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ORIGINAL RESEARCH

AOC1 Contributes to Tumor Progression by Promoting the AKT and EMT Pathways in Gastric Cancer

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Background: AOC1 is a copper-containing amine oxidase that is responsible for catalyzing the deamination of polyamines, which produces reactive oxygen species. Previous studies have demonstrated that polyamines are involved in the regulation of proliferation, migration, and apoptosis of cells. However, very little is known about the functions and regulatory mechanisms of AOC1 in tumors.

Methods: Based on GEPIA data, we found that AOC1 was significantly upregulated in human gastric cancer tissues. We knocked down AOC1 in human AGS and MKN45 cells using siRNA transfection, then utilized qRT-PCR assay and Western blot to verify the effectiveness of AOC1 knockdown in gastric cancer cells.

Results: Function analysis demonstrated that knockdown of AOC1 inhibited the proliferation, invasion, and migration of human gastric cancer cells. Flow cytometry detection suggested that AOC1 knockdown induced apoptosis in human gastric cancer cells. Mechanism investigation suggested that AOC1 knockdown increased the ratio of Bax/Bcl2 and induced activation of the caspase cascade. Furthermore, the AKT signaling pathway was inactivated when AOC1 was silenced, including downregulated phosphorylation level of AKT and expression of downstream effectors, Cyclin D1, and p70S6K. Finally, we found that knockdown of AOC1 inhibited the epithelial–mesenchymal transition (EMT) in human gastric cancer by increasing the expression of epithelial markers E-cadherin, as well as decreasing mesenchymal marker N-cadherin, SNAIL and Slug.

Conclusion: Our study suggests that AOC1 functions as an oncogene in human gastric cancer by activating the AKT signaling pathway and EMT process and maybe a target of 6-mercaptopurine, which provides new insight in the clinical use of AOC1 in gastric cancer therapy.

Keywords: AOC1, proliferation, invasion and migration, apoptosis, AKT, epithelial-mesenchymal transition

Introduction

Gastric cancer is ranked as the fifth most common malignancy and the third leading cause of cancer-related deaths worldwide.[1–3] Due to the low sensitivity and specificity of early diagnostic biomarkers, gastric cancer patients are frequently diagnosed at an advanced stage, which is accompanied by malignant hyperplasia, extensive infiltration, lymph node metastasis or distant metastasis.[2,3] Despite great advances in surgical resection, chemotherapy, and radiotherapy, the prognosis of gastric cancer patients remains poor due to the high frequency of post-treatment recurrence and metastasis.[4]

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Scientists have thoroughly investigated the molecular mechanisms of gastric cancer, including mutations and aberrant expression of oncogenes or tumor suppressor genes, as well as genome abnormalities, but useful clinical applications are still very rare.[5,6] Therefore, the elucidation of new mechanisms related to the pathogenesis of gastric cancer is crucial for the development of effective targeted therapy for human gastric cancer.

Amine oxidases refer to a class of enzymes that catalyze the deamination of amine groups to produce aldehydes, ammonia, and hydrogen peroxide. Depending on the structure and reactivity of various substrates (mono-, di-, or polyamines), amine oxidases are divided into four classes consisting of monoamine oxidases (MAO) such as MAO-A and MAO-B, polyamine oxidases, lysyl oxidases, and copper-containing amine oxidases.[7] Increasing evidences have shown that amine oxidases are important biological regulators of apoptosis and tumor progression by means of polyamine homeostasis and reactive oxygen species.[8,9]

