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Anlotinib inhibits the progress of colorectal cancer cells by antagonizing VEGFR/JAK2/STAT3 axis

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Abstract. – OBJECTIVE: Anlotinib, a novel tyrosine kinase receptor inhibitor (TKI), targets multi-targets, including vascular endothelial growth factor receptor (VEGFR). Increasing evidence suggests that anlotinib exhibits effective anti-tumor activity in various cancer types, such as liver cancer. However, the biological function of anlotinib in the treatment of colorectal cancer (CRC) remains largely unknown. This investigation aims to investigate the function and possible molecular mechanism of anlotinib in CRC therapy.

MATERIALS AND METHODS: Human colorectal cancer cells (HCT-116 and LOVO) were cultured and treated with anlotinib alone or combined with cisplatin (DDP). Thereafter, CCK8 assay, CyQUANT NF assay, and colony formation were used to determine the cytotoxicity property and cell proliferation of colorectal cancer. To evaluate the invasion and metastasis of colorectal cancer cells, we conducted wound healing and trans-well assay. Hoechst33342 fluorescence staining and Flow Cytometry analysis were applied for apoptosis detection. Real-time qPCR and Western blot were used to measure the mRNA or protein level.

RESULTS: Our results showed anlotinib alone or combined with cisplatin inhibited cell proliferation, migration, and invasion and activated apoptosis in colorectal cancer cells. Furthermore, we found that anlotinib inhibiting the phosphorylation level of VEGFR, Janus Kinase 2 (JAK2), and Signal Transducer and Activator of Transcription 3 (STAT3). Combination chemotherapy of anlotinib with cisplatin is more sensitive to colorectal cancer.

CONCLUSIONS: These findings suggested that anlotinib might benefit colorectal cancer therapy by antagonizing VEGFR/JAK2/STAT3 signaling. Our study may provide new insights into novel molecular therapeutic strategies for colorectal cancer.

Key Words:
Anlotinib, Cisplatin, VEGFR, STAT3, Colorectal cancer.

Introduction

Colorectal cancer (CRC) has been proved as the third most commonplace reason for the deaths caused by cancer around the world. Also, it is predicted that CRC will be of higher and higher incidence over time[1]. As for the deaths due to CRC, metastasis is the most important reason[2,3]. At present, the standard treatment of CRC is adjuvant chemotherapy after surgery, which usually involves in platinum-based drugs[4]. In spite of the fact that the Cisplatin (DDP) has been recognized as a first-rate chemotherapeutic drug for the treatment of solid tumors, which assumes an irreplaceable role of DDP in the therapy for CRC[5,6], the treatment is still accompanied by drug resistance and reduced effectiveness[7-9]. It is a pressing need to develop a more effective targeted drug for CRC treatment alone or combined with chemotherapy.

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The literature indicated that cisplatin, a chemotherapeutic agent for colorectal cancer, cisplatin can be more effective through a combination approach[10]. Anlotinib (AL3818), as a third-line broad-spectrum anticancer drug, has been testified to be capable of improving the prognosis of those patients who suffer the advanced Non-Small-Cell Lung Cancer (NSCLC)[11-14]. For the medullary thyroid carcinoma, which is either locally advanced or metastatic, osteosarcoma, as well as liver cancer, anlotinib also showed effective anti-tumor activity[15-17]. As a Tyrosine Kinase Inhibitor (TKI), the anlotinib targeted on several genes, including Fibroblast Growth Factor Receptor (FGFR) and Vascular Endothelial Growth Factor Receptor (VEGFR). Many researches demonstrated that the increased expression of Vascular Endothelial Growth Factor A (VEGFA) is related to the development of tumor and the prognosis of CRC[18-20]. VEGFR2, as a receptor for VEGFA, activates a number of downstream pathways, such as JAK2/STAT3 signaling, which affects cell proliferation, migration, and tubular formation. Some studies[21,22] showed that several VEGFR2 inhibitors inhibited tumor angiogenesis.

We hypothesized anlotinib alone or combined with platinum could improve the treatment of CRC. We found that anlotinib could inhibit cell proliferation, invasion and migration, and promote apoptosis through VEGFR/JAK2/STAT3 signaling pathway. This investigation may provide a new thought for the clinical treatment of CRC.

Material and Methods

Cell Culture and Treatments

Human colorectal cancer cells (HCT-116 and LOVO) were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) supplied with 100 μg/ml streptomycin, 100 U/ml penicillin (Solarbio, Beijing, China), and 10% Fetal Bovine Serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA) at 37°C with 5% CO_2. Anlotinib was a gift from Chia Tai Tianqing Pharmaceutical company (Nanjing, Jiangsu, China). Cisplatin (DDP) was purchased from Jiangsu Haosen Pharmaceutical Co., Ltd (Lianyungang, Jiangsu, China). Anlotinib was dissolved in Dimethyl Sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) and stored in dark at -20°C. Cancer cells were treated with a serial of concentration (0, 2, 4, 8, and 16 μM) of anlotinib or DDP (0, 2, 4, 8, and 16 μM). For the combination treatment, 3 μM anlotinib and 8 μM DDP were applied.

Cell Viability and Cell Proliferation Assay

Cytotoxicity property and cell proliferation of anlotinib and/or DDP against HCT-116 and LOVO cells were determined by CCK-8 (Cell Counting Kit-8) assay (Dojindo, Molecular Technologies, Kumamoto, Japan) and CyQUANT NF assay (Thermo Fisher Scientific, Waltham, MA, USA), respectively. Briefly, 5×10^3 colorectal cancer cells for each well were seeded in a 96-well plate and treated with different concentrations of anlotinib and/or DDP after 24, 48, or 72 hours. For cytotoxicity detection, after incubated with CCK-8 agents for 2 hours, absorbance at 450 nm was detected. The median lethal dose (LD50) was calculated using SPSS 23.0 (IBM, Armonk, NY, USA). For cell proliferation analysis, cells were labeled with CyQUANT NF reagent. After incubated with dye binding solution for 10 min, the fluorescence intensity was measured by a fluorescence microplate reader. The median inhibitory concentration (IC50) was then calculated by SPSS 23.0 (IBM, Armonk, NY, USA).

