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Original Article

Anlotinib suppresses MLL-rearranged acute myeloid leukemia cell growth by inhibiting SETD1A/AKT-mediated DNA damage response

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Abstract: Leukemias driven by chromosomal translocation of the mixed-lineage leukemia (MLL) gene are highly prevalent in hematological malignancy. The poor survival rate and lack of effective targeted therapy for patients with MLL-rearranged (MLL-r) leukemias emphasize an urgent need for improved knowledge and novel therapeutic approaches for these malignancies. The present study aimed to investigate the potential effectiveness and mechanism of Anlotinib, a novel receptor tyrosine kinase inhibitor, in MLL-r acute myeloid leukemia (AML). The findings revealed that Anlotinib significantly inhibited the growth of MLL-r AML cells in both *in vivo* and a murine xenograft model. RNA sequencing identified that multiple genes involved in DNA damage response were responsible for Anlotinib activity. To further elucidate the correlation between the DNA damage response induced by Anlotinib and MLL fusion, Gene Expression Profiling Interactive Analysis (GEPIA) was conducted. It revealed that Anlotinib impaired DNA damage response via inhibiting SETD1A and AKT. In conclusion, Anlotinib exerts anti-leukemia function by inhibiting SETD1A/AKT-mediated DNA damage response and highlights a novel mechanism underlying Anlotinib in the treatment of MLL-r AML.

Keywords: Anlotinib, MLL-rearranged acute myeloid leukemia, DNA damage, SETD1A/AKT

Introduction

Cytogenetic abnormalities are closely associated with clinical features and therapeutic responses in acute myeloid leukemia (AML) [1]. The mixed-lineage leukemia (*MLL1*) gene (also known as *KMT2A*) on chromosome 11q23 is disrupted in a unique group of leukemia, with a prevalence of approximately 10% [2]. The clinical outcome of patients carrying MLL rearrangement remains extremely poor, while the response rate reported in adult MLL-rearranged (MLL-r) AML is about 40% [3]. Although intense chemotherapy might reduce the risk of relapse, it is associated with long-term adverse effects and a high rate of treatment-related mortality [4]. Therefore, effective and less toxic therapy is urgently required to treat this subset of AML.

The normal *MLL1* gene encodes a 500-kDa nuclear protein with multiple functional domains and binding partners, which are recognized as transcriptional deregulators of distinct *HOX* genes in normal hematopoietic differentiation [5]. In addition to *MLL1*, five more MLL family members (*MLL2*, *MLL3*, *MLL4*, *SETD1A*, and *SETD1B*) are found in mammals [6]. All of the MLL proteins physically associate with other protein factors to form large macromolecular complexes that stimulate the H3K4 methyltransferase activity of MLL proteins [6]. Leukemia-associated translocations involving

11q23 have been shown to generate in-frame fusions of the *MLL1* gene with >80 different partner genes [7]. According to the report, the five most frequent fusion partners of *MLL* are *AF4* (~36%), *AF9* (~19%), *ENL* (~13%), *AF10* (~8%), *PTD* (5%), which together represent more than 80% of the MLL fusion proteins (MLL-FPs) found in MLL-r leukemia patients [7]. MLL-FPs have different chromatin-modifying activities than normal MLL proteins. The catalytic SET domain of wild-type MLL that harbors H3K4 methyltransferase activity is lost in MLL-FPs [2, 6]. Moreover, the N-terminal truncation of MLL alone is not sufficient to transform cells [2]. These findings argue for a non-catalytic function of MLL and redundancies, such that other MLL homologs might contribute to leukemogenesis. Hence, unexpected and major roles for wild-type MLL and its paralogs (MLL2 and SETD1A) have been observed in MLL-r leukemia [8-10]. Therefore, drugs targeting MLL homolog binding partners have been identified as potential therapeutic tools against MLL-r leukemia [11-16]. In addition, several inhibitors of signaling pathways, such as PI3K/AKT/mTOR and MAPK, show promising anti-leukemia activity for the treatment of MLL-r leukemia [17, 18].

Anlotinib, a new oral small-molecule receptor tyrosine kinase (RTK) inhibitor, is designed to primarily inhibit multi-targets, including VEGFR1, VEGFR2/KDR, VEGFR3, c-Kit, PDGFR-α, and the fibroblast growth factor receptors (FGFR1, FGFR2, and FGFR3) [19]. The data from the preclinical study have demonstrated that Anlotinib has anti-tumor activity across a broad spectrum of advanced cancers with little side effects [19]. Thus, Anlotinib has been approved in China for the treatment of patients with advanced or metastatic non-small cell lung cancer [20]. It is also undergoing phase II and/or III clinical development for various sarcomas and carcinomas in China, USA, and Italy [21]. In addition to angiogenesis and associated targets, new mechanisms and targets have been reported for Anlotinib inhibitory action on tumor growth [22, 23]. Since Anlotinib can inactivate both AKT and ERK pathways, it could be speculated to possess a potential anti-leukemia activity.

