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

Jinzhu Chen
The First Hospital of Longhai

Juan Feng
Department of Hematology, The First Affiliated Hospital of Xiamen University and Institute of Hematology, School of Medicine, Xiamen University, Xiamen, 361003, P.R. China. Key laboratory of Xiamen for diagnosis and treatment of hematological malignancy, X

Zhihong Fang
Department of Hematology, The First Affiliated Hospital of Xiamen University and Institute of Hematology, School of Medicine, Xiamen University, Xiamen, 361003, P.R. China. Key laboratory of Xiamen for diagnosis and treatment of hematological malignancy, X

Jing Ye
Department of Hematology, The First Affiliated Hospital of Xiamen University and Institute of Hematology, School of Medicine, Xiamen University, Xiamen, 361003, P.R. China. Key laboratory of Xiamen for diagnosis and treatment of hematological malignancy, X

Qinwei Chen
Department of Hematology, The First Affiliated Hospital of Xiamen University and Institute of Hematology, School of Medicine, Xiamen University, Xiamen, 361003, P.R. China. Key laboratory of Xiamen for diagnosis and treatment of hematological malignancy, X

Qiuling Chen
Department of Hematology, The First Affiliated Hospital of Xiamen University and Institute of Hematology, School of Medicine, Xiamen University, Xiamen, 361003, P.R. China. Key laboratory of Xiamen for diagnosis and treatment of hematological malignancy, X

Kai Chen
The First People's Hospital of Foshan (The Affiliated Foshan Hospital of Sun Yat-sen University), Foshan, Guangdong, China, 528000

Xiaoming Xiong
Department of Hematology, The First Hospital of Longhai, Fujian, 363199, P.R. China

Xuan Pu
Department of Biology, Case Western Reserve University, Cleveland, OH, USA

Guoweo Li
Department of Hematology, Huizhou Municipal Central Hospital, Huizhou, 516001, P.R, China

Haihan Song
Department of Immunology, DICAT Biomedical Computation Centre, Vancouver, BC, Canada
Bing Xu (xubingzhangjian@126.com)
The First Affiliated Hospital of Xiamen

Research

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

Posted Date: March 11th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-16791/v1

Abstract

Background: Leukemias driven by chromosomal translocation of the mixed-lineage leukemia gene (MLL) are highly prevalent in hematological malignancy. The poor survival rate and lack of an 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. In this study, we investigated the potential effectiveness and mechanism of Anlotinib, a novel receptor tyrosine kinase inhibitor, in MLL-r acute myeloid leukemia(AML).

Methods: MLL-r AML cell lines were treated with different concentration of Anlotinib, then cell viability, apoptosis and cell cycle were analyzed. Next, the anti-leukemia effect of Anlotinib was further evaluated in vivo in a xenograft model of AML-carrying MLL-rearrangement. Finally, we performed RNA-seq analysis, Gene Set Enrichment Analysis (GSEA) and western bolting to explore the underlying mechanism of the anti-leukemia effect of Anlotinib.

Results: Our findings revealed that Anlotinib significantly suppresses the growth, promotes robust apoptosis and induces G2/M arrest in MLL-r AML cells. Moreover, Anlotinib effectively inhibited the growth of MLL-r AML cells in a xenograft murine model. In mechanism, we find that the inhibitive role of Anlotinib in MLL-r AML could be largely attributed to the dysfunction of DNA damage and repair. Furthermore, we confirmed that Anlotinib impaired DNA damage response via inhibiting SETD1A and AKT.

Background

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 dose intensification of chemotherapy might reduce the risk of relapse, it is however associated with the long-term adverse effects and a high rate of treatment-related mortality[4]. Therefore, a more effective and less toxic therapy is urgently needed to treat this subset of AML.

The normal MLL1 gene encodes an approximately 500-kDa nuclear protein with multiple functional domains and binding partners, which was historically 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 larger macromolecular complexes, which stimulates 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 to more than 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 represents 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. Thecatalytic SET domain of wild type MLL that harbors H3K4 methyltransferase activity is lost in MLL-FPs[2,6]. Moreover, N-terminally truncated 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. For this reason, unexpected and major roles for wild-type MLL and its paralogs (MLL2 and SETD1A) have been observed in MLL-r leukemia[8,9,10]. Therefore, drugs targeting MLL homolog binding partners have been identified as potential therapeutic tools against MLL-r leukemia[11,12,13,14,15,16]. In addition, several inhibitors of signaling pathways such as PI3K/AKT/mTOR and MAPK show a promising antileukemia activity for treatment of MLL-r leukemia[17,18].

Anlotinib, a new oral small-molecule receptor tyrosine kinases(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]. Amazing results obtained from the preclinical study have demonstrated that Anlotinib has anti-tumor activity across a broad spectrum of advanced cancers with little side effects[19]. For this reason, Anlotinib has been approved in China for the treatment of patients with advanced or metastatic non-small cell lung cancer[20], and also is undergoing phase II and/or III clinical development for various sarcomas and carcinomas in China, USA and Italy[21]. Besides angiogenesis 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 pathway, it is reasonable to assume that Anlotinib might also have a potential antileukemia activity.

In this study, we investigated the antitumor effects of Anlotinib in MLL-r leukemia and explored its potential mechanisms and downstream targets.

Materials And Methods

Reagents.

Anlotinib was purchased from Chia Tai Tianqing Pharmaceutical Group Co Ltdand dissolved in dimethyl sulfoxide (DMSO; Invitrogen, Carlsbad, CA, USA) as 10 mM stock solution for storage at -20℃. It was thawed and diluted to the required concentrations with cell culture medium prior to experiments.

Cell culture.

Human AML cell lines Molm-13 and MV4-11 carrying 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 assay of cell viability was performed as reported previously[16]. Briefly, 2×10^4/well cells 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. The cells were incubated for additional 2 h, followed by analyzing the absorbance at 450 nm with a microplate reader (ELx800, BioTek, Winooski,VT, USA). Data from three independent experiments in triplicate were presented as percentage of viable cells by comparing to untreated control. IC50 values were determined using the SPSS 20.0 software.

Analysis of apoptosis.