Copper/TPQ-containing amine oxidases (CAOs) are responsible for catalyzing the deamination of primary amines in organisms, and their molecular structure contains a copper ion and a 2,4,5-trihydroxyphenylalanine quinone (TPQ) cofactor. Human CAOs include three protein-coding genes (AOC1-3) and copper containing 4, pseudogene (AOC4P) as a lncRNA. CAOs have been found to participate in the regulation of a variety of pathological and physiological processes such as cell proliferation, differentiation, survival, glucose uptake, and immune regulation.[10] Currently, several studies have implicated the involvement of CAOs in tumor progression. For example, Woo Young Sun et al report that in breast cancer, stromal AOC3 expression is correlated with a high histological grade and that patients with AOC3 negativity tend to have a shorter survival time and lymph node metastasis.[9] Tong-Hong Wang et al. report that AOC4P inhibits hepatocellular carcinoma proliferation and metastasis by acting as a competitive endogenous RNA for vimentin and further suppressing the EMT process.[11] AOC1 functions as a secreted diamine oxidase for the degradation of polyamines (such as putrescine and histamine), which are highly expressed in the kidneys, placenta, intestine, and lungs with lower levels in the brain.[12] Karin M. Kirschner et al report that AOC1 is involved in embryonic kidney morphogenesis and is transcriptionally regulated by the Wilms tumor transcription factor, WT1.[12] However, to the best of our knowledge, there has not been a study investigating the role of AOC1 in tumors. In this study, we first investigated the expression of AOC1 in human gastric cancer tissues by searching the Gene Expression Profiling Interactive Analysis (GEPIA) website. Next, we identified the function and action mechanism of AOC1 in human gastric cancer cell lines, AGS, and MKN45. Our study revealed that AOC1 played important roles in the proliferation and movements of human gastric cancer cells by regulating the AKT signaling pathway and EMT process.

Materials and Methods

Cell Culture and Transfection

Human gastric cancer cell lines, AGS, and MKN45 were purchased from the Cell Bank of The Chinese Academy of Sciences (Shanghai, China). Cells were maintained in the RPMI-1640 medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Invitrogen, Burlington, Canada) at 37°C in an atmosphere of 5% CO_2. After incubation for 24 h, the cells were collected and AOC1 expression was detected by Western blot. siRNAs targeting AOC1 (si-AOC1) were designed and conducted by Genewiz (Beijing, China). A scrambled siRNA was used as the negative control (NC). When the cells were cultured to 60% confluence, si-AOC1 or siNC (final density of 50 nM) was transiently transfected into AGS or MKN45 cells using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. After transfection for 24 or 48 hrs, cell function analysis was further performed.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

After transfection for 24 hrs, total RNA of human gastric cancer cells was extracted using TRIzol reagent (Invitrogen, USA). A reverse transcription reaction was then performed to produce a strand of cDNA using a First Strand cDNA Synthesis kit (Fermentas, Waltham, MA, USA). AOC1 mRNA expression was tested using a FTC-3000 Real-Time Quantitative Thermal Cycler (Funglyn Biotech Inc., Shanghai, China) with β-actin as an internal control. AOC1 mRNA expression was quantified using the $2^{-\Delta\Delta Ct}$ method. The experiment was performed in triplicate. The primers used in qRT-PCR were as follows:

AOC1-F:5′ -TGTCCACGCAACCTTCTACA- 3′
AOC1-R:5′ -ACTGGGTCTGCTCAAGTGTG- 3′
β-actin-F: 5′ -CCCGAGCCGTGTTTCCT- 3′
β-actin-R: 5′ -GTCCCAGTTGGTGACGATGC- 3′

Western Blot

Human AGS and MKN45 cells were transfected with siNC or si-AOC1 for 48 hrs. The cells were collected, then the total protein was extracted using RIPA buffer (Solarbio, Beijing, China) containing 1% proteinase inhibitor cocktail (Roche Diagnostics). Then, the proteins were denatured by incubating at 95°C for 5 mins. 20-μg protein from each group was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), which was then electroblotted onto a PVDF membrane (PerkinElmer, Waltham, MA). Protein bands were blocked in TBST buffer (TBS buffer containing 0.01% Tween-20) and 5% bovine serum albumin (BSA) for 2 hrs, incubated with the primary antibodies overnight at 4°C. After being washed four times by TBST buffer, protein bands were further incubated with the corresponding secondary antibodies for 1 hr at room temperature. Primary antibodies against AOC1 (Cat#16338-1-AP, 1:1000), GAPDH (Cat# 60004-1-Ig, 1:10,000), Bax (Cat# 50599-2-Ig, 1:6000), Bcl2 (Cat# 12789-1-AP, 1:1000), Caspase-9 (Cat# 10380-1-AP, 1:300), Caspase-3 (Cat# 19677-1-AP, 1:10,000), AKT (Cat# 60203-2-Ig, 1:5000), p-AKT (Cat# 66444-1-Ig, 1:2000), Cyclin D1 (Cat# 60186-1-Ig, 1:10,000), p70S6K (Cat# 66638-1-Ig, 1:3000), E-cadherin (Cat# 60335-1-Ig, 1:5000), N-cadherin (Cat# 66219-1-Ig, 1:5000) and SNAIL (Cat# 26183-1-AP, 1:1000) were purchased from ProteinTech (Rosemont, IL, USA).