In this study, we investigated the anti-tumor effects of Anlotinib in MLL-r leukemia and explored the underlying mechanisms and downstream targets.

Materials and methods

Reagents

Anlotinib was purchased from Chia Tai Tianqing Pharmaceutical Group Co. Ltd and solubilized in dimethyl sulfoxide (DMSO; Invitrogen, Carlsbad, CA, USA) as 10 mM stock and diluted to the required concentrations with cell culture medium before the experiments.

Cell culture

Human AML cell lines Molm-13 and MV4-11 carried MLL rearrangement were purchased from ATCC (Rockefeller, MD, USA) and cultured at 37°C in a 5% CO_2 incubator in Iscove's modified Dulbecco's medium (IMDM) and RPMI-1640 medium (HyClone, Thermo Scientific, Logan, UT, USA), respectively, and supplemented with 10% fetal bovine serum (FBS; Gibco, Thermo Scientific, Grand Island, NY, USA).

Cell viability analysis

The cell viability assay was performed, as reported previously [16]. Briefly, 2×10^4 cells/well were seeded in 100 μL medium on 96-well plates and treated with indicated concentrations of Anlotinib for 24, 48, and 72 h. Then, 10 μL of the Cell Counting Kit-8 (CCK-8) reagent (Dojindo, Kumamoto, Japan) was added to each well, and the reaction was incubated for an additional 2 h. The absorbance was measured at 450 nm on a microplate reader (ELx800, BioTek, Winooski, VT, USA). Data from three independent experiments in triplicate were presented as a percentage of viable cells in comparison to untreated control. IC50 values were determined using the SPSS 20.0 software.

Analysis of apoptosis

The assay was performed as reported previously [16]. Briefly, the cells were cultured and treated with Anlotinib for 24, 48, and 72 h, as described above. Cell apoptosis was determined by double staining with Annexin V-FITC and PI (eBioscience, Thermo Scientific, San Diego, CA, USA), according to the manufacturer's instructions. Then, the data were analyzed by flow cytometry (FACS Fortessa, BD Biosciences, Franklin Lakes, NJ, USA) using the FACS C6 software.

Analysis of cell cycle

Molm-13 and MV4-11 cells were cultured in 6-well plates and treated with Anlotinib for 8 h and 24 h. Then, the cells were collected and washed with phosphate-buffered saline (PBS), followed by fixation in ethanol (70%). After overnight incubation at 4°C, the cells were stained with PI and examined by flow cytometry.

Western blot analysis

An equivalent of 2×10^5 cells/well was treated with Anlotinib for 24 and 48 h and analyzed by Western blotting using the indicated primary and secondary HRP-conjugated antibodies (Abcam, Cambridge, UK). The blots were detected by visualization using the ECL Western Blotting Detection Kit (GeneFlow, Staffordshire, UK). The antibodies included anti-POLD1 (sc-17776, SantaCruz, CA, USA), anti-POLD2 (HPA-026745, Atlas Antibodies AB, Sweden), anti-POLD3 (A301-244A-M, Bethyl, USA), anti-SETD1A (ab70378, Abcam), anti-AKT (ab8805, Cell Signaling Technology, USA), and anti-GAPDH (D16H11, Cell Signaling Technology).

RNA sequencing

Cells were incubated with Anlotinib for 12 h, following which, total RNA was isolated, as described previously [24]. RNA sequencing (RNA-seq) was then carried out commercially (Sangon Biotech, Shanghai, China). GSEA was used to enrich the signaling pathway involved in differentially expressed genes. The gene set is c2.cp.kegg.v6.0.symbols.gmt [curated].

Animal study

Animal studies were performed according to the Xiamen University Animal Guideline and approved by the Animal Welfare Committee. Briefly, a total of 5×10^6 Molm-13 cells were inoculated subcutaneously into nude mice. After 3 days, the animals were randomly divided into vehicle control and Anlotinib groups (n=5/group), and then treated with either vehicle (PBS) or Anlotinib (6 mg/kg/day) by oral gavage for 9 days. Tumor size and body weight were measured daily. Subsequently, the animals were sacrificed at the end of drug treatment and the tumor size was measured as 1000 mm [3]. All tumors were removed, measured, and weighed. The tumor volume was calculated using the formula: $V = (L\times W^2)/2$, where L is the longest and W the shortest diameter of the tumor.