The assay was performed as reported previously[16]. In brief, cells were cultured and treated with Anlotinib for 24, 48, and 72 h in a same condition as described above. Cell apoptosis was determinated by double staining with Annexin V-FITC and PI (eBioscience, Thermo Scientific, San Diego, CA, USA) following the manufacturer's instruction. The data were then analyzed by flow cytometry (FACS Fortessa, BD Biosciences, Franklin Lakes, NJ, USA) with using the FACS C6 software.

Analysis of cell cycle.

Molm-13 and MV4-11 cells were grown in 6-well plates and treated with Anlotinib for 8 h and24 h. Then 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.

Cells with 2×10^5/well were treated with Anlotinib for 24, and48 h, and then subjected to western blot analysis using indicated primary antibodies and secondary HRP-conjugated antibodies (ab150121,Abcam, Cambridge, UK). Blots were detected by visualization using the ECL Western Blotting Detection Kit (GeneFlow, Staffordshire, UK). Antibodies included anti-POLD1(sc-17776, SantaCruz,USA), anti-POLD2(HPA026745, Atlas Antibodies AB,Sweden),anti-POLD3(A301-244A-M, Bethyl,USA), anti-SETD1A (ab70378, Abcam, Cambridge, UK), anti-AKT (ab8805, Cell Signaling Technology,USA),and anti-GAPDH(D16H11,Cell Signaling Technology,USA).

RNA sequencing.

Cells were incubated with Anlotinib for 24, after which total RNA was isolated as described previously[24]. RNA sequencing (RNA-seq) was then carried out via a commercially available service (Sangon Biotech, Shanghai). GSEA was used to enrich the signal pathways 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. In brief, a total of 5×10^6 Molm-13 cells were inoculated subcutaneously into nude mice. After 3 days, mice were randomly divided into vehicle control and Anlotinib groups (n = 5, each group), and then treated respectively with either vehicle (PBS) or Anlotinib (6 mg/kg/day) by oral gavage for 9 days. Tumor size and body weight were measured daily. Mice were sacrificed at the end of drug treatment or tumor size reached 1000 mm^3. All tumors was removed, measured, and weighted. 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 softwares. The differences between two groups were analyzed using the two-tailed Student's t test. $P < 0.05$ was considered as statistically significant.

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 Fig. 1A and B, Anlotinib markedly suppressed cell growth of MLL-r AML cell lines in a dose-dependent manner. Growth inhibition was dramatically enhanced at 72 h in MV4-11, whereas no significant increase in viability reduction was observed as time extension in Molm13. The IC50 values of Anlotinib were varied in two cell lines, with 2.28 μM at 24 h, 1.69 μM at 48 h, 1.88 μM at 72 h for Molm13 and 6.42 μM at 24 h,6.68 μM at 48 h, 2.02 μM at 72 h for MV 4–11. These results indicate that Molm13 is likely 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 ($P < 0.01$, Fig. 1C-F). However, no significant increase in apoptosis was detected at 48 h compared with 24 h after treatment. These data suggest that 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 also associated with cell cycle arrest, we analyzed cell cycle distribution upon Anlotinib treatment.At the early phase (8 hours) after Anlotinib administration, a dose-dependent cell cycle arrest in G2/M checkpoint was observed in both Molm-13 and MV4-11 (Fig. 2A and C). This effect was continued until the late phase (24 hours) of Anlotinib treatment (Fig. 2B and D).

The regimen using Anlotinib is active in vivo in a xenograft model of AML-carrying MLL- rearrangement.

Then, the anti-tumor activity of the regimen using Anlotinib was examined in a mouse xenograft model established by subcutaneous inoculation with Molm-13 cells. After 3 days, mice were randomly divided into vehicle control group and Anlotinib treatment group. Anlotinib was administered orally once a day at a dose of 6 mg/kg/day for 9 days. During the drug administration, tumor volumes and mouse body weights were measured every day to evaluate the antitumor efficacy and side effects of Anlotinib. Our results showed that Anlotinib significantly reduced tumor burden of mice bearing MLL-r AML cells, as reflected by a remarkable decrease of tumor mass and weight (Fig. 3A-C). Otherwise, there was no notable distinction between the mouse body weights of the Anlotinib and control groups (Fig. 3D), suggesting a good tolerance for the in vivo use of Anlotinib. Together, these findings indicate 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 tumor suppression role for Anlotinib in MLL-r AML, we performed RNA-seq analysis and found a different pattern of gene expression between Anlotinib treated group and control group (Fig. 4A).These differentially expressed genes(DEGs)were further analyzed with GSEA to identify their biological functions. The results showed that Anlotinib can affect many biological processes including spliceosome, DNA repair, metabolism, cell cycle and so on (Fig. 4B). Among them, genes involved in DNA damage and repair pathway were particularly enriched (Fig. 4C). Through Venn diagrams, we identified five critical genes for the DNA damage response (Fig. 4D). They are mainly composed of POLD1、POLD2、POLD3、LIG1 and PCNA which were downregulated by Anlotinib(Fig. 4E),and these findings were confirmed by western blotting(Fig. 4F). Our results indicate that the inhibitive role of Anlotinib in MLL-r AML could be largely attributed to the dysfunction of DNA damage and repair.

Anlontinib impairs DNA damage responses by downregulating SETD1A and AKT.

Recent studies have shown that MLL-fusion-driven leukemia requires SETD1A and AKT for 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 that are highly relevant for the biology of MLL-fusion proteins[27]. Thus, we sought to revealthe molecular logic of how 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 with POLD1, POLD2, and POLD3(Fig. 5A-H). These results further confirmed that SETD1A and AKT are required to regulate the expression of DNA repair specific genes in MLL-r leukemia. For this reason, we wondered whether Anolotinib is able to target SETD1A and AKT. As showed in Fig. 5I, both SETD1A and AKT were significantly downregulated in Anlotinib treatment group. Collectively, these results demonstrate that Anlotinib inhibit MLL-r leukemia cell growth through targeting SETDA1 and AKT mediated DNA damage response.