Colony-Forming Assay

HCT-116 and LOVO cells were seeded in a six-well plate at 3×10^5 cells/ml and incubated to 80% confluency. After treated with anlotinib and/or DDP for 24 hours, viable cells were harvested and then incubated in a 60 mm culture dish at 2×10^3 cells/3 ml for 12 days. Cells were stained with crystal violet and observed by an IX71 inverted microscope (Olympus, Tokyo, Japan).

Cell Migration and Invasion Assays

Transwell chambers (Corning, Corning, NY, USA) coated with or without Matrigel were applied to analyze cell invasion and migration, respectively. Briefly, cells (1×10^5 cells/well) were treated with anlotinib and/or DDP for 24 hours and then were incubated in the upper chamber with 200 μL of serum-free DMEM for 24 hours. The lower chamber filled with the complete medium was fixed with Paraformaldehyde and then dyed with crystal violet. The invasive or migrated cells were then counted under an IX71 inverted microscope (Olympus, Tokyo, Japan).

Wound healing assays are used to determine collective cell migration. After reached 90% confluence, cells were scraped and then were treated with anlotinib and/or DDP. The distance between the edges of the wound was measured under an IX71 inverted microscope (Olympus, Tokyo, Japan).

RNA Extraction, cDNA Synthesis, and qPCR

RNA extraction reagent and RevertAid First Strand cDNA Synthesis Kit were purchased from Thermo Fisher Scientific company (Waltham, MA, USA). The relative mRNA expression was evaluated by quantitative PCR in ABIPRISM® 7900HT Fast Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). For each gene, $2^{-\Delta\Delta CT}$ was calculated to determine the relative expression. The primers were listed in **Supplementary Table I**.

Analysis of Apoptosis

To investigate the apoptosis of colorectal cancer cells after treatment of anlotinib, we used Annexin V-PE (BD Biosciences, San Jose, CA, USA) to label apoptosis cells. Collected colorectal cancer cells were resuspended and labeled with 5 µl Annexin V-PE and 5 µl 7-AAD. The gross majority of classical apoptotic hallmarks were examined by a flow cytometer (Beckman Coulter, Brea, CA, USA).

Apoptotic nuclear morphology was visualized by the Hoechst33342 staining. After treated with anlotinib and/or DDP for 24 hours, HCT-116 cells were fixed and then stained with Hoechst33342 solution (Solarbio, Beijing, China). Cells were observed under an IX71 fluorescence microscope (Olympus, Tokyo, Japan).

Bioinformatics Analysis

An interactive web-based gene expression analysis tool, GEPIA (http://gepia.cancer-pku.cn) was used to analyze the difference of VEGFA mRNA between colorectal cancer and normal tissues and the association of VEGFA with the cancer progress and prognosis[23]. The Human Protein Atlas was used to identify tumor-type specific protein expression[24]. Metascape (http://metascape.org) was used to provide typical batch annotation and gene-GO term enrichment analysis[25]. GO annotation and KEGG pathway enrichment information of genes were also performed by DAVID online program (https://david.ncifcrf.gov)[26].

Western Blot Analysis

Cell lysates were run on 10% or 12% SDS polyacrylamide gel to separate proteins, which then be transferred to Nitrocellulose (NC) membrane. After incubated with the specified primary antibody, and then corresponding horseradish peroxidase (HRP) conjugated secondary antibody, NC membrane was developed by enhanced chemiluminescence (ECL) luminescence reagents (GE Healthcare, Amersham, Buckinghamshire, UK). β-actin was applied as reference control. All antibodies were purchased from Abcam (Eugene, OR, USA).

Statistical Analysis

Data were analyzed using GraphPad Prism 7.0 software (GraphPad Software, La Jolla, CA, USA) and SPSS 23.0 program (IBM, Armonk, NY, USA). One-way ANOVA or Student's *t*-test method were applied to analyze the differences among groups. All tests were two-sided tests. *p*-value less than 0.05 was designed as statistical significance.

Results

Anlotinib May Improve the treatment of CRC by Blocking VEGFA

It has been well known that anlotinib has an inhibitory effect on the binding of VEGFA to VEGFR. In this study, we assessed the VEGFA expression level in CRC and normal tissues and found an elevated mRNA and protein level in CRC tissues (Figure 1A, B). GEPIA analysis showed that higher VEGFA expression was related to Malignant CRC staging (Figure 1C) and poor disease-free survival of CRC patients (Figure 1D). After treating CRC cells with anlotinib, we found that Vascular Endothelial Growth Factor A (VEGFA), Platelet-Derived Growth Factor A (PDGFA), Fibroblast Growth Factor Receptor 2 to 4 (FGFR2 to 4), Fibroblast Growth Factor 1 (FGF1), Fibroblast Growth Factor 2 (FGF2), Stem Cell Factor (SCF), and Cluster of Differentiation 117 (CD117/C-KIT) were significantly down-regulated (Figure 1E). Anlotinib may improve CRC treatment by inhibiting the effect of VEGFA. Collectively, our results indicated that anlotinib inhibited the phosphorylation of VEGFR2 in CRC cells (Figure 1F).