Statistical analysis

All statistical analyses were carried out using the SPSS 23.0 and GraphPad Prism 6.0 software. The differences between the two groups were analyzed using the two-tailed Student's t-test. $P<0.05$ indicated statistical significance.

Results

Anlotinib effectively suppresses the growth and induces robust apoptosis of MLL-rearranged AML cells

To assess the therapeutic action of Anlotinib in MLL-r AML, we first investigated its growth inhibition effect against two MLL-r AML cell lines (Molm13 and MV4-11) using the CCK8 assay. As shown in **Figure 1A** and **1B**, Anlotinib markedly suppresses cell growth of MLL-r AML cell lines in a dose-dependent manner. The growth inhibition was dramatically enhanced at 72 h in MV4-11 cells, whereas no significant increase was detected in the reduced viability in Molm13 cells in a time-dependent manner. The IC50 values of Anlotinib were varied in the two cell lines: 2.28 μM at 24 h, 1.69 μM at 48 h, and 1.88 μM at 72 h in Molm13 cells and 6.42 μM at 24 h, 6.68 μM at 48 h, and 2.02 μM at 72 h in MV 4-11 cells. These findings indicated that Molm13 is more susceptible to Anlotinib than MV4-11.

To further verify the anti-leukemia efficacy of Anlotinib, we analyzed cell apoptosis by Annexin V/7-AAD double staining at 24 h and 48 h. In line with the results of viability, Anlotinib treatment resulted in significantly enhanced apoptosis in a dose-dependent manner ($P<0.01$, **Figure 1C-F**). However, no significant increase was detected in apoptosis at 48 h as compared to that after 24 h post-treatment. These data suggested that the induction of apoptosis might contribute to the encouraging anti-leukemia effect of Anlotinib.

Anlotinib induces G2/M arrest in MLL-rearranged AML cells

To explore whether the cytotoxicity of Anlotinib is associated with cell cycle arrest, we analyzed the cell cycle distribution upon Anlotinib treat-

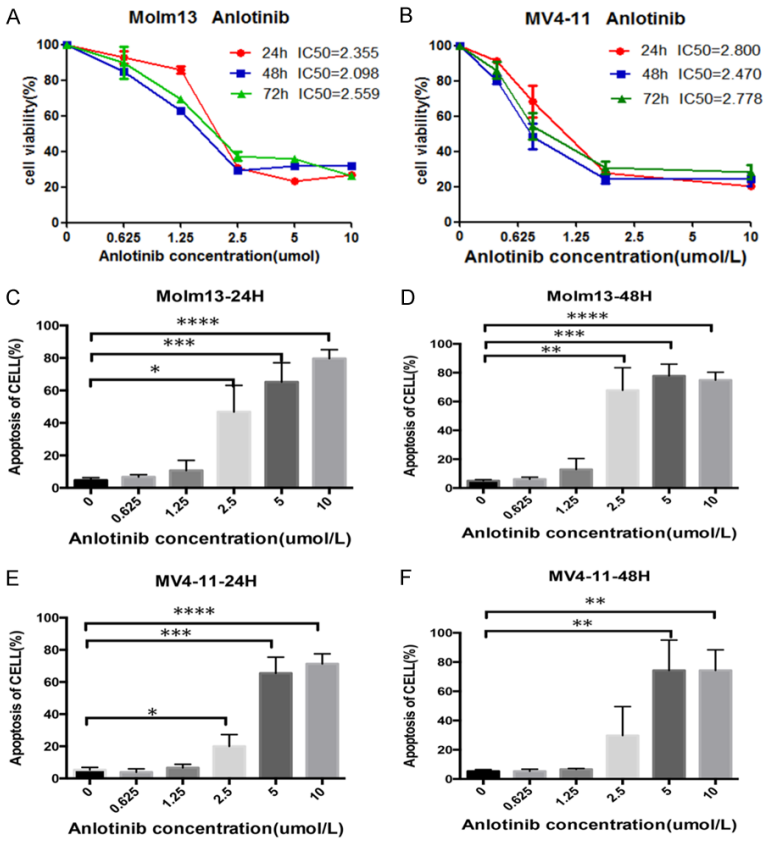

Figure 1. Anlotinib suppresses the growth and induces robust apoptosis of MLL-rearrangement AML cells. Molm-13 (A, C, D) and MV4-11 (B, E, F) cells were separately treated with designated concentrations of Anlotinib for 24, 48, or 72 h, after which cell viability was analyzed by a CCK-8 kit, and the percentage of apoptotic cells was examined by Annexin V/PI double staining. The experiments were carried in triplicate.

ment. At the early phase (8 h) after Anlotinib administration, a cell cycle arrest at the G2/M checkpoint was observed in both Molm-13 and MV4-11 cells in a dose-dependent manner (**Figure 2A** and **2C**). This effect was continued until the late phase (24 h) of Anlotinib treatment (**Figure 2B** and **2D**).