Discussion

Leukemias driven by MLL-rearrangement are highly prevalent in acute leukemia with a dismal prognosis[2,3,4]. The poor survival rate and lack of an 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]. Recently, a considerable amount of studies have revealed that MLL and its chaperones are required for genomic integrity in MLL-r leukemia[10,27,28,29,30,31] and lead to chemoresistance[32]. For example, SETD1A is required for survival of AML cells depending on its FLOS domain but not the enzymatic SET domain[10]. The FLOS domain of SETD1A acts as a cyclin-K-binding site that is responsible for chromosomal recruitment of cyclin K and for DNA-repair-associated gene expression[10]. In addition, SETD1A is found functionally found to interact with the DNA damage repair protein RAD18 to safeguard genome stability[30]. These data suggest a pivotal role of 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, inhibited MLL-r AML cell growth and progression in both in vitro and in vivo preclinical models by targeting DNA damage response.

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[19,20,21]. Although it was designed to primarily inhibit receptor tyrosine kinase related to tumor vasculogenesis[34], amounts of evidences show that Anlotinib suppresses tumor cells growth via inhibition of multiple pathways and targets[19,20,21]. Some of its 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 field of indications for Anlotinib to the treatment of MLL-r AML by inducing apoptosis and cell cycle arrest (Fig. 1 and Fig. 2). Moreover, our functional analysis on RNA sequencing data unravel an important role of DNA damage response for Anotinib antitumor activity (Fig. 4),which in line with cell cycle checkpoint arrest in G2/M by Anotinib(Fig. 2). In our experimental setting, we confirmed that Anlotinib is able to induce dysfunction of DNA damage response as manifested by downregulating DNA damage response genes (POLDs) (Fig. 4). Our results provide a novel insight into the mechanism and clinical application of Anlotinib.

DNA polymerase delta (POLD), a member of family B polymerases in eukaryotes, is essential for the leading and lagging strand synthesis[38,39,40], which 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 the other POLD subunits[42]. Multiple studies suggest that the abnormality of POLD1 is related to the poor prognosis of many tumors[43,44]. However, its role in AML is little known. Here, we disclose POLD1 as a prognostic predictor in AML by GEPIA, where patients with high POLD1 expression have an inferior survival (data not shown). In our research, Anlotinib could downregulate the expression of POLD1 and its subunits, highlighting a potential application of Anlotinib to the subgroup of AML with high POLD1 expression.

Since SETD1A and AKT are both required for appropriate expression of DNA damage response[10,25,26], we further unravel the relationship between SETD1A/AKT and POLD family proteins by using bioinformatics (Fig. 5). Importantly, our results point to a molecular logic for SETD1A and AKT to control POLD family genes expression. Although much work is still needed to define the exact mechanisms by which SETD1A/AKT interact POLDs, according to our current findings, Anlnotib hampers DNA damage response via inhibition of SETD1A and AKT in MLL-r AML cells.

Conclusion

Taken together, our work shows that Anlotinib effectively suppresses the growth and induces robust apoptosis in MLL-r AML cells, probably through inhibiting SETD1A and AKT mediated DNA damage response. The present study also highlights a novel mechanism and a promise of using Anlotinib in the treatment of MLL-r leukemia.

Abbreviations

MLL: mixed-lineage leukemia gene; MLL-r: MLL-rearranged;AML: acute myeloid leukemia; GSEA: Gene Set Enrichment Analysis; MLL-FPs: MLL-Fusion Proteins; RTK: receptor tyrosine kinases;DMSO: dimethyl sulfoxide;IMDM: Iscove's modified Dulbecco's medium; FBS: fetal bovine serum; CCK-8: Cell Counting Kit-8; PBS: phosphate buffered saline; RNA-seq: RNA sequencing; DEGs: differentially expressed genes; GEPIA: Gene Expression Profiling Interactive Analysis; POLD: DNA polymerase delta; DEGs: differentially expressed genes

Declarations

Ethics approval and consent to participate

All animal study procedures were approved by the Animal Care and Use Committee of the first Affiliated Hospital of Xiamen University.

Consent for publication

No applicable

Availability of data and materials

The data generated during this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

Fundings

This work was supported by the National Natural Science Foundation of China (81770126, 81770161,81570156, and 81800163), Beijing Medical and Health Foundation(F3100C), Foundation of health and family planning Commission of in Fujian Province of China（2017-2-99）

Authors' contributions

Conception and design: CJZ, FJ, FZH and XB. Acquisition, analysis and interpretation of data: CJZ, FJ, FZH, CQW, CQL, CK, XXM, PX, LGW and SHH. Animal model analysis and data collection: CJZ,YJ and CQW. Writing, and revision of the manuscript: CJZ, FJ, FZH,LGW,SHH and XB.

Acknowledgements

Not applicable.

Authors' information

[1]Department of Hematology, The First Hospital of Longhai, Fujian, 363199, P.R. China. [2]Department of Hematology, The First Affiliated Hospital of Xiamen University and Institute of Hematology, School of Medicine, Xiamen University, Xiamen, 361003, P.R. China. [3] Key laboratory of Xiamen for diagnosis and treatment of hematological malignancy, Xiamen, 361003, P.R. China. [4]The First People's Hospital of Foshan (The Affiliated Foshan Hospital of Sun Yat-sen University), Foshan, Guangdong, 528000, P.R. China. [5]Department of Biology, Case Western Reserve University, Cleveland, OH, USA. [6]Department of Hematology, Huizhou Municipal Central Hospital, Huizhou ,516001, P.R, China. [7]Department of Immunology, DICAT Biomedical Computation Centre, Vancouver, BC, Canada.

References

1. Kuykendall A, Duployez N, Boissel N, Lancet JE, Welch JS. Acute myeloid leukemia: the good, the bad, and the ugly. American Society of Clinical Oncology educational book. Am Soc Clin Oncol Annual Meeting 2018;38:555–73.
2. Zeisig BB, Kulasekararaj AG, Mufti GJ, So CW. SnapShot: acute myeloid leukemia. Cancer cell 2012;22:698.
3. Winters AC,Bernt KM,MLL-Rearranged Leukemias-An Update on Science and Clinical Approaches. Front Pediatr 2017;5:4.
4. Sheridan C. First new drug approval for AML in 15 years. Nature biotechnology 2017;35:696–8.
5. Kawagoe H, Humphries RK, Blair A, Sutherland HJ, Hogge DE. Expression of HOX genes, HOX cofactors, and MLL in phenotypically and functionally defined subpopulations of leukemic and normal human hematopoietic cells.Leukemia 1999;13: 687-98.