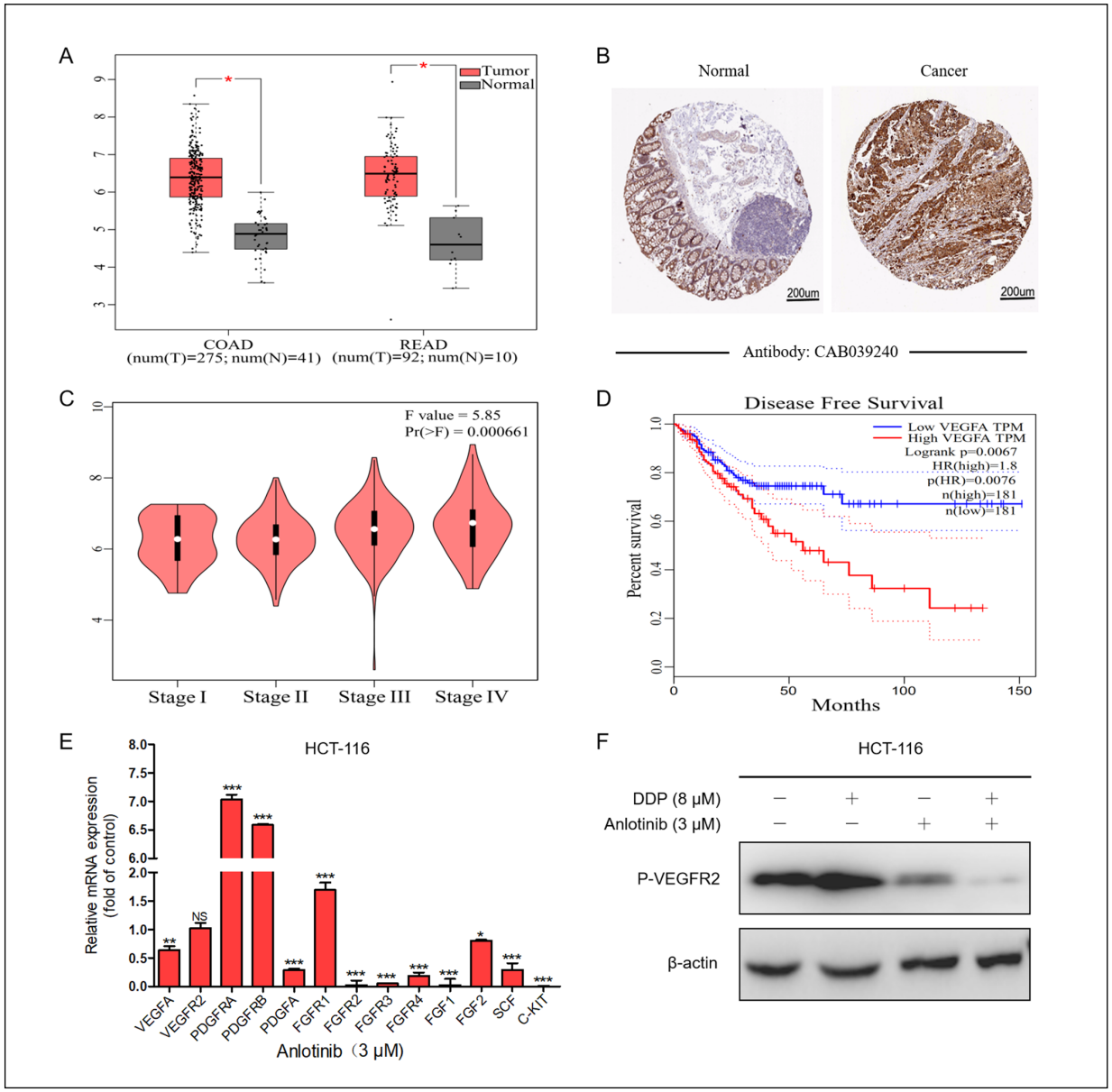

Figure 1. Anlotinib improves the treatment of colorectal cancer by blocking the effects of VEGFA. **A,** The expression of VEGFA in colon adenocarcinoma (COAD), rectum adenocarcinoma (READ), and normal tissues based on TCGA database. **B,** The expression of VEGFA by immunohistochemistry in normal and colorectal cancer tissues (Human Protein Atlas). **C,** VEGFA expression and TNM staging of Colorectal cancer based on TCGA. **D,** Effect of VEGFA expression level on colorectal cancer patient survival based on GEPIA. **E,** The mRNA level of Anlotinib's target and effector genes in HCT-116 cells treated with anlotinib. **F,** The P-VEGFR2 proteins were detected by Western blot. Repeat three times for each experiment. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, NS: no significance.

Anlotinib Inhibits Cell Viability and Proliferation in Human Colorectal Cancer Cells

CCK-8 and CyQUANT cell proliferation assays were applied to determine the influence of anlotinib on the viability and proliferation of colorectal cancer cells. CCK-8 experiments showed that anlotinib combined with DDP had higher cytotoxicity than single drug. Anlotinib combined with DDP are toxic to CRC HCT-116 and LOVO cells in a dose and time-dependent manner (Figure 2A-F).

The CyQuant cell proliferation results showed that anlotinib markedly inhibited the proliferation

