6\),\(^7\),\(^8\),\(^9\), highlighting the urgent need to develop new therapeutic approaches for treating this disease, especially for chemotherapy-resistant and relapsed patients.

An important breakthrough in the treatment of non-small cell lung cancer (NSCLC) is the discovery of epidermal growth factor receptor (EGFR) activating mutations as an effective therapeutic target and the successful development of EGFR tyrosine kinase inhibitors (EGFRi)\(^10\),\(^11\). First generation drugs, represented by erlotinib and gefitinib, reversibly interfere with ATP binding to EGFR. Second and third generation drugs, including afatinib, osimertinib and rociletinib, covalently and irreversibly inhibit kinase by binding to an active cysteine residue in the EGFR\(^12\),\(^13\). In 2007 and 2008, two independent cases reported EGFRi treatment induced complete remission of AML in patients with concomitant lung cancer\(^14\),\(^15\). These case reports were followed by \textit{in vitro} studies in which EGFRi displayed anti-proliferative or pro-differentiation effects against AML\(^16\),\(^17\),\(^18\),\(^19\), demonstrating EGFR monotherapy as a promising treatment for AML. It is believed that these compounds function in an EGFR-independent pathway because EGFR is not expressed in AML according to multiple studies\(^14\),\(^16\),\(^18\),\(^20\). Therefore, much efforts
have been implemented to identify the “off-target” of EGFR inhibitors in AML cells and a couple of non-receptor tyrosine kinases including spleen tyrosine kinase (SYK) and Janus kinase 1 (JAK1) have been suggested to function downstream of EGFRi\textsuperscript{16,18}. However, no evidence of direct binding by which these molecules induce phenotypic alterations in AML has been revealed. While multiple clinical trials using erlotinib or afatinib in AML have yielded conflicting, but mostly negative clinical results\textsuperscript{21,22,23,24}. Effective use of EGFRi in the treatment of AML patients has been stymied due to the absence of a method identifying the patients that might optimally benefit from this approach. Using an unbiased screen for molecular targets of EGFRi in myeloid leukemia cells, we discovered that phosphorylation of the CD34 protein was inhibited by osimertinib and this represents a mechanism for the anti-survival effects of EGFRi on AML cells. We show herein that CD34 expression on leukemia stem cells identifies a patient subset which might gain the greatest benefit from treatment with these compounds.

Results

EGFR inhibitors induced apoptosis of both acute and chronic myeloid leukemia stem/progenitor cells.

To test the effects of EGFRi on leukemic stem/progenitor cells, we isolated CD34\textsuperscript{+} leukemic stem/progenitor cells from AML patients by FACS and then treated CD34\textsuperscript{+} AML cells in culture with AZD9291 (osimertinib), a third generation covalent EGFR inhibitors. The percentage of apoptosis was measured using Annexin V/PI assay. As shown in Fig. 1a, treatment with osimertinib from 0.5 µM dose-dependently induced apoptosis of CD34\textsuperscript{+} leukemic stem/progenitor cells. Similar effects were also observed from other covalent EGFR inhibitors including afatinib and rociletinib (Fig. 1b)\textsuperscript{11,25}. Surprisingly, the EGFR inhibitors also displayed remarkable apoptosis-inducing effects on primary CD34\textsuperscript{+} cells sorted from CML patients (Fig. 1c), which population is believed to be responsible for relapse of CML due to insensitivity to BCR-ABL inhibitor imatinib\textsuperscript{6}, demonstrating that EGFR inhibitors kill stem/progenitor cells of myeloid leukemia.

We next evaluate the effect of EGFR inhibitors on CD34\textsuperscript{−} leukemia cells purified from AML and CML patients. As depicted in Fig. 2a, exposure of CD34\textsuperscript{−} cells from AML and CML individuals to different EGFR inhibitors did not induce significant cell death compared to its CD34\textsuperscript{+} counterparts, suggesting a specificity of EGFR-inhibitors towards CD34\textsuperscript{+} cells. Moreover, incubation of the bone marrow mononuclear cells (BMMCs) from AML samples with osimertinib demonstrated that CD34\textsuperscript{+} cells within the bulk population were more susceptible than CD34\textsuperscript{−} population (Fig. 2b).