Anlotinib is actively used in vivo in a xenograft model of AML-carrying MLL rearrangement

The anti-tumor activity of the regimen using Anlotinib was examined in a mouse xenograft model established by subcutaneous inoculation of Molm-13 cells. After 3 days, the mice were randomly divided into vehicle control group and Anlotinib treatment group. Anlotinib was administered orally once a day at 6 mg/kg/day for 9 days. During drug administration, the tumor volumes and body weights of the mice were measured daily to evaluate the anti-tumor efficacy and side effects of Anlotinib. Our data showed that Anlotinib significantly reduced the tumor burden of mice bearing MLL-r AML cells, as reflected by a remarkable decrease in tumor mass and weight (**Figure 3A-C**). On the other hand, no notable difference was observed between the mouse body weights of the Anlotinib and control groups (**Figure 3D**), suggesting tolerance for the *in vivo* use of Anlotinib. Together, these findings indicated that the regimen of Anlotinib is safe and effective *in vivo* against MLL-r AML.

Inactivation of DNA damage response contributes to the anti-leukemia effect of Anlotinib

To delineate the molecular basis of the tumor suppression role for Anlotinib in MLL-r AML, we performed RNA-seq analysis and identified a different pattern of gene expression between Anlotinib-treated and control groups (**Figure 4A**). These differentially expressed genes (DEGs) were further analyzed with GSEA to deduce their biological functions. These results showed that Anlotinib affects several biological processes, including spliceosome formation, DNA repair, metabolism, and cell cycle (**Figure 4B**). Among these, genes involved in DNA damage and repair pathways were enriched (**Figure 4C**). Herein, we identified five critical genes (**Figure 4D**), *POLD1*, *POLD2*, *POLD3*, *LIG1*, and *PCNA*, for the DNA damage response, using the Venn diagram. These genes were downregulated by Anlotinib (**Figure 4E**), which was confirmed by Western blotting (**Figure 4F**). These findings indicated that the inhibitory role of Anlotinib in MLL-r AML could be largely attributed to the dysfunction of DNA damage and repair.

Anlotinib impairs DNA damage responses by downregulating SETD1A and AKT

Recent studies have shown that MLL-fusion-driven leukemia requires SETD1A and AKT for

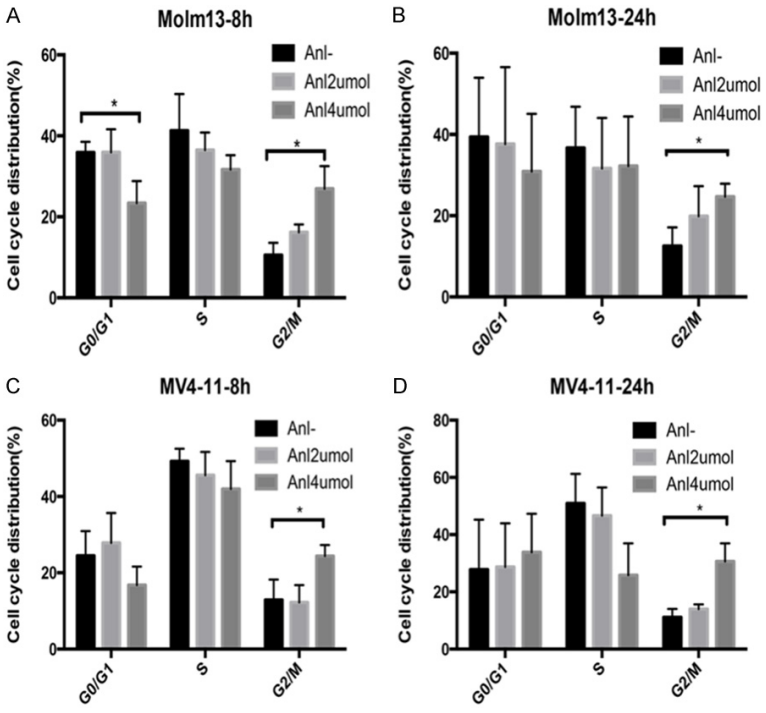

Figure 2. Anlotinib causes a dose-dependent arrest of G2/M phase cell cycle distribution in MLL-r cells. The experiments were carried out in triplicate and showed that the percentage of cells in the G2/M phase increased significantly in a dose-dependent manner, resulting in G2/M cell cycle arrest.