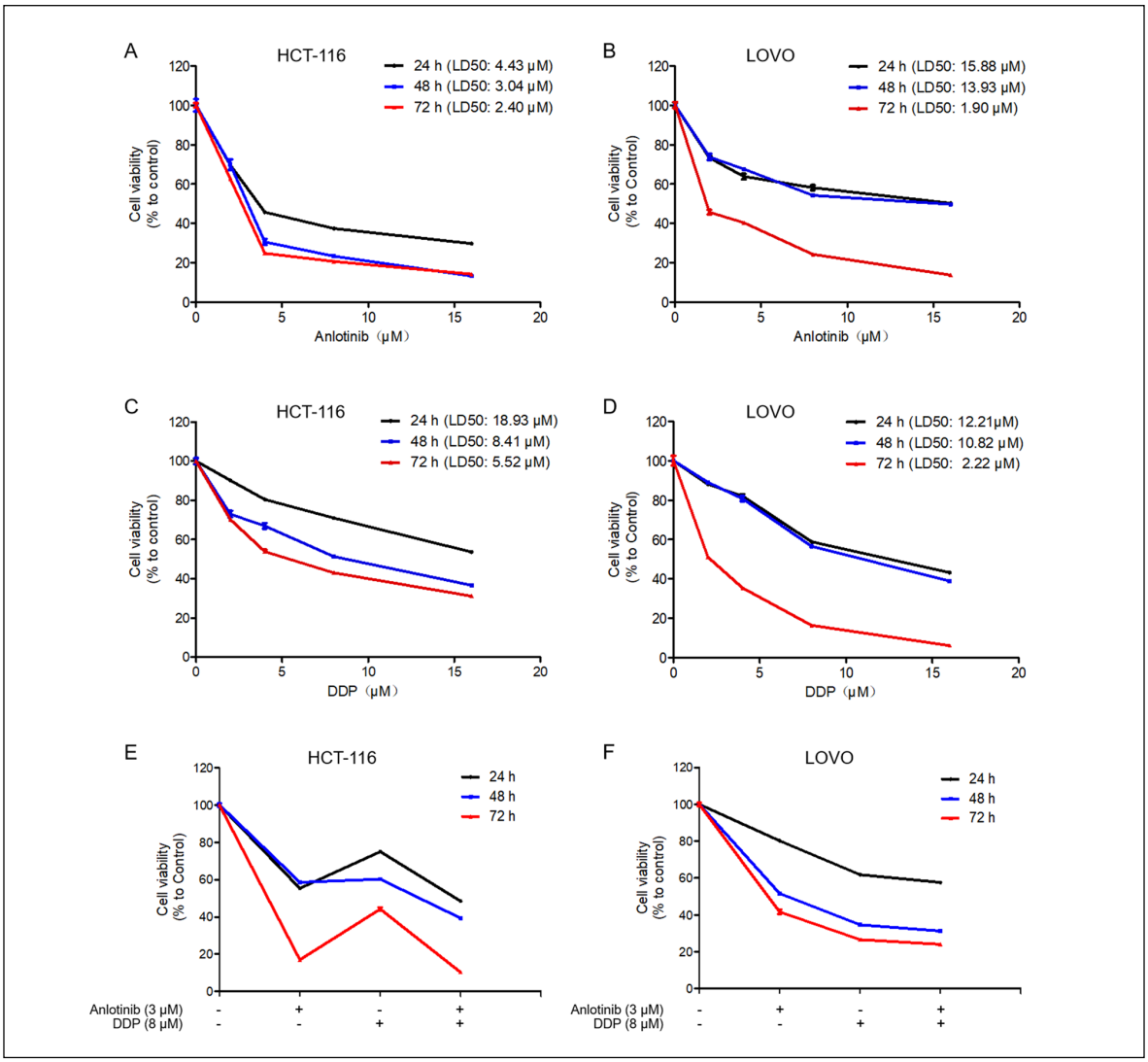

Figure 2. Cell viability analysis of colon cancer cells treated with anlotinib and/or DDP. **A-F,** HCT-116 or LOVO cells were treated with anlotinib and/or DDP at 2 μM to 16 μM concentrations. IC50 was calculated for each group using SPSS. Results were obtained from three separate experiments.

of CRC cells, which was associated with concentration and duration of action (Figure 3A-F). The value of IC50 indicated that anlotinib had a stronger inhibitory effect on colorectal cells than DDP. Clonal formation experiments also confirmed the inhibitory effect of anlotinib alone or in combination with DDP on the proliferation of colorectal cells. Moreover, the combination group was more effective (Figure 3G-J).

Anlotinib Modulates CRC Migration and Invasion In Vitro

To validate whether anlotinib affects tumor migration and invasion, the aforementioned colorectal cancer cells were treated with anlotinib and/or DDP. There was a marked decrease in the edge closure speed of the wound in the cells, especially in those with the combination treatment of anlotinib and DDP (Figure 4A-D). The transwell experiments presented similar results that the capabilities of the invasion and migration were significantly suppressed after treated with anlotinib and/or DDP (Figure 4E-H).

Anlotinib Induces the Apoptosis of CRC

After analyzing the gene expression data from the TCGA database, we found that the PARP1 was up-regulated and caspase-3 is de-