The primary antibody against Slug (Cat#9585, 1:1000) was purchased from Cell Signaling Technology (Boston, MA, USA). All secondary antibodies were purchased from ProteinTech. The protein bands were visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce; Thermo Fisher Scientific, Inc.). The intensity of the protein bands was determined using ImageJ software.

Cell Proliferation Analysis

Cell proliferation analysis was performed using a Cell Counting Kit-8 (CCK-8; Sigma, USA) following the manufacturer's instructions. Briefly, human gastric cancer cells were diluted into 3×10^4 cells/mL and added into a 96-well plate at 100 μL per well. The cells were then transfected with si-AOC1 or siNC. At 0 hr, 24 hrs, 48 hrs, and 72 hrs, 10 μL of CCK-8 agent was added to each well for another 1.5 hrs of incubation. Finally, the absorbance was measured on a microplate reader at 450 nm (Thermo Fisher, USA).

Colony formation assay was performed by seeding about 500 cells into a 6-cm cell culture dish. The medium was replaced with fresh medium every 3 days. After culture for 14 days, the cells were fixed with 3 mL of methanol for 10 mins and then stained with 0.1% crystal violet for 10 mins. After being washed and dried in the air, the cell clones were photographed and counted.

Transwell Assay

Cell invasion and migration were analyzed by a 24-well transwell chamber with 8 μm pores (Corning, NY, USA). After transfection for 48 hrs, human AGS and MKN45 cells were collected by trypsin digestion and made into a cell suspension with a density of $5x10^5$ cells/mL. For the migration assay, 100 μL of cell suspension was added into the upper chambers and 200 μL of medium with 10% FBS was added into the lower chamber as a chemoattract. After incubation for 24 hrs in a 37°C incubator, the residual cells on the upper surface of the transwell membrane were removed by a cotton swab. The migrated cells on the lower surface of the transwell membrane were fixed with methanol, washed with PBS and stained with 0.1% crystal violet for 10 mins. For the invasion assay, the transwell membranes were pre-coated with Matrigel (BD Biosciences, San Jose, CA, USA). Cells on the lower surface of the transwell membrane were photographed with a light microscope and counted in five random fields.

Cell Apoptosis Analysis

Cell apoptosis analysis was performed using an Annexin V/PI apoptosis kit (BestBio, Shanghai, China). Briefly, AGS or MKN45 cells were transfected with siNC or si-AOC1 for 48 hrs and then collected by trypsin digestion. After centrifugation at 1000 rpm for 5 mins, cells were resuspended in binding buffer to a density of $1x10^6$ cells/mL. Then, 1 mL of cell suspension was incubated with 5 μL Annexin V (1 μg/mL) for 15 mins and 5 μL PI (1 μg/mL) for 5 mins. The incubation steps were all performed in the dark. Finally, the percentage of apoptotic cells was determined using flow cytometry.

Statistical Analysis

All statistical analysis was performed using Prism 7 (GraphPad Software). The differences between two groups were compared using unpaired two-tailed Student's *t*-test or one-way analysis. All experiments were performed in triplicate. $P<0.05$ was considered significant.