the appropriate expression of DNA damage response genes to safeguard genomic integrity [10, 25, 26]. Moreover, MLL-fusion proteins engage a large number of distinct protein-protein interactions relevant to the biology of MLL-fusion proteins [27]. Thus, we sought to reveal the molecular etiology underlying the modular protein-protein interactions between SETD1A/AKT and DNA damage response genes using two online databases: Gene Expression Profiling Interactive Analysis (GEPIA) and STRING (https://string-db.org/). Our bioinformatics analysis showed that both SETD1A and AKT were strongly correlated to POLD1, POLD2, and POLD3 (**Figure 5A-H**). These results further confirmed that SETD1A and AKT regulate the expression of DNA repair-specific genes in MLL-r leukemia. Hence, we investigated whether Anlotinib targets SETD1A and AKT. As shown in **Figure 5I**, both SETD1A and AKT were significantly downregulated in the Anlotinib treatment group. Collectively, these results demonstrated that Anlotinib inhibits MLL-r leukemia cell growth by targeting SETDA1- and AKT-mediated DNA damage response.

Discussion

Leukemias driven by MLL rearrangement are highly prevalent in acute leukemia with a dismal prognosis [2-4]. The poor survival rate and lack of effective targeted therapy for patients with MLL-r leukemias emphasize an urgent need for improved knowledge and novel therapeutic approaches for these malignancies. Oncogenic MLL-fusion proteins often hijack essential molecular mechanisms during leukemogenesis [6]. A large number of studies have shown that MLL and its chaperones are required for genomic integrity in MLL-r leukemia [10, 27-31], which lead to chemoresistance [32]. For example, SETD1A is required for the survival of AML cells depending on the FLOS domain but not the enzymatic SET domain [10]. The FLOS domain of SETD1A acts as a cyclin-K-binding site responsible for chromosomal recruitment of cyclin K and DNA-repair-associated gene expression [10]. In addition, SETD1A interacts with the DNA damage repair protein RAD18 to safeguard genome stability [30]. These data suggested a pivotal role in the DNA damage response for MLL-fusion-driven leukemia. Therefore, targeting DNA repair enzymes represent promising therapeutic strategies for the treatment of AML, especially MLL-driven leukemias [33]. In the present study, we showed that a novel RTK inhibitor, Anlotinib, inhibits MLL-r AML cell growth and progression by targeting DNA damage response in both *in vitro* and *in vivo* preclinical models.

Anlotinib has a broad spectrum of inhibitory action on tumor angiogenesis and growth and has been approved for the treatment of several advanced refractory solid tumors [19-21]. Although it was designed to inhibit receptor tyrosine kinase related to tumor vasculogenesis [34], accumulating evidence showed that Anlotinib suppresses tumor cell growth via inhi-