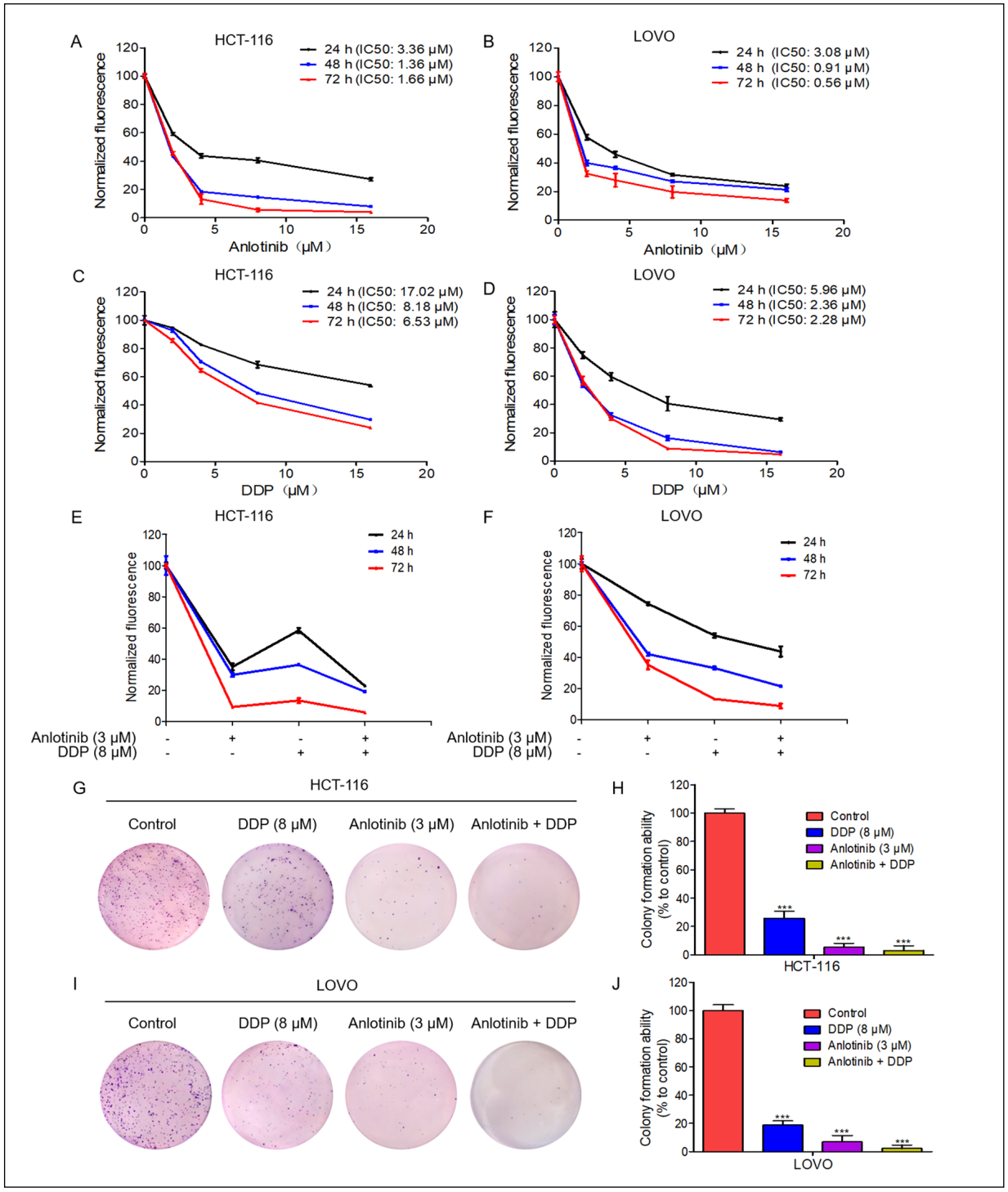

Figure 3. Cell proliferation analysis of colon cancer cells treated with anlotinib and/or DDP. HCT-116 and LOVO cells were treated with anlotinib and/or DDP. **(A-F)** CyQUANT NF cell proliferation assay and **(G-J)** colony formation experiment (magnification: 40×) were used to assess cell proliferation. Repeat three times for each experiment. The significance of various treatments compared to control is indicated by asterisks, ***$p < 0.001$.

regulated in CRC tissues compared to normal colorectal tissues (Figure 5A-D). Anlotinib significantly increased the mRNA levels of proapoptotic genes (CASP2, CASP3, CASP7, CASP8, CASP9, CASP10, BAX, BIM, and BID) in HCT-116 and LOVO cells. It is worth noting

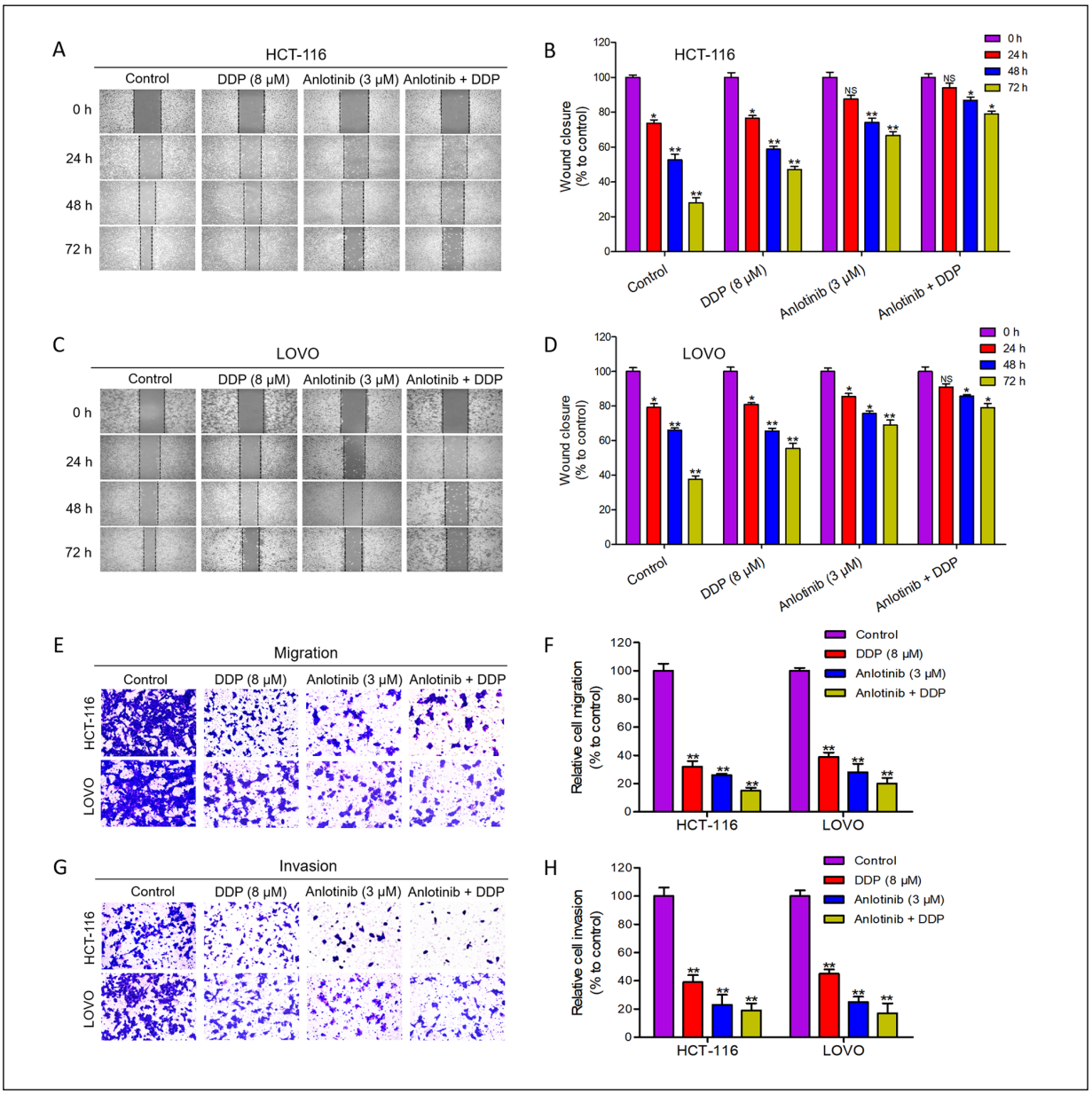

Figure 4. The migration and invasion analysis of colorectal cancer cells with anlotinib. **A-F,** Migration detection by wound healing experiments (magnification: 40×) and transwell experiments (magnification: 200×). **G, H,** Cell invasion detection by transwell assay (magnification: 200×). Representative images of wound closure were taken at 0, 24, 48, and 72 h. The transwell experiment was performed after 24 hours of intervention. Repeat three times for each experiment. Significance of various treatments compared to control is indicated by asterisks, *$p < 0.05$, **$p < 0.01$, NS: no significance.

that there is a decreased expression of apoptosis inhibitory gene (bcl2, bcl2L1, bcl2L2) and DNA repair gene poly ADP-ribose polymerase 1 (PARP1) (Figure 5E). In addition, the proteins of cleaved PARP (the degradation of the PARP) and cleaved caspase 3 (the Caspase-3 activated form) were significantly increased in cancer cells treated with anlotinib alone or in combination with DDP (Figure 5F).