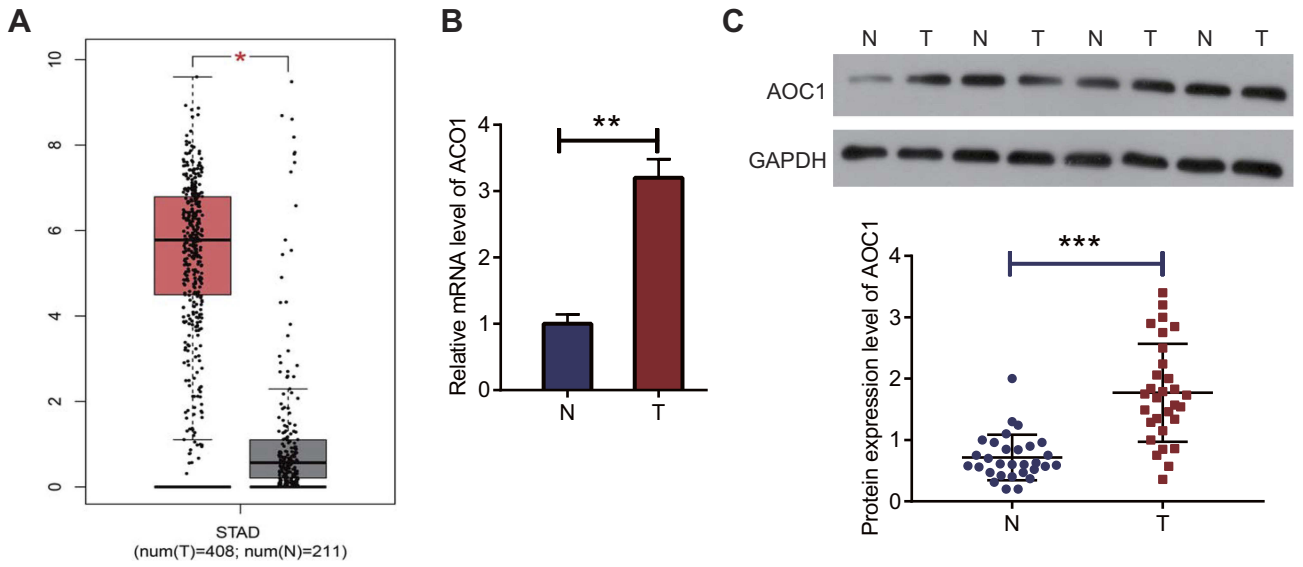

Figure 1 AOC1 is highly expressed in gastric cancer tissues compared with normal tissues. (**A**) The red and gray boxes indicate gastric cancer and normal tissues, respectively. Data were obtained from the GEPIA website, including 408 gastric cancer samples and 211 controls. The y-axis indicated the log2-transformed gene expression levels. qPCR (**B**) and western blot (**C**) were performed to detect AOC1 expression in the tumor and paracancerous tissues of 30 patients with gastric cancerthe. *P<0.05; **P<0.01; ***P<0.001.