Osimertinib Induced Loss Of Y329 Phosphorylation Of Cd34

To reveal the mechanism of EGFRi, we first looked for the expression of EGFR on CD34\textsuperscript{+} leukemia stem/progenitors cells from AML and CML patients. Taking the EGFR\textsuperscript{+} lung carcinoma cell line A549 as a positive control, we conducted western blot, immunofluorescent staining and flow cytometry analysis using antibody against EGFR or Y1068-phosphorylated EGFR, an active form of EGFR\textsuperscript{26}. In accordance
with previous observations\textsuperscript{14,18,20}, EGFR protein in CD34\textsuperscript{+} leukemia stem/progenitors cells were barely detectable (Supplementary Fig. 1).

Next, we investigated EGFR-independent mechanisms for the inhibitory effects of EGFRi in CD34\textsuperscript{+} leukemia stem/progenitor cells. We carried out quantitative proteomic analyses of phospho-tyrosine(Y) peptides to explore intracellular signaling events in response to osimertinib. CD34\textsuperscript{+} cells isolated from 3 AML individuals were pooled together and treated with osimertinib followed by analysis of tyrosine-phosphorylated sites targeted by osimertinib using liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS). Previously reported large-scale phosphoproteomic studies indicated that off-targets of EGFR inhibitors are preferentially intracellular non-receptor tyrosine kinases including Syk and Lyn\textsuperscript{12,18,27,28}. As expected, the group of proteins annotated as tyrosine protein kinases was significantly overrepresented as being under-phosphorylated in the phosphoproteomes of osimertinib-treated cells (Fig. 3a, Supplementary Table 1). Notably, phosphorylation of a significant portion of membrane proteins was inhibited upon osimertinib exposure, which was further supported by STRING analysis (Fig. 3a and 3b, Supplementary Table 2). Meanwhile, motif analysis revealed the osimertinib targeted “-E-X-X-pY” motif, which is enriched (fold increase 4.66 vs 3.88) in downregulated phospho-tyrosine sites compared to all regulated sites (right panel, Fig. 3b). Among the 23 proteins containing the “-E-X-X-pY” motif (Supplementary Table 3), there are 7 protein tyrosine kinases and unexpectedly, one membrane molecule CD34, the marker of stem/progenitor cells that lacks tyrosine kinase activity (right panel, Fig. 3b)\textsuperscript{29,30}. A remarkable loss of Y329 phosphorylation of CD34 after osimertinib exposure was observed by phosphoproteomic analysis (Fig. 3a and Supplementary Fig. 2). Next we validated the downregulation of Y329 phosphorylation of CD34 (Fig. 3d), but not changes in the protein level of CD34 (Fig. 3c) by osimertinib treatment using parallel reaction monitoring-based targeted MS (PRM-MS) in 5 AML patient samples as well as CD34-expressing KG-1 and Kasumi-1 leukemia cell lines (Fig. 3c-e, Supplementary Fig. 2–8). This finding was recapitulated in an independent set of AML specimens using a custom-designed antibody against the phosphorylated Y329 residue of CD34(CD34-pY329) (Supplementary Fig. 9). Taken together, these data demonstrate that treatment with osimertinib inhibits phosphorylation of Y329 in CD34.

**EGFR covalent inhibitors directly bind with CD34 at cysteine 199**

We next investigated how EGFR inhibitors associate with CD34. One common feature of leukemia stem/progenitors cells from AML and CML patient samples is the expression of CD34. Moreover, EGFRi do not kill CD34\textsuperscript{−} leukemia cells. We proposed that CD34 might be a non-tyrosine kinase target of EGFRi. To test this hypothesis, we incubated purified recominant CD34 with osimertinib or afatinib, two covalent EGFRi, followed by MS analysis. We got 44% sequence coverage of CD34 protein and identified all the 6 cysteines within the cysteine-rich domain in CD34 sequence. Notably, the m/z ratio of the C199-containing peptide TSSCAEFKK (Fig. 4a) was measured as 1,056.49 in the absence of osimertinib and 1,498.74 in the presence of osimertinib. For afatinib binding assay, the m/z ratio of the same C199-containing peptide was measured as 1,484.63 and 1,056.49 with/without afatinib (Fig. 4b). The
calculated mass shift was consistent with the addition of one molecule of osimertinib or afatinib respectively. Clearly, EGFR covalent inhibitors bind the C199 residue of CD34 in vitro.