6. Chan AKN, Chen CW,MLL Rewiring the Epigenetic Networks in -Rearranged Leukemias: Epigenetic Dysregulation and Pharmacological Interventions. Front Cell Dev Biol 2019;7: 81.
7. Meyer C, Burmeister T, Gröger D, Tsaur G, Fechina L, Renneville A et al. The MLL recombinome of acute leukemias in 2017. Leukemia 2018;32: 273-84.
8. Liang K, Volk AG, Haug JS, Marshall SA, Woodfin AR, Bartom ET et al. Therapeutic Targeting of MLL Degradation Pathways in MLL-Rearranged Leukemia. Cell 2017;168:59-72.
9. Chen Y, Anastassiadis K, Kranz A, Stewart AF, Arndt K, Waskow C et al. MLL2, Not MLL1, Plays a Major Role in Sustaining MLL-Rearranged Acute Myeloid Leukemia.Cancer Cell, 2017;31: 755-70.
10. Hoshii T, Cifani P, Feng Z, Huang CH, Koche R, Chen CW et al. A Non-catalytic Function of SETD1A Regulates Cyclin K and the DNA Damage Response.Cell, 2018;172: 1007-21.
11. Borkin D, He S, Miao H, Kempinska K, Pollock J, Chase J et al. Pharmacologic Inhibition of the Menin-MLL Interaction Blocks Progression of MLL Leukemia In Vivo.Cancer Cell. 2015;27:589-602.
12. Cao F, Townsend EC, Karatas H, Xu J, Li L, Lee S et al. Targeting MLL1 H3K4 methyltransferase activity in mixed-lineage leukemia.Mol Cell. 2014;53:247-61.
13. Funato K, Major T, Lewis PW, Allis CD, Tabar V. Use of human embryonic stem cells to model pediatric gliomas with H3.3K27M histone mutation.Science. 2014;346:1529-33.
14. Grembecka J, He S, Shi A, Purohit T, Muntean AG, Sorenson RJ et al. Menin-MLL inhibitors reverse oncogenic activity of MLL fusion proteins in leukemia. Nature chemical biology 2012;8:277-84.
15. Kühn MW, Song E, Feng Z, Sinha A, Chen CW, Deshpande AJ et al. Targeting Chromatin Regulators Inhibits Leukemogenic Gene Expression in NPM1 Mutant Leukemia. Cancer Discov.2016;6:1166-81.
16. Ye J, Zha J, Shi Y, Li Y, Yuan D, Chen Q et al. Co-inhibition of HDAC and MLL-menin interaction targets MLL-rearranged acute myeloid leukemia cells via disruption of DNA damage checkpoint and DNA repair. Clinical Epigenetics 2019;11:137
17. Kampen KR, Ter Elst A, Mahmud H, Scherpen FJ, Diks SH, Peppelenbosch MP et al. Insights in dynamic kinome reprogramming as a consequence of MEK inhibition in MLL-rearranged AML. Leukemia 2014;28: 589-99.
18. Sandhöfer N, Metzeler KH, Rothenberg M, Herold T, Tiedt S, Groiß V et al. Dual PI3K/mTOR inhibition shows antileukemic activity in MLL-rearranged acute myeloid leukemia. Leukemia 2015;29: 828-38.
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: 120.
20. Han B, Li K, Wang Q, Zhang L, Shi J, Wang Z et al. Effect of Anlotinib as a Third-Line or Further Treatment on Overall Survival of Patients With Advanced Non-Small Cell Lung Cancer: The ALTER 0303 Phase 3 Randomized Clinical Trial. JAMA Oncol 2018;4: 1569-75.
21. Syed YY.Anlotinib: First Global Approval. Drugs 2018;78: 1057-62.
22. He C, Wu T, Hao Y,Anlotinib induces hepatocellular carcinoma apoptosis and inhibits proliferation via Erk and Akt pathway. Biochem Biophys Res Commun. 2018;503: 3093-9.

23. Tang L, Yu W, Wang Y, Li H, Shen Z. Anlotinib inhibits synovial sarcoma by targeting GINS1: a novel downstream target oncogene in progression of synovial sarcoma. Clin Transl Oncol 2019;21: 1624-33.
24. Li Y, Wang Y, Zhou Y, Li J, Chen K, Zhang L et al. Cooperative effect of chidamide and chemotherapeutic drugs induce apoptosis by DNA damage accumulation and repair defects in acute myeloid leukemia stem and progenitor cells. Clin Epigenetics 2017; 9: 83.
25. Song M2, Bode AM, Dong Z, Lee MH. AKT as a Therapeutic Target for Cancer. Cancer Res. 2019;79: 1019-31.
26. Biechonski S, Gourevich D, Rall M, Aqaqe N, Yassin M, Zipin-Roitman A et al. Quercetin alters the DNA damage response in human hematopoietic stem and progenitor cells via TopoII- and PI3K-dependent mechanisms synergizing in leukemogenic rearrangements. Int J Cancer 2017;140: 864-76.
27. Skucha A, Ebner J, Schmöllerl J, Roth M, Eder T, César-Razquin A et al. MLL-fusion-driven leukemia requires SETD2 to safeguard genomic integrity. Nat Commun 2018;9: 1983.
28. Lu F, Wu X, Yin F, Chia-Fang Lee C, Yu M, Mihaylov IS et al. Regulation of DNA replication and chromosomal polyploidy by the MLL-WDR5-RBBP5 methyltransferases. Biol Open 2016;5: 1449-60.
29. Herbette M, Mercier MG, Michal F, Cluet D, Burny C, Yvert G et al. The C. elegans SET-2/SET1 histone H3 Lys4 (H3K4) methyltransferase preserves genome stability in the germline. DNA Repair (Amst.) 2017;57: 139-50.
30. Alsulami M, Munawar N, Dillon E, Oliviero G, Wynne K, Alsolami M et al. SETD1A Methyltransferase Is Physically and Functionally Linked to the DNA Damage Repair Protein RAD18. Mol Cell Proteomics 2019;18: 1428-36.
31. Wood K, Tellier M, Murphy S. DOT1L and H3K79 Methylation in Transcription and Genomic Stability. Biomolecules 2018;8: pii: E11.
32. Mar BG, Chu SH, Kahn JD, Krivtsov AV, Koche R, Castellano CA et al. SETD2 alterations impair DNA damage recognition and lead to resistance to chemotherapy in leukemia. Blood 2017;130: 2631-41.
33. Morgado-Palacin I, Day A, Murga M, Lafarga V, Anton ME, Tubbs A et al. Targeting the kinase activities of ATR and ATM exhibits antitumoral activity in mouse models of MLL-rearranged AML. Sci Signal 2016;9: ra91.
34. 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: 105.
35. Jung CL, Kim HJ, Kim DH, Huh H, Song MJ, Kim SH. CKIT mutation in therapy-related acute myeloid leukemia with MLLT3/MLL chimeric transcript from t(9;11)(p22;q23). Ann Clin Lab Sci.2011;41:193-6.
36. Chen YP, Lin HJ, Chen JS, Tsai MY, Hsieh HP, Chang JY et al. CDKN1A-mediated responsiveness of MLL-AF4-positive acute lymphoblastic leukemia to Aurora kinase-A inhibitors. Int J Cancer 2014;135: 751-62.
37. Goldenson B, Crispino JD.The aurora kinases in cell cycle and leukemia. Oncogene 2015;34: 537-45.