Consistently, Hoechst33342 staining observed strong bright blue fluorescence with morphological features of condensed or fragmented nuclei in HCT-116 cells with drug treatment (anlotinib alone or in combination with DDP), suggesting early and later apoptosis (Figure 5G-J).

We also tested the effect of anlotinib and/or DDP on apoptosis by flow cytometry and found that 3 μM of anlotinib significantly induced early

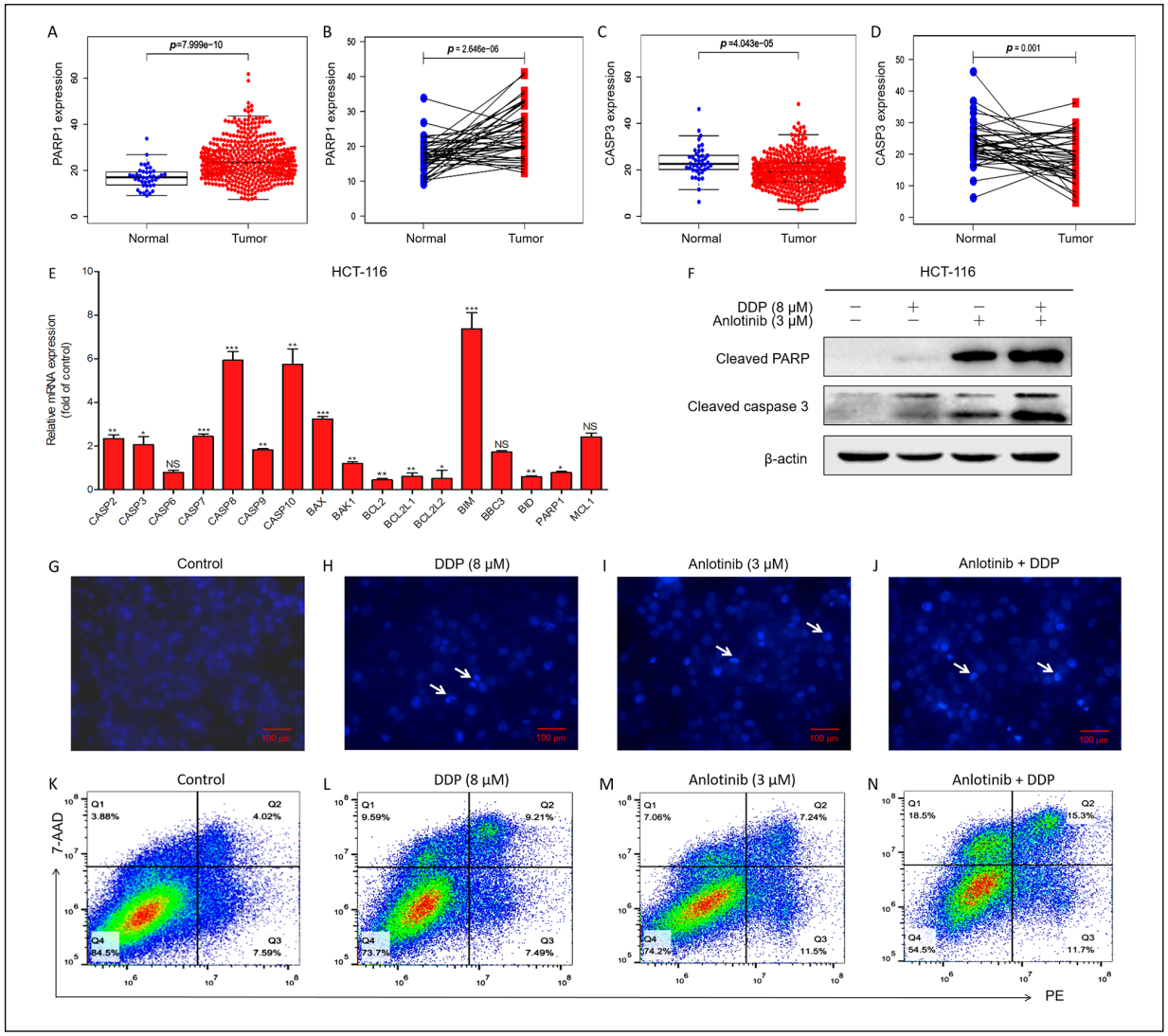

Figure 5. Anlotinib significantly induced apoptosis in colorectal cancer cells. HCT-116 and LOVO cells were treated with anlotinib and/or DDP for 48 hours. **A-D,** The expression of PARP1 and caspase-3 in paired and unpaired samples were analyzed based on the TCGA RNA-seq data. **E,** The mRNA level of apoptotic genes in HCT-116 cells treated with anlotinib. **F,** The cleaved PARP and cleaved-caspase3 proteins were detected by Western blot. **G-J**, Effect of anlotinib and/or DDP on the nuclear morphology of CRC cells after treatment (48 h) was detected by Hoechst 3332 staining. Apoptotic bodies are indicated by white arrows. **K-N,** Flow cytometry was used to detect the probability of apoptosis cells induced by anlotinib and/or DDP in colorectal cancer cells. Triple experiments for each. Significance of various treatments compared to control is indicated by asterisks, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, NS: no significance.

and later apoptosis of CRC cells compared to control and DDP group. It was noteworthy that the combination of anlotinib with DDP produced more apoptosis cells compared to single drug (Figure 5K-N).