To clarify the role of CD34 in mediating the apoptotic effect induced by osimertinib, we depleted CD34 in primary CD34^+ AML cells, KG-1 and Kasumi-1 cell with the CRISPR-Cas9 system. As shown in Fig. 4c, knockout of CD34 caused primary leukemia cells less sensitive to osimertinib, which could be restored by re-expression of CD34^WT but not CD34^C199S (Fig. 4d), indicating that the binding to C199 of CD34 is essential in mediating the effect of osimertinib.

**Osimertinib inhibits phosphorylation of STAT3 through impairing the interaction between CD34 and Src**

As leukemia cell maintenance and growth depends upon kinase-mediated signal cascade, we next investigated the kinase signals that are associated with modification of CD34 in response to osimertinib. We treated primary CD34^+ cells with osimertinib and probed several major signaling pathways. Constitutive signal transducer and activator of transcription 3(STAT3) activation were observed in CD34^+ AML patient samples (Fig. 4e) as well as KG-1 and Kasumi-1 leukemia cell lines (Fig. 4g), but not in purified CD34^- primary leukemia cells (Fig. 4f). Osimertinib significantly suppressed STAT3 activation without altering the protein level of STAT3 (Fig. 4e). Phosphorylation of ERK (p44/42) and AKT was not altered upon osimertinib treatment (Fig. 4e and 4g). Moreover, osimertinib treatment did not induce significant downregulation of STAT3 in CD34^C199S-expressing KG-1 cells compared to its WT counterpart (Fig. 4h), indicating that STAT3 is the major kinase that is responsible for apoptosis-inducing effect of osimertinib. In support of this hypothesis, STAT3 inhibitor stattic induced synthetic lethality with osimertinib (Fig. 4i and 4j).

STAT3 can be activated by growth factor receptors or cytokine receptors, usually via kinases such as janus-activated kinase (JAK) or proto-oncogene tyrosine-protein kinase Src, leading to increased cell survival. To clarify the intracellular signals between CD34 and activated STAT3, we pooled CD34^+ cells from 3 AML individuals, treated with/without osimertinib and conducted endogenous immunoprecipitation–mass spectrometry (IP–MS) assays using an antibody to CD34. STAT3 was not observed in the CD34-immunoprecipitate, suggesting absence of direct interaction between CD34 and STAT3 (Supplementary Table 4). Among the CD34-interacting proteins, Src was identified to be downregulated upon osimertinib treatment (Fig. 5a, Supplementary Table 4) and was further validated in KG-1 and Kasumi-1 cells as well as primary CD34^+ cells using antibody against CD34 (Fig. 5b-d). The association between Src and CD34 was further confirmed through IP assay using an antibody to Src (Fig. 5b and 5c) and immunofluorescent staining (Fig. 5e and 5f). More importantly, the endogenous interaction between CD34 and Src was impaired upon osimertinib treatment (Fig. 5g), providing a molecular basis for osimertinib-induced downregulation of STAT3 phosphorylation. Of note, Crk-like protein(CRKL), a previously reported CD34-interacting adaptor protein, was not immunoprecipitated by anti-CD34 antibody in our system (data not shown). Collectively, we proposed that the binding of osimertinib to CD34 impaired its interaction with Src, leading to impaired activation of STAT3 that is
necessary for the maintenance of CD34\(^+\) leukemia cells. Supporting this finding, phosphorylation of Src was apparently suppressed by osimertinib (Fig. 4e-g) and Src inhibitor saracatinib\(^36\) synergistically induced apoptosis with osimertinib in primary CD34\(^+\) cells (Fig. 5h).

**Effect of Osimertinib on CD34\(^+\) cells in PDX preclinical models**

To assess the clinical relevance of these findings, the *in vivo* effects of osimertinib on CD34\(^+\) leukemia stem/progenitor cells were further evaluated by patient-derived xenograft (PDX) AML and CML models. We sorted CD34\(^+\) cells from AML or CML patients and exposed them to osimertinib for 48 hours before transplantation into sub-lethally irradiated NSG mice (Fig. 6a). Human CD45\(^+\) cells were detectable by flow cytometry in bone marrow 16 weeks post-transplantation, representing successful engraftment of human leukemia stem cells. Percentage of CML CD34\(^+\) cells were largely reduced in osimertinib-treated group (Fig. 6c). Osimertinib clearly reduced engraftment of CD34\(^+\) cells as indicated by decreased numbers of CD45\(^+\) and CD33\(^+\) cells in the bone marrow of both AML and CML PDX models (Fig. 6b-c).