38. Chilkova O, Stenlund P, Isoz I, Stith CM, Grabowski P, Lundström EB et al. The eukaryotic leading and lagging strand DNA polymerases are loaded onto primer ends via separate mechanisms but have comparable processivity in the presence of PCNA. Nucleic Acids Res 2007; 35:6588-97.
39. Greenough L, Menin JF, Desai NS, Kelman Z, Gardner AF. Characterization of family D DNA polymerase from Thermococcus sp. 9 degrees N. Extremophiles 2014; 18:653-64.
40. Henneke G, Flament D, Hubscher U, Querellou J, Raffin JP. The hyperthermophilic euryarchaeota Pyrococcus abyssi likely requires the two DNA polymerases D and B for DNA replication. J Mol Biol 2005; 350:53-64.
41. Podust VN, Chang LS, Ott R, Dianov GL, Fanning E. Reconstitution of human DNA polymerase delta using recombinant baculoviruses: the p12 subunit potentiates DNA polymerizing activity of the four-subunit enzyme. J Biol Chem 2002; 277:3894-901.
42. Zhou Y, Meng X, Zhang S, Lee EY, Lee MY. Characterization of human DNA polymerase delta and its subassemblies reconstituted by expression in the MultiBac system. PLoS One 2012; 7:e39156.
43. Cui Y, Keles S, Charbonnier LM, Julé AM, Henderson L, Celik SC et al. Combined Immunodeficiency due to a loss of function mutation in DNA Polymerase. J Allergy Clin Immunol 2020;145:391-401.
44. Wang F, Zhao Q, Wang YN, Jin Y, He MM, Liu ZX et al. Evaluation of POLE and POLD1 Mutations as Biomarkers for Immunotherapy Outcomes Across Multiple Cancer Types. JAMA Oncol. 2019. doi:10.1001/jamaoncol.2019.2963