Prediction of Possible Pathways for Cancer Treatment with Anlotinib

VEGFR2, FGFR1, PDGFR, and C-KIT are the major target genes of anlotinib. To assess the functional features of anlotinib, we evaluated the genes regulated by VEGFR2, FGFR1, PDGFR, and C-KIT from the KEGG database and obtained 194 overlap genes among them (Figure 6A). Then the functional enrichment analysis of 194 overlap genes was performed using Metascape and DAVID. Results indicated that these correlated genes were mainly involved in pathways related to proliferation, death, apoptosis, migration, proteoglycans, and cell signaling transduction, which

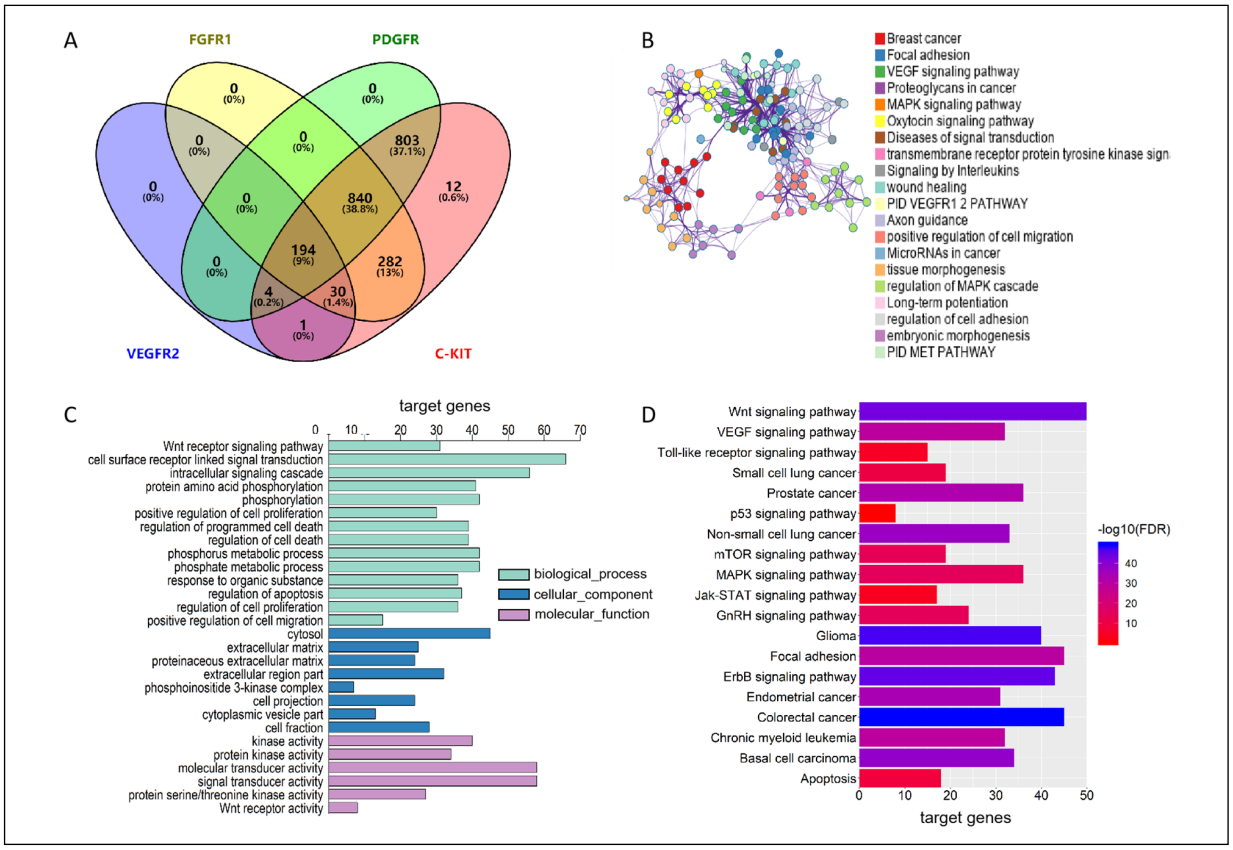

Figure 6. Functional and pathway enrichment analyses of anlotinib-associated target genes. **A,** Overlap of VEGFR2, FGFR1, PDGFR, and C-KIT associated genes. **B,** Functional enrichment analysis of the overlap genes by Metascape. **C,** GO analysis of the overlap genes. **D,** Enriched pathways of the overlap genes in KEGG. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

participated in tumor initiation and progression (Figure 6B, C). The ensuing KEGG pathways bar plot showed that enrichment was significant in CRC and played a vital role in multiple pathways, such as Wingless-type (wnt), VEGF, toll-like receptor, Protein P53 (P53), Mechanistic Target of Rapamycin Kinase (mTOR), Mitogen-activated Protein Kinase (MAPK), and JAK/STAT signaling pathways (Figure 6D).

Anlotinib Improves the Treatment of CRC by Inhibiting JAK2/STAT3 Signaling

Bioinformatic prediction suggested that anlotinib may affect multiple signaling pathways in the development of CRC. We detected the mRNA level of hub genes in these pathways after incubation with anlotinib for 48 hours (Figure 7A). We found that anlotinib could affect the expression of hub genes involved in JAK2/STAT3, ERK/JNK/P38 mediated kinase pathway, AKT-β catenin pathway, etc. Since JAK2 and STAT3 mRNA was significantly down-regulated after treatment with anlotinib, we then detected the proteins of JAK2/STAT3 signaling pathway in CRC cells with anlotinib and/or DDP treatment. The results showed that anlotinib inhibited phosphorylation of JAK2 and STAT3, but not JAK2 and STAT3 protein (Figure 7B).

Discussion

The incidence of CRC in China has increased year by year[27]. The main reason is the metastasis before radical surgery[28]. Therefore, the key to the prevention and treatment of CRC is to control the metastasis of tumors. Clinically, the major treatment for CRC is surgery and chemotherapy. Chemotherapy is an effective treatment for patients with CRC who cannot undergo surgery. However, the traditional chemotherapy agents not only have a large side reaction, but also produce chemo-