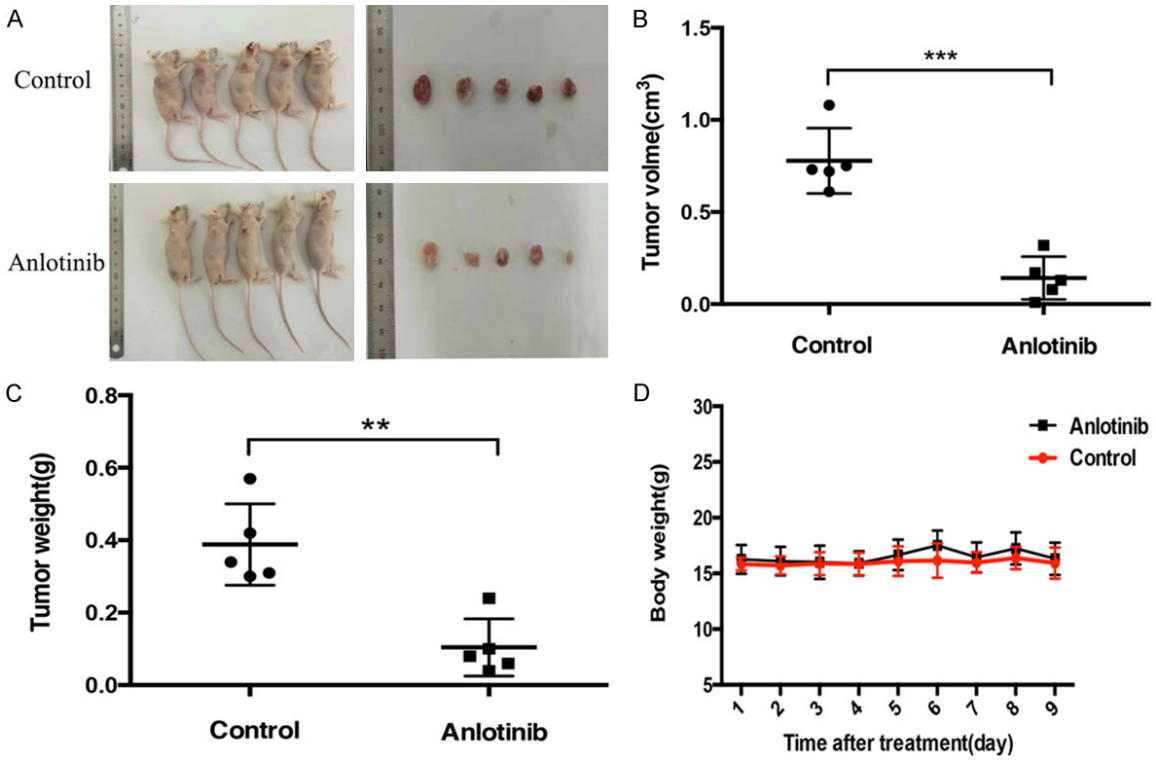

Figure 3. Anlotinib suppresses tumor growth in a xenograft model of MLL-r AML. (A-C) Mice were sacrificed after drug treatment for 9 days, and then the images of mice and tumors were captured (A). Volume (B) and weight (C) of tumors were measured and calculated. Values indicated that the mean ± SEM for five mice/group (***P*<0.01). (D) Mice were weighed daily after tumor cell inoculation.

bition of multiple pathways and targets [19-21]. Some of these targets, such as c-Kit [35], Aurora-B [36, 37], AKT [18], and MEK pathway [17], are involved in MLL-r leukemia. In this context, our study extends the indications for Anlotinib to the treatment of MLL-r AML via apoptosis and cell cycle arrest (**Figures 1** and **2**). Moreover, our functional analysis on RNA sequencing data unravels a major role of DNA damage response for Anlotinib anti-tumor activity (**Figure 4**), consistent with the Anlotinib-induced cell cycle arrest at G2/M checkpoint (**Figure 2**). We also confirmed that Anlotinib induces the dysfunction of DNA damage response manifested by downregulating DNA damage response genes (POLDs) (**Figure 4**). These results provide a novel insight into the mechanism and clinical application of Anlotinib.

DNA polymerase delta (POLD) is a member of family B polymerases in eukaryotes and is essential for the leading and lagging strand synthesis [38-40]. It includes four subunits in mammals: POLD1-4 [41]. POLD1 functions as the catalytic subunit, which plays critical roles in DNA-replicate and DNA-repair processes, and POLD2 serves as a scaffold by interacting with POLD1 and other POLD subunits [42]. Multiple studies suggested that the abnormality of POLD1 is related to the poor prognosis of many tumors [43, 44]. However, its role in AML is not yet clarified. Herein, we described POLD1 as a prognostic predictor in AML by GEPIA, where patients with high POLD1 expression showed a poor survival (data not shown). Also, Anlotinib downregulated the expression of POLD1 and its subunits, highlighting its potential application to the subgroup of AML with high POLD1 expression.

Since both SETD1A and AKT are required for the appropriate expression of DNA damage response [10, 25, 26], we further unraveled the correlation between SETD1A/AKT and POLD family proteins using bioinformatics (**Figure 5**). Importantly, our results point to a molecular etiology of SETD1A and AKT to control the expression of POLD family genes. Although much work is necessary to define the exact mechanisms that underlie the interaction between SETD1A/