**Osimertinib Therapy In Cd34-high AML Patients**

Based on our analyses that CD34\(^+\) leukemia stem/progenitors cells can be targeted by covalent EGFR inhibitors, we reasoned that the controversial outcomes of clinical trials might be due to difference of the expression of CD34\(^+\) across clinical samples such that administration of EGFR inhibitors might only be of benefit to the subsets of AML with high percentages of CD34\(^37\). We next purified the CD34\(^+\) cells and measured the level of CD34 expression on leukemia cells using quantitative proteomics (Fig. 7a). We observed that CD34 expression on AML CD34\(^+\) cells were higher than CD34\(^+\) cells from healthy donors. The group of patients (n = 15) in the upper quartile was designated as “CD34-high” population among all the AML (n = 59). Furthermore, osimertinib treatment on normal CD34\(^+\) cells displayed less toxicity compared to its AML counterpart (Fig. 6d), suggesting a therapeutic window. Next, after obtaining informed consent, two CD34-high patients who have failed to respond to any available therapy was initiated on 80 mg once daily of osimertinib.

Patient 1, a 47-year-old woman was diagnosed with AML-M0 with a peripheral white blood cell (WBC) count of 223×10\(^9\)/L, and morphologically 90% of which were myoblasts. The percentage of CD34\(^+\) blasts was 80.3% (Fig. 7b). After diagnosed with AML-M0, induction chemotherapy with HA (homoharringtonine and cytosine arabinoside) was begun, but on day 21 of chemotherapy interval bone marrow showed 97% blasts indicating refractory disease. Her disease was refractory to a second induction on a clinical trial (ChiCTR1800019049) using FLAG (fludarabine, cytarabine and filgrastim) combined with decitabine, and then a third line treatment with CLAG (cladribine, cytarabine and G-CSF) with 34% blasts in the bone marrow on day 28. Finally, she rejected any further chemotherapy for the intolerable myotoxicity, and requested oral drugs to expect an accidental prolongation for her life. Considering the status of multiple drug resistance and the potential efficacy for AML from the case reports, she was voluntarily started on osimertinib (off-label, non-protocol) 80 mg once daily (day 0) after evaluating the response of her CD34\(^+\)
BMMC to osimertinib in vitro (Fig. 1a, AML-#4). Her WBC count dropped sharply from \(24.9 \times 10^9/L\) to \(7.1 \times 10^9/L\) at day 2 and remained below \(5 \times 10^9/L\) till day 17 (Fig. 7b). A bone marrow biopsy demonstrated 5% of blast in the bone marrow on day 14 (Fig. 7d).

Patient 2, a 69-year-old man was diagnosed with AML-M2a, when he presented with dyspnea, dizziness, and a WBC count of \(68.3 \times 10^9/L\) with 50.1% of marrow blasts CD34+. He developed pneumonia of left upper lobe. Given his poor performance status, he rejected any chemotherapy and voluntarily started on osimertinib 80 mg once daily (day 0) and the WBC count dropped from \(24.9 \times 10^9/L\) to \(3.1 \times 10^9/L\) on day 3 and remained below \(5 \times 10^9/L\) till day 9 (Fig. 7c and 7e).

In parallel, the level of Y329-phosphorylated CD34(pCD34-Y329) decreased significantly after administration of osimertinib in both patients (Fig. 7f). Collectively, these data suggested an opportunity to evaluate osimertinib’s usage for treating AML patients from the subgroup with high percentage of CD34 and warrants clinical investigation in the treatment of refractory/resistant AML.

**Discussion**

CD34 is a heavily glycosylated sialomucin-type transmembrane phosphoglycoprotein, first identified on stem/progenitor cells\(^{29,38}\). Recent evidences demonstrate a wider context of CD34 expression including muscle satellite cells, corneal keratocytes, interstitial cells, epithelial progenitors, and vascular endothelial progenitors\(^{38}\). Clinically, sorting CD34+ cells is used to enrich donor hematopoietic stem cells for allogeneic stem cell transplantation. Besides its well-known role as stem cell marker, the function of CD34 is poorly understood. The most well-defined role of CD34 is in cyto-adhesion as a binding partner of L-selectin\(^ {39,40,41,42}\). However, controversial evidence also supports a role of CD34 in blocking cell adhesion\(^ {43}\). Structurally, CD34 possesses an extracellular domain containing mucin domains which bears multiple O-linked and N-linked carbohydrates and is followed by a cysteine-rich domain with undermined function. The cytoplasmic domain contains a consensus site for tyrosine phosphorylation but lacks enzymatic domain motifs (Fig. 7g). It is therefore speculated that CD34 transduces signals through binding adaptor proteins such as CRKL to modulate biological processes including adhesion\(^ {34}\).