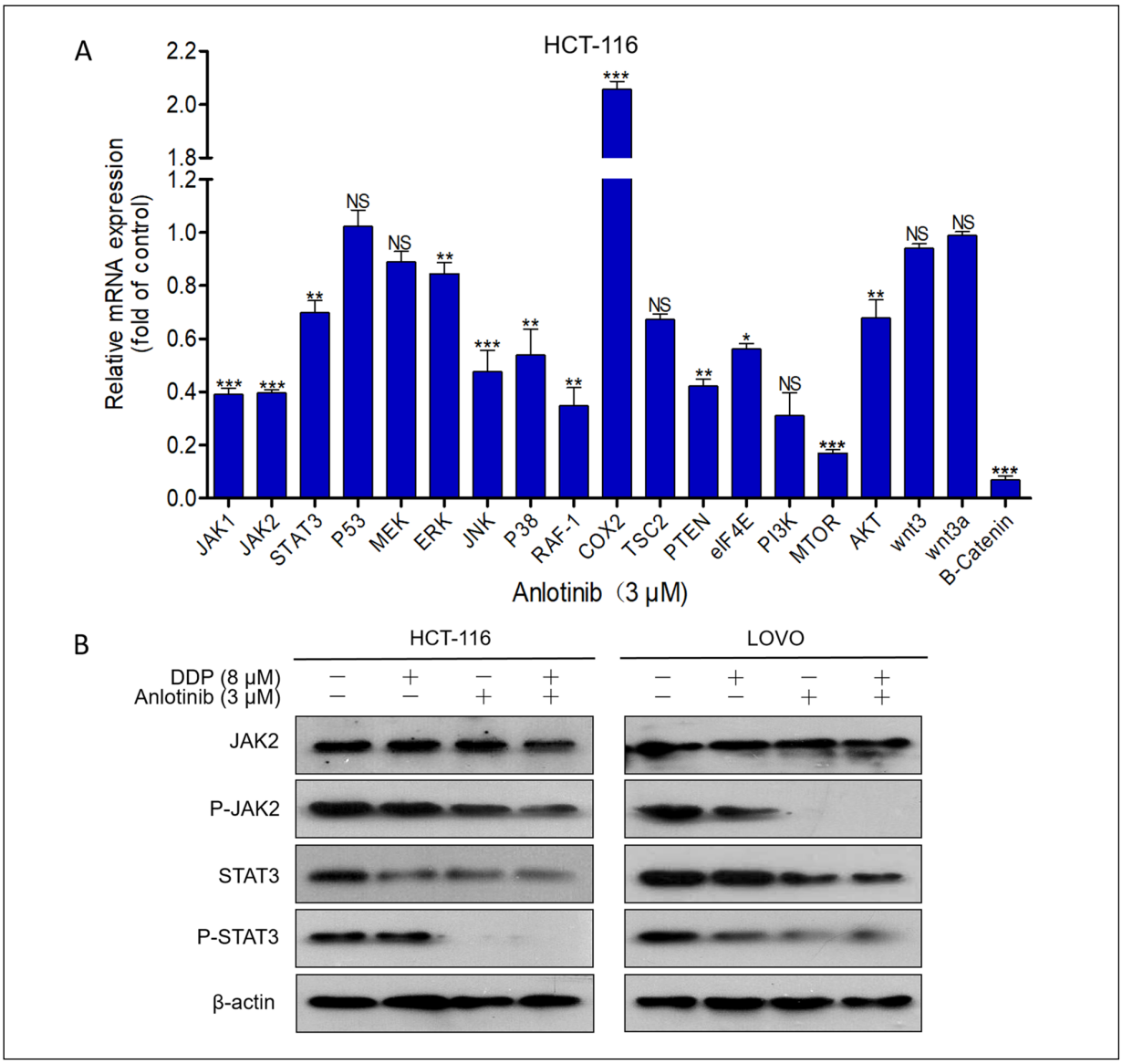

Figure 7. Anlotinib inhibits the activation of JAK2/STAT3 signaling. **A,** mRNA level of Hub genes of predicted pathways in HCT-116 cells incubated with anlotinib for 48 hours. **B,** Protein expression in JAK2/STAT3 signaling pathway after anlotinib and/or DDP treatment. Repeat three times for each experiment. Significance of various treatments compared to control is indicated by asterisks, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, NS: no significance.

therapy resistance through multiple mechanisms, which make chemotherapy less effective[29]. Novel effective agents which targeted on special gene, significantly prolonged the survival time of patients with advanced colorectal diseases[30]. Combination therapy of traditional chemotherapy with targeted therapy has become an important treatment strategy for advanced CRC[31]. Targeted drugs are a new class of anti-tumor drugs that inhibit tumor growth by acting with a specific target gene.

Anlotinib, as an inhibitor of tyrosine kinase receptor, has been applied as a novel systemic chemotherapy for advanced or metastatic NSCLC[32]. Anlotinib presented on significant therapeutic effects on osteosarcoma[16], hepatocellular carcinoma[17], and recurrent glioblastoma[33] via blockade of VEGFR2, Mesenchymal-epithelial transition factor (MET) or suppression of angiogenesis and Extracellular Regulated Protein Kinases (ERK)/Protein Kinase B (PKB/AKT) pathways. Similar to these findings, our data

showed anlotinib inhibited the proliferation, metastasis of Colorectal cancer. Our study provided new evidence that anlotinib might be a promising way to treat CRC.

We demonstrated that anlotinib inhibited the phosphorylation of VEGFR2. After interacting with VEGFA, VEGFR is activated and sends signals into cells to promote cell growth and migration through intracellular signaling cascades, such as JAK/STAT signaling pathway. Other targeted therapies, such as fruquintinib, which blocked the part of VEGFR have been used as a third-line drug to treat CRC[34]. The inhibition of VEGFR helps to reduce cancer growth by blocking downstream signal. In VEGFR2 mediated cascades, JAK/STAT pathway participates in proliferation, apoptosis, and angiogenesis of CRC[35-37]. Recently, several reports showed that JAK2/STAT3 signaling pathway is involved in tumor progression by mediating the regulation of VEGFA[38,39]. STAT3 is a key transcriptional activation biomarker in tumor antigen therapy[40]. Our results supported that anlotinib inhibited the phosphorylation level of JAK2 and STAT3, but not JAK2 and STAT3 in both LOVO and HCT-116 cells. It is clear that STAT3 plays an important role in the development of CRC[41,42]. The activation of STAT3 in tumor cells has been proved to enhance self-renewal and promote tumor progression in colorectal cancer[37].

Conclusions

In summary, our study revealed a previously unknown mechanism of anlotinib in the treatment of colorectal cancer. The present data illustrated a scheme that anlotinib inhibited cell proliferation, invasion, migration and activated apoptosis by inhibiting VEGFR/JAK2/STAT3 signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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