Figures

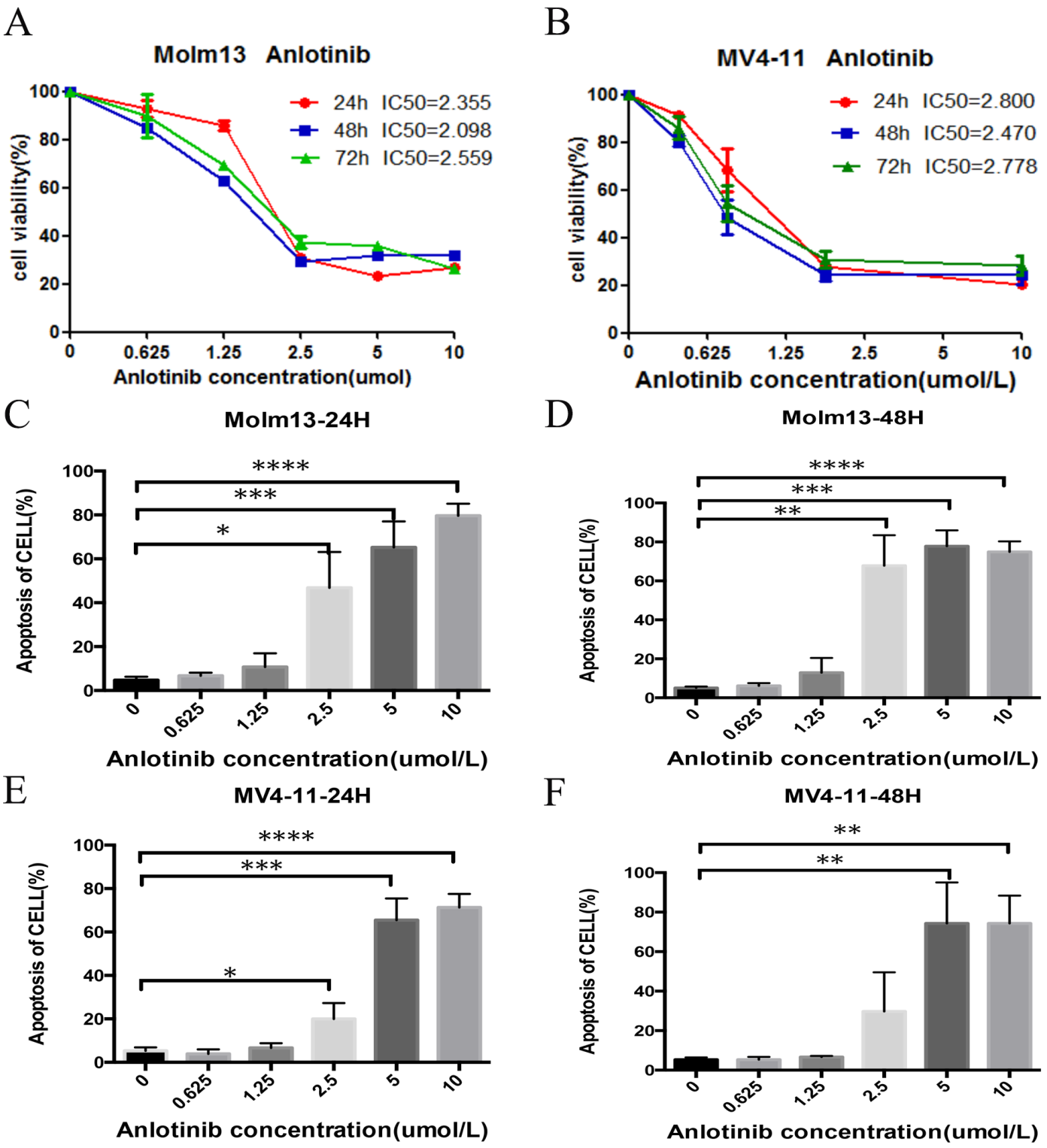

Figure 1

Anlotinib effectively suppresses the growth and induces robust apoptosis of MLL-rearragement AML cells. Molm-13(A□C□D) and MV4-11 (B□E□F) cells were separately treated with designated concentrations of Anlotinib for 24h□48h or 72h, 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 experi¬ments were carried in triplicate).

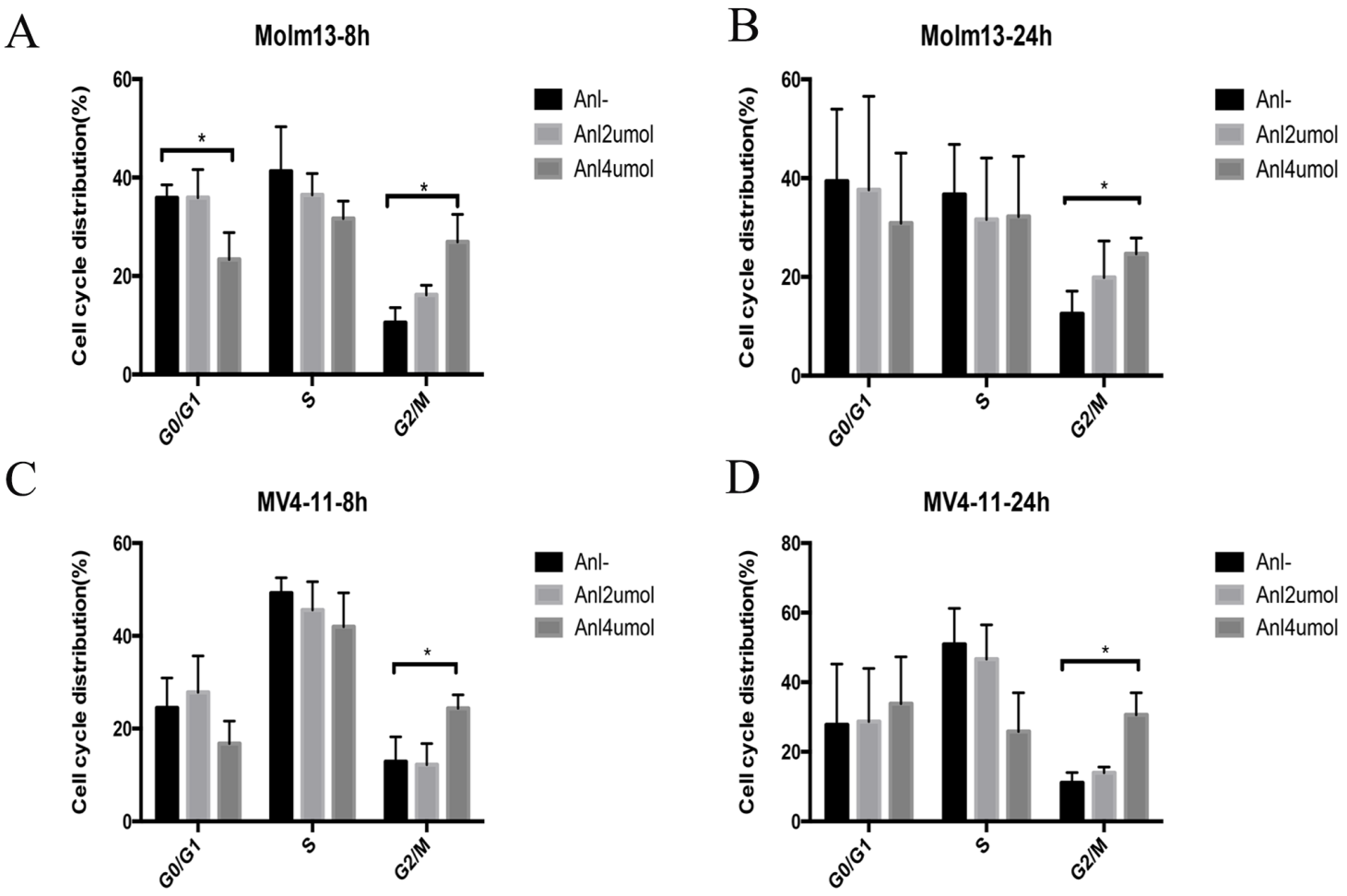

Figure 2

Anlotinib leads to dose-dependent arrest of G2/M phase cell cycle distribution in MLL-r cells. The experi¬ments were carried in triplicate and showed that the percentage of cells in G2/M phase increased significantly in a dose-dependent manner, resulting in G2/M cell cycle arrest.