In this study, we described three previously unknown characteristics of CD34. First, the cysteine rich domain is the target of covalent tyrosine kinase inhibitors. Recently developed orally administrated covalent inhibitors including ibrutinib, afatinib and osimertinib have displayed multiple advantages versus conventional reversible inhibitors including the potential for more sustained target engagement and prolonged pharmacodynamic effects in cancers\(^ {14,44}\). To the best of our knowledge, this is the first evaluation of administration of the third generation EGFR inhibitors on AML cells and patients. More importantly, our results provide a rational for targeting this domain in developing CD34-associated drug development in a wide range of clinical context including stem cell mobilization. Second, we defined a signal pathway that is mediated by intracellular domain of CD34 contributing to stem cell survival. A new phosphorylation event and binding partner of CD34 was identified in the membrane fraction of AML cells. Our result also suggested that leukemia stem/progenitor cells are vulnerable to Src/STAT3 perturbation.
and support more pre-clinical and clinical evaluation of EGFRi in AML treatment. However, we have not yet determined the mechanism of how Y329- phosphorylation is affected by extracellular C199 residue, and further investigation is necessary. Thirdly, CD34 can serve as a therapeutic target for eradicating stem/progenitor cells while sparing the healthy counterpart. Interestingly, the two original cases of EGFR-inhibitor responsive AML that occurred in NSCLC were both diagnosed as M1, with 88% and 81% of blasts CD34+ \textsuperscript{14,15}.

The clinical response of two AML patients to anecdotal osimertinib monotherapy suggested significant anti-leukemia activity, particularly as these patients had experienced multiple rounds of chemotherapy or had poor performance status precluding treatment with conventional induction regimens. Osimertinib therapy may serve as a bridge to allogenic hematopoietic stem cell transplantation for patients with refractory AML, although transplant was not performed on the two patients in this study. Unexpectedly, EGFR covalent inhibitors also present activity against stem/progenitor cells in CML. Administration of imatinib and second generation of TKI has transformed CML from a fatal malignancy to a manageable disease with lifelong therapy\textsuperscript{6,45,46}. However, CML leukemia stem cells are independent of BCR-ABL for survival and rare resistance to imatinib remains a significant clinical challenge. The selective killing of CD34\textsuperscript{+} cells by osimertinib suggested that synergistic use of imatinib with osimertinib may provide an attractive approach to target BCR-ABL-independent mechanism of resistance.

In summary, our work reveals that EGFR covalent inhibitors target CD34 with significant anti-myeloid leukemia activity. Despite CD34 expression being used as a marker on hematopoietic stem/progenitor cells, the data presented herein reveal an unanticipated and novel function for CD34 in the maintenance of hematopoietic stem/progenitor cells and define a biomarker for the selection of the AML patients that may benefit from EGFR inhibitor therapy.

**Methods**

**Patients samples and cells**

Bone marrow samples were collected from leukemia patients diagnosed according to French-American-British classification at Department of Hematology of the Second Hospital of Dalian Medical University. Informed consent was obtained from all patients in accordance with the Declaration of Helsinki, and all manipulations were approved by the Medical Science Ethic Committee of Dalian Medical University. Donors for allogeneic bone marrow transplantation were used to purify normal healthy CD34\textsuperscript{+} cells. KG-1 and Kasumi-1 were authenticated. Details are available in Supplementary material.

**Reagents And Antibodies**

Osimertinib (HY-15772), afatinib (HY-10261), rociletinib (HY-15729), stattic (HY-13818), saracatinib (HY-10234) were obtained from MedChemExpress (MCE). Puromycin was from Merck/Millipore. Antibodies against the following proteins were used: CD34 (Abcam, ab81289), EGFR (Abcam, ab52894), phosphorylated Y1068-EGFR (Abcam, ab40815), phospho-STAT3 (Cell Signaling Technology, #9145),
phospho-AKT (Cell Signaling Technology, 4056), phospho-MAPK (Cell Signaling Technology, #4370), phospho-AKT (Cell Signaling Technology, #4060), phosphor-Src (Cell Signaling Technology, #6943), Src (Proteintech, 60315; Abcam, ab231081), and β-actin (Cell Signaling Technology, #4970). Secondary antibodies for immunofluorescence staining are Alexa Fluor 488 and Alexa Fluor 555 (Invitrogen Molecular Probes, A11008 and A31572).