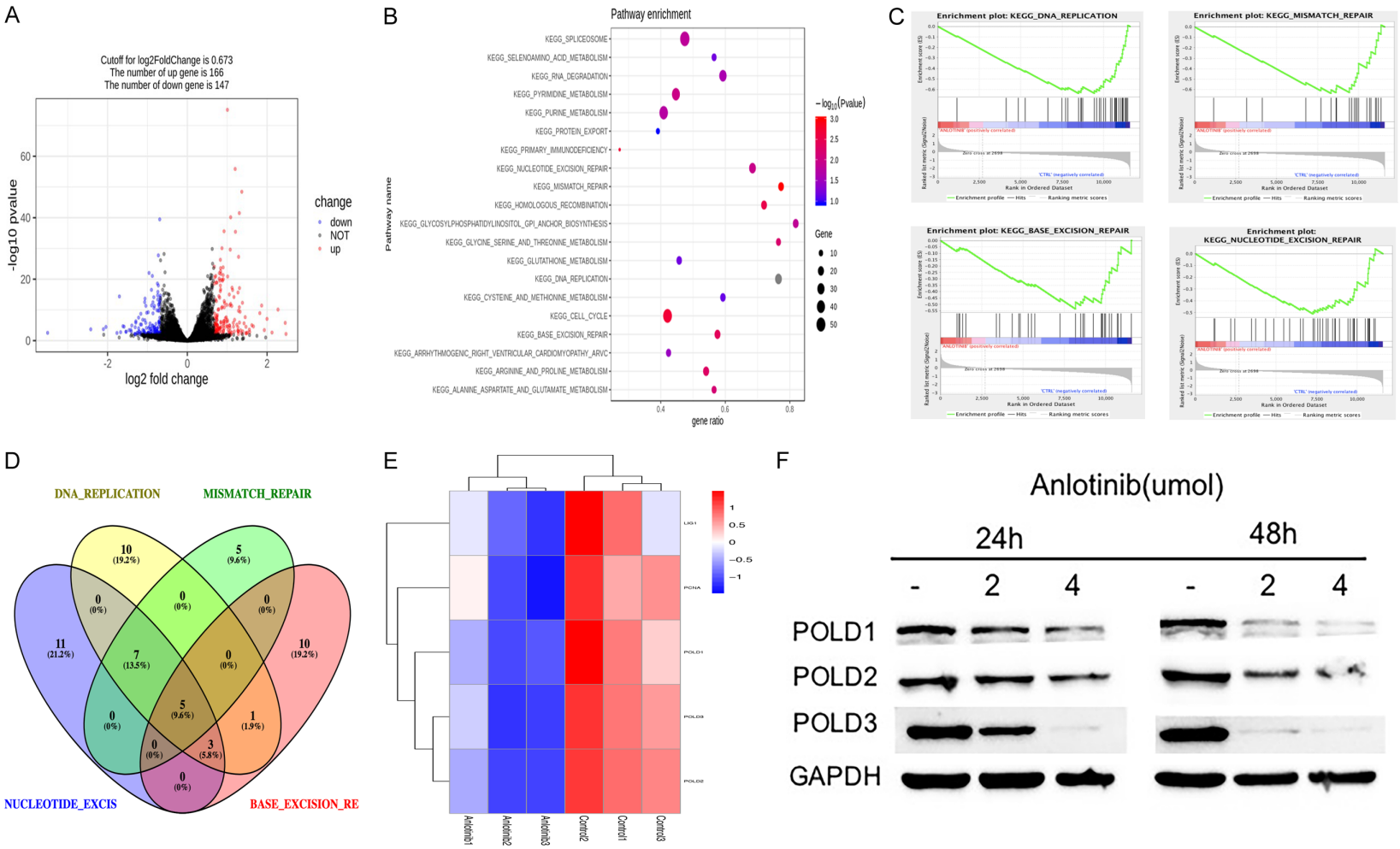

Figure 4. Inactivation of DNA damage response contributes to the anti-leukemia effect of Anlotinib. (A) RNA sequencing was performed in the Anlotinib-treated Molm-13 and control groups: Volcano map shows the number of different genes. (B) GSEA was used to analyze the RNA-seq data: A bubble chart shows the top 20 downregulated pathways enriched by GSEA. (C) Four representative pathways are related to DNA damage and repair. A Venn diagram (D) and a heatmap (E) shows five common genes among four representative pathways. (F) The expression of the POLD family was confirmed by Western blotting.