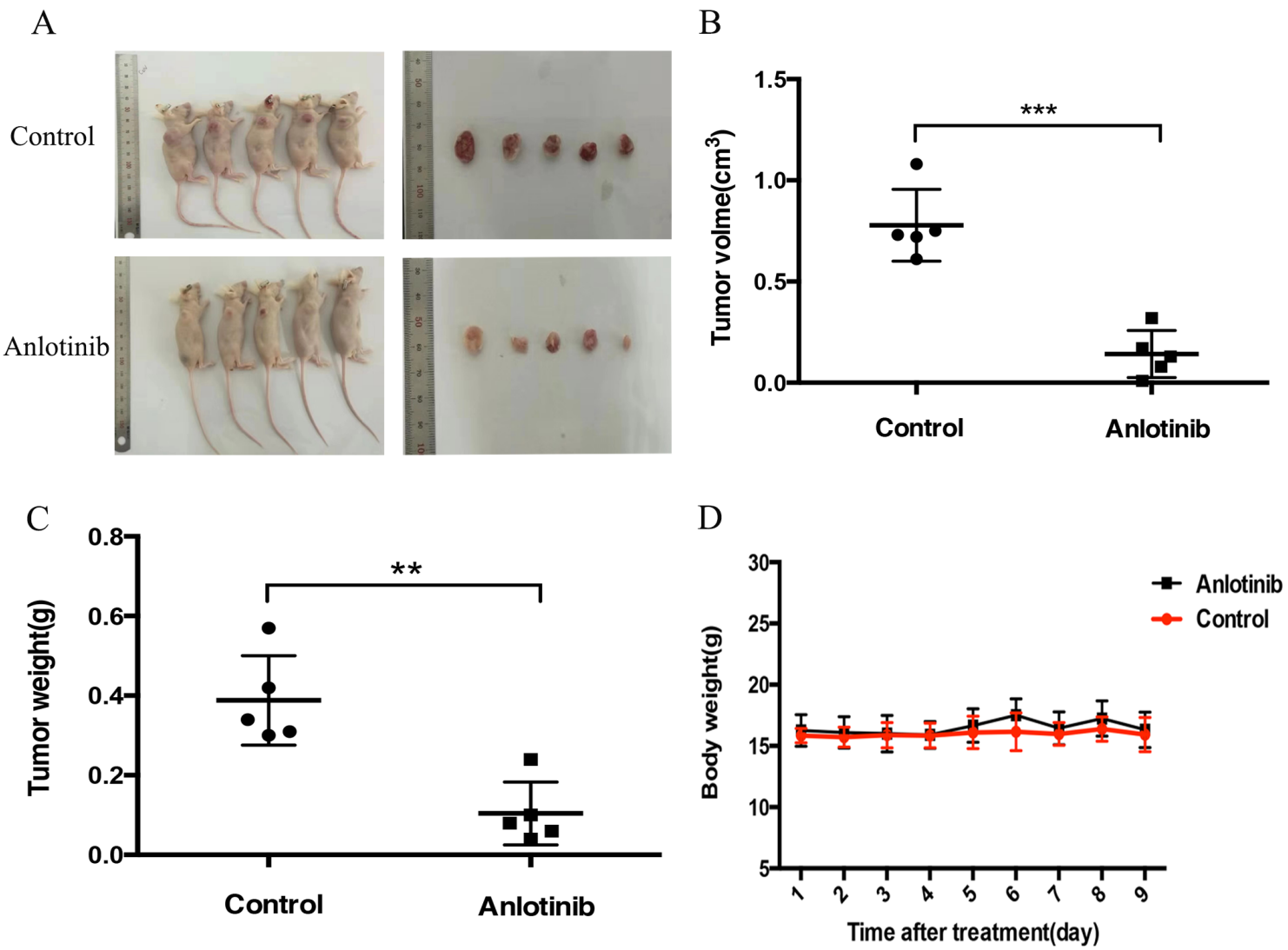

Figure 3

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 indicate mean ± SEM for 5 mice/each group (**P < 0.01). (D) Mice were weighed daily after tumor cell inoculation.