**AML And CML Patient-derived Xenograft Models**

Primary CD34+ AML or CML cells were cultured with osimertinib for 48h and were transplanted via tail vein into female 8-10-week-old sub-lethally irradiated (2.5Gy) NOD.Cg-PrkdcscidIl2rgtm1Wjl/SzJ NSG mice (The Jackson Laboratory). Human cells were assessed using anti-human CD45 (Invitrogen, 11045942), anti-mouse CD45 (Invitrogen, 12045182), anti-human CD34 (Invitrogen, 17034942). anti-human CD33 (Invitrogen, 48033742) by flow cytometry. Animal handling was approved by the committee for humane treatment of animals at Shanghai Jiao Tong University School of Medicine.

**Statistical analysis**

Statistical analyses between the control and treatment groups were performed by standard two-tailed Student's t-test. All experiments were repeated at least three times. A value of p < 0.05 was considered to be statistically significant.

**Declarations**

**Conflict of Interest:**

All authors concur with this submission and declare no competing financial interests.

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**Author contributions**

Y.L., E.K.W. and J.S.Y. designed research; L.X., L.-X. Y, H.-C.L., Y.J. and Y.L. performed research; J.-S.Y., Z.-J.K., C.-T.Z., B.-B.G. and Y.J. contributed new reagents, collected subject samples and managed subject information; E.K.W., L.X., Y.L., J.-S.Y. and X.-H.Z. analyzed data; and Y.L., E.K.W. and J.-S.Y. wrote the paper.

**Data availability**
The data supporting the finding of this study are available in the supplementary tables and figures. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomcentral.proteomexchange.org) via the iProX partner repository with the dataset identifier PXD023328.

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**Figures**
EGFR inhibitors induce apoptosis of CD34+ leukemia cells. a, CD34+ cells magnetically purified from BMMC of AML or CML patients were treated with indicated concentrations of osimertinib for 72 hours. Cell apoptosis were measured by AnnexinV/PI staining. Representative flow cytometry plots of CD34+ cells from 4 AML individuals were shown. Cell viability measured by trypan blue (left, n=10 patient samples) and quantified apoptosis (right, n=10 patient samples) were shown on the bottom. Ctrl represents control that without osimertinib treatment. b,c Primary CD34+ cells were treated with 1 µM afatinib, osimertinib or rociletinib for 72 hours. Representative flow cytometry plots, cell viability measured by trypan blue and quantified apoptosis were shown on the right. ** and *** indicated p value against 0 or control(ctrl) group < 0.005 and < 0.001 respectively.
Figure 2

Covalent EGFR inhibitors selective kill CD34+ AML and CML cells. a, Primary CD34- cells were treated with 2 µM afatinib, osimertinib or rociletinib for 72 hours. Cell apoptosis were measured by AnnexinV/PI staining. Representative flow cytometry plots, cell viability measured by trypan blue and quantification of apoptosis were shown on the bottom. b. BMMCs of 2 AML patients were treated with 2 µM osimertinib for 72 hours and cell apoptosis were measured by AnnexinV/PI assay co-staining with CD34-APC.
Figure 3