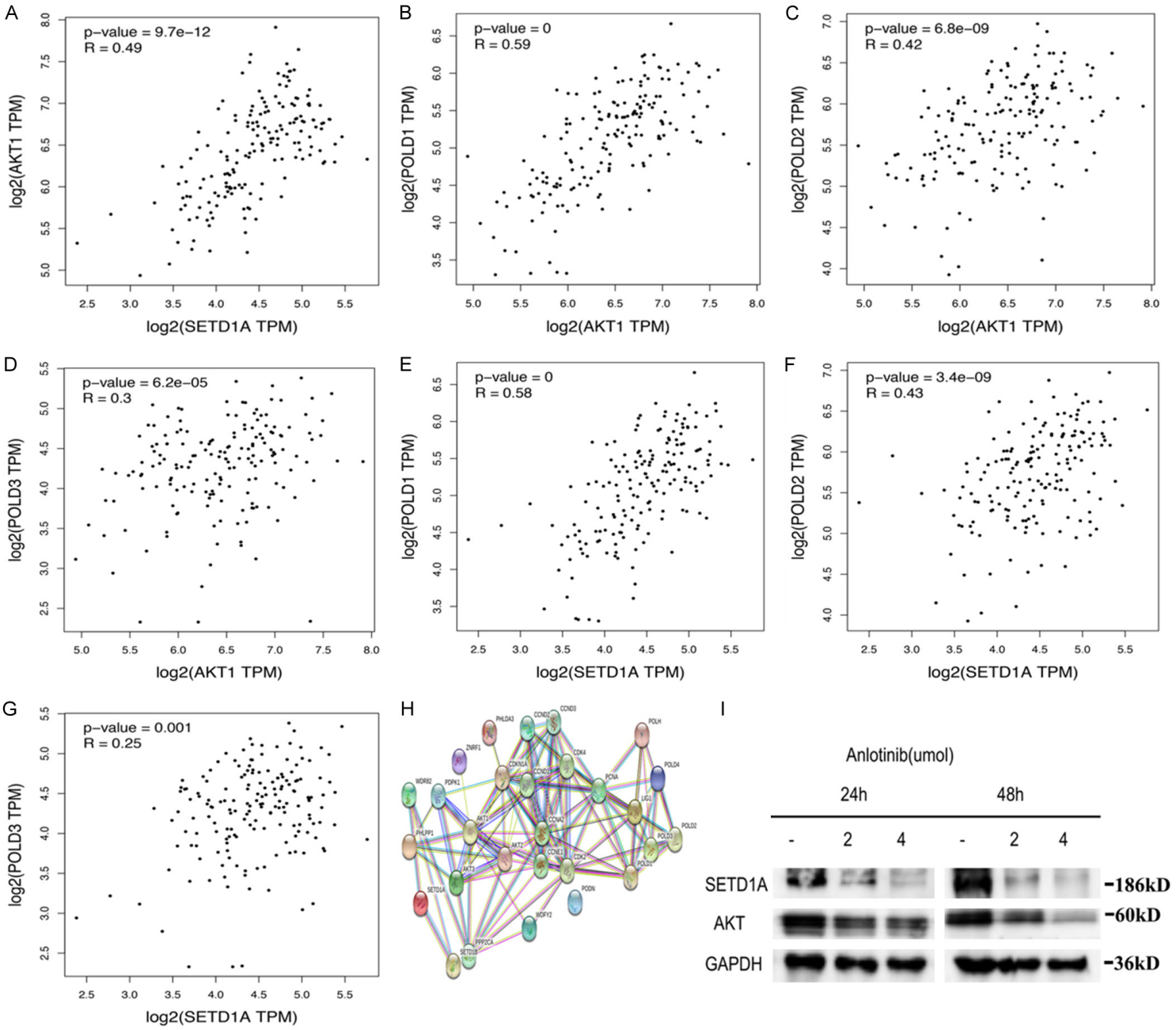
A
p−value = 9.7e−12
R = 0.49
log2(AKT1 TPM)
log2(SETD1A TPM)
B
p−value = 0
R = 0.59
log2(POLD1 TPM)
log2(AKT1 TPM)
C
p−value = 6.8e−09
R = 0.42
log2(POLD2 TPM)
log2(AKT1 TPM)
D
p−value = 6.2e−05
R = 0.3
log2(POLD3 TPM)
log2(AKT1 TPM)
E
p−value = 0
R = 0.58
log2(POLD1 TPM)
log2(SETD1A TPM)
F
p−value = 3.4e−09
R = 0.43
log2(POLD2 TPM)
log2(SETD1A TPM)
G
p−value = 0.001
R = 0.25
log2(POLD3 TPM)
log2(SETD1A TPM)
H
I
Anlotinib(umol)
24h
48h
-
2
4
-
2
4
SETD1A
AKT
GAPDH
-186kD
-60kD
-36kD

Figure 5. Anlotinib impairs DNA damage responses by downregulating SETD1A and AKT. The correlation analysis by GEPIA database revealed (A) SETD1A and AKT1 (*P*=9.7e-12 R=0.49), (B) AKT1 and POLD1 (*P*=0 R=0.59), (C) AKT1 and POLD2 (*P*=6.8e-09 R=0.42), (D) AKT1 and POLD3 (*P*=6.2e-05 R=0.3), (E) SETD1A and POLD1 (*P*=0 R=0.58), (F) SETD1A and POLD2 (*P*=3.4e-09 R=0.43), (G) SETD1A and POLD3 (*P*=0.001 R=0.25). The network (H) contains 28 nodes, including five hub genes, and the correlation is also illustrated. (I) Western blotting showed that SETD1A/AKT axis was significantly downregulated in Anlotinib-treated Molm-13 cells.

AKT and POLDs, according to our current findings, Anlotinib hampers DNA damage response via inhibition of SETD1A and AKT in MLL-r AML cells.

Taken together, the current study shows that Anlotinib suppresses the growth and induces robust apoptosis in MLL-r AML cells, putatively by inhibiting SETD1A- and AKT-mediated DNA damage response. This study also highlights a novel mechanism and deems Anlotinib as a potential candidate in the treatment of MLL-r leukemia.

Disclosure of conflict of interest

None.

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