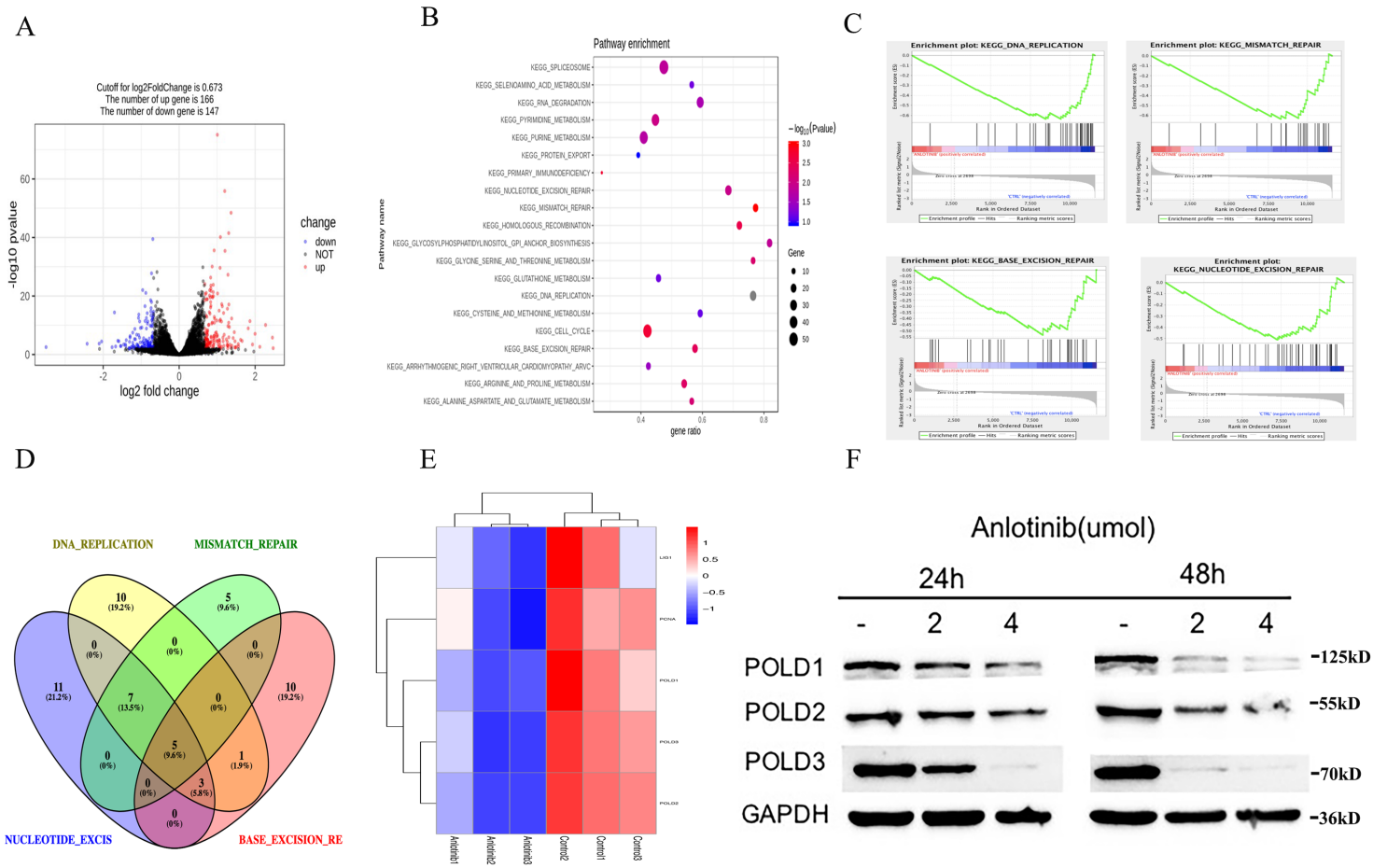

Figure 4

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 group: Volcano map shows the number of different genes. (B) GSEA was used to analysize the RNA-seq data: A bubble chart shows top 20 down-regulated pathways enriched by GSEA. (C)Four representative pathways related to DNA damage and repair is shown. A Venn diagram(D) and A heatmap(E) shows five common genes among four representative patheways. (F) The expression of POLD family was confirmed by western blotting.

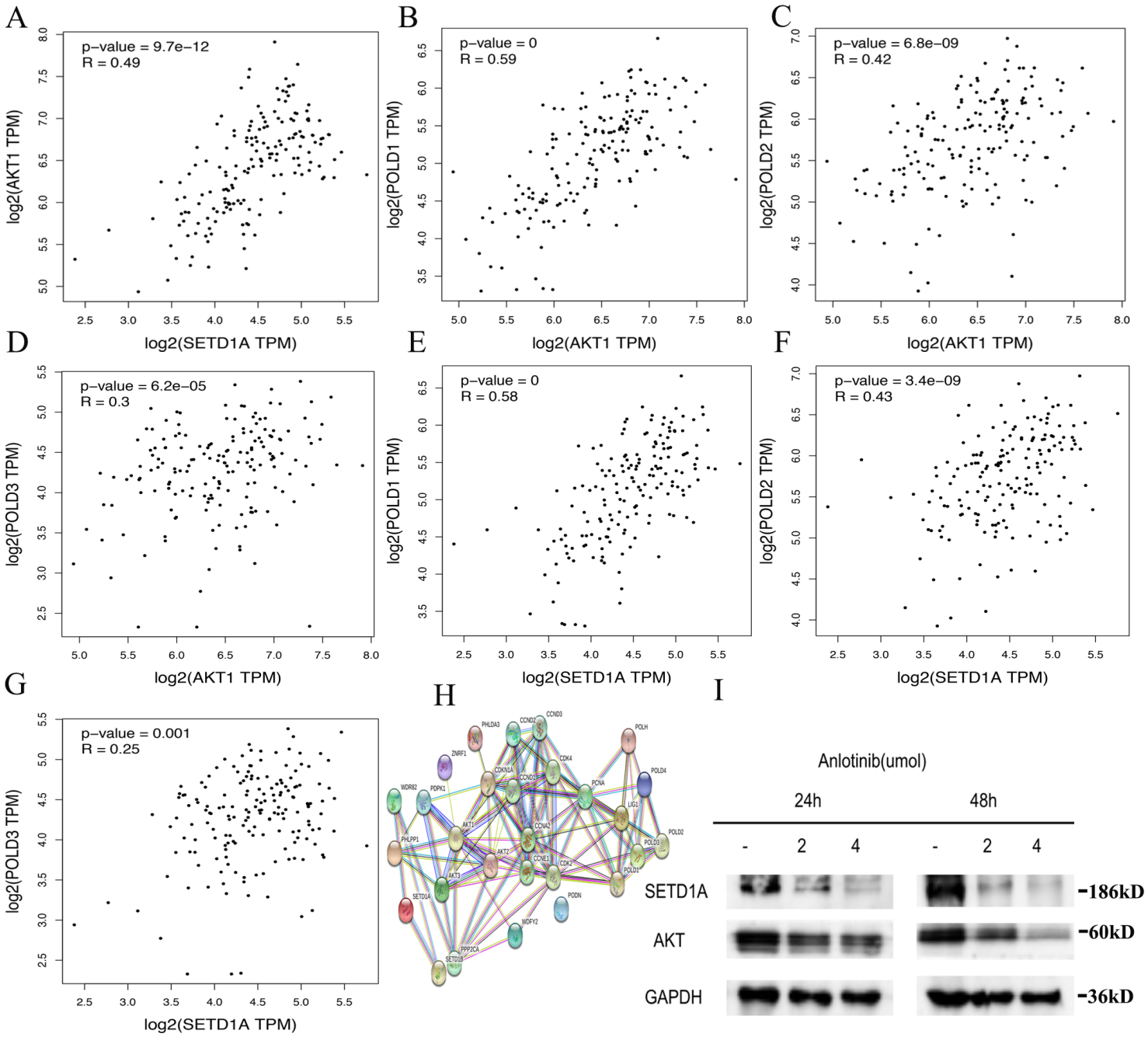

Figure 5

Anlontinib impairs DNA damage responses by downregulating SETD1A and AKT. Correlation analysis by GEPIA database shows in (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 5 hub genes and the relationship of them is also illustrated. (I) Western bloting showed that SETD1A/AKT axis was significantly down regulated in Anlotinib treated Molm-13 cells.