Osimertinib downregulates level of tyrosine phosphorylation of CD34. a, Heatmap of levels of the indicated phosphorylated tyrosine sites in osimertinib-treated and untreated (ctrl) CD34+ cells. The CD34+ cells were purified from 3 AML individuals and pooled together followed by treatment with 2 µM osimertinib for 48 hours. Tyrosine-phosphorylated peptides were enriched and analyzed by MS. The colors in the map represent the quantitative value (normalized total spectra) according to the Scaffold_4.3.3. b, Phospho-proteome changes observed in osimertinib-treated CD34+ cells analyzed by String database. The edge indicates known interaction between two proteins. Sequence logo represents the motif clustering from the tyrosine phosphorylation data set. c,d, Intensity histogram of non-phosphorylated (c) and phosphorylated (d) Y329-containing peptide of CD34 protein quantified by PRM-MS assays. The osimertinib treated or untreated CD34+ cells were collected and the cell lysates were immunoprecipitated with anti-CD34 or IgG antibodies followed by PRM-MS analysis. e, Level of Y329-phosphorylation of CD34 quantified by PRM-MS assays in AML cell lines and 5 primary AML CD34+ samples. Data are representative of three independent experiments.
Osimertinib and afatinib directly bind with CD34 protein and inhibits STAT3 phosphorylation in CD34+ cells. a,b, MS/MS analysis of the cysteine 199 (C199) tryptic peptide for recombinant CD34 protein incubated with (top) or without (bottom) osimertinib(a) or afatinib(b) for 30 min. c, CD34 was knocked out by CRISPR-Cas9 system in CD34+ cells purified from AML patients (n=3) and were exposed to 2 μM osimertinib for 72h. Apoptosis were measured by flow cytometry. ** indicated p value against scramble group < 0.005. d, KG-1 and Kasumi-1 cells were infected with CD34-targeting CRISPR-Cas9 to deplete
endogenous CD34 and then infected with plasmids encompassing WT or C199S mutant CD34. These cells were treated with 2 μM osimertinib for 72 hours and apoptosis were determined by flow cytometry. ** indicated p< 0.005 between the line-pointed group. e-g, Immunoblot analysis showing activity of the STAT3, AKT, ERK, Src in response to osimertinib (2 μM, 72h) in primary CD34+ or CD34- cells(e,f) or KG-1 and Kasumi-1 cell (g). The experiment was performed twice with similar results. h, Immunoblots analysis of STAT3 activity in Kasumi-1 cells re-constitutively expressing WT or C199S mutant CD34. i,j, Primary CD34+ AML cells were treated with 2 μM osimertinib, 1 μM stattic or osimertinib plus stattic(combo) and apoptosis was measured by AnnexinV/PI staining. Representative flow cytometry plots(i) and quantified apoptotic cells(j) were shown. ** indicated p< 0.005 between the line-pointed group.

Figure 5

![Figure 5](image-url)
Figure 5

Osimertinib impairs interaction between CD34 and Src. a, IP-coupled LC-MS/MS assay using anti-CD34 antibody in Kasumi-1 cells. String database and gene ontology (GO) analysis were applied. The edge indicates known interaction between two proteins. b-d, IP assay was conducted using antibodies against Src or CD34 in KG-1, Kasumi-1 and primary CD34+ AML cells. e,f, Immunofluorescent staining of Src and CD34 in KG-1 and Kasumi-1. Three representative images are shown. g, IP assay was conducted using antibodies against CD34 in primary CD34+ AML cells treated with/without osimertinib. h, CD34+ cells purified from AML patients (n=3) were treated with 2 µM osimertinib, 2 µM saracatinib or osimertinib plus saracatinib (combo) and apoptosis were measured by AnnexinV/PI staining. ** indicated p< 0.005 between the line-pointed group.
Figure 6

Effects of osimertinib in human AML and CML PDX models and normal CD34+ cells. a, Diagram of experimental design. b,c Representative analyses of human CD45 and CD34 (hCD45 and hCD34, respectively) expression in mouse bone marrow were used to assess engraftment of AML(b) or CML(c) cells. Percentages of human CD45+ and CD33+ cells in the bone marrow at 16 weeks were shown on the right panel. d, CD34+ cells purified from BMMCs of healthy donors were treated with indicated
concentrations of osimertinib for 72 hours. Cell apoptosis were quantified by AnnexinV/PI staining. Flow cytometry plots were shown. Cell viability measured by trypan blue and quantified apoptosis (n=3) were shown on the right.

Figure 7

Osimertinib-induced response in CD34+% high AML. a. BMMC from healthy donors and AML were collected and CD34+ and CD34- cells were purified followed by quantitative proteomics analysis of CD34 expression. n=31 for donor CD34-, n=61 for donor CD34+, n=17 for AML CD34-, n=59 for AML CD34+. The red dotted line showed the position of upper quartile. b,c, White blood cell(WBC) counts and flow cytometry analysis of CD34 expression of two AML patients treated with osimertinib. Day 0 was defined as the day that osimertinib was started. d,e, Microscopic appearance of peripheral blood (PB) and bone marrow(BM) smear. f, Western blot analysis of pY329-CD34 of BMMC at the indicated time of osimertinib treatment. g, A model proposing the mechanism by which osimertinib induces apoptosis through binding CD34.
Supplementary Files

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