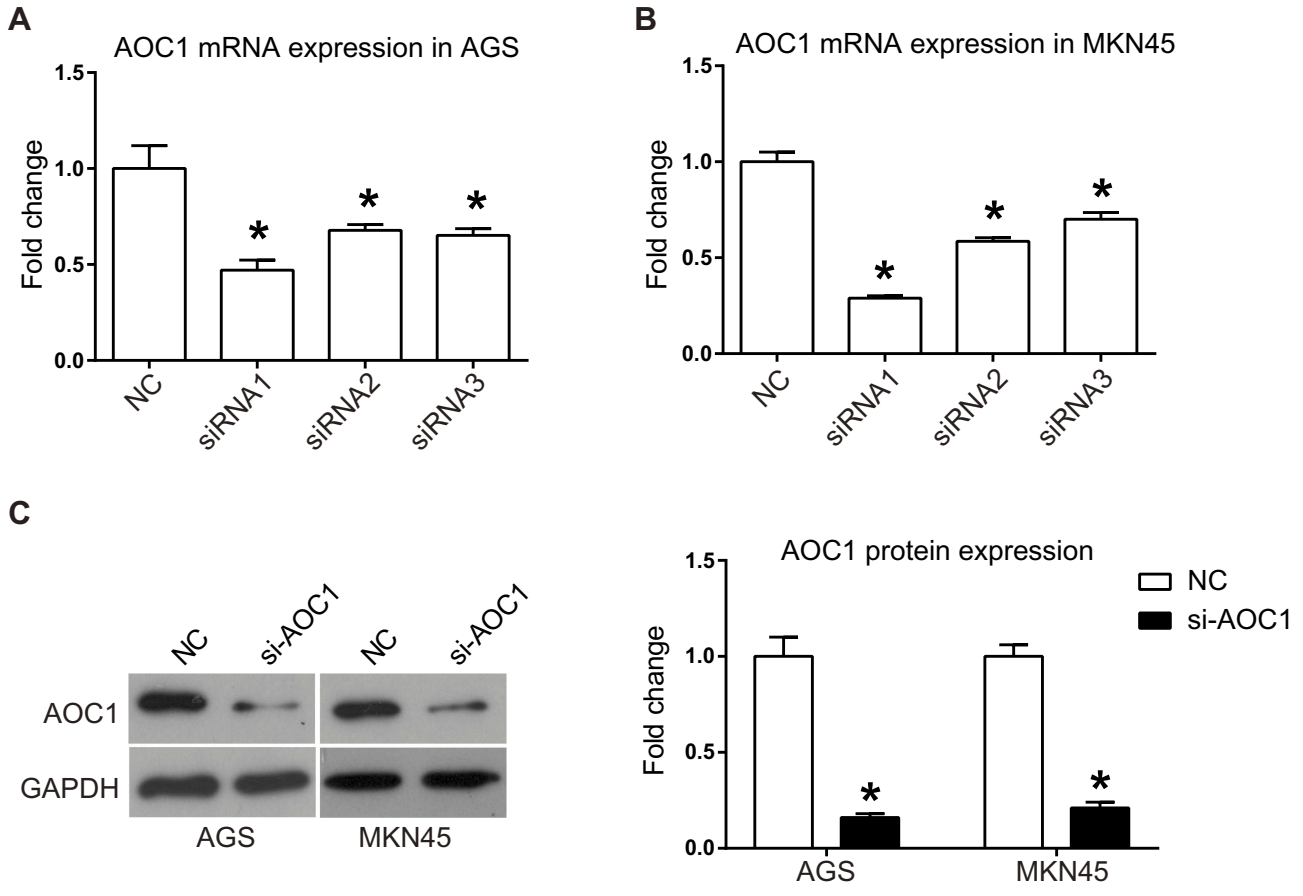

Figure 2 AOC1 expression is effectively knocked down by siRNA transfection in human gastric cancer cells. qRT-PCR assay was used to detect the interference efficiencies of siRNAs targeting AOC1 on mRNA levels in human (**A**) AGS and (**B**) MKN45 cells. (**C**) Western blot validated the effectiveness of AOC1 knockdown on protein levels. A scrambled siRNA was used as the negative control (NC). All experiments were performed in triplicate, independently. *P<0.05.

Results

AOC1 Is Highly Expressed in Human Gastric Cancer Tissues

GEPIA is an online server integrating gene RNA expression data from the Cancer Genome Atlas (TCGA) and the Genotype-Tissue Expression (GTEx) dataset,[13] which is very beneficial for tumor-related gene function research. In order to investigate the involvement of AOC1 in human gastric cancer, we searched its expression on the GEPIA website. As shown in Figure 1A, AOC1 expression was significantly increased in human stomach adenocarcinoma (STAD) tissues compared with normal tissues, which suggested that AOC1 might play an important role in the progression of human gastric cancer. Therefore, we collected the tumor and paracancerous tissues of 30 patients with gastric cancer and detected the expression of AOC1 by qPCR and Western blot. Compared with paracancerous tissues (1±0.1), AOC1 mRNA was overexpressed in gastric cancer tissues (3.2±0.2); additionally, the protein levels of AOC1 in tumor tissues (1.77±0.15) were significantly higher than that in paracancerous tissues (0.72±0.06) (Figure 1C).

Downregulation of AOC1 Inhibits the Proliferation of Human Gastric Cancer Cells

Since AOC1 was found to be upregulated in human gastric cancer tissues, we further investigated the function of AOC1 in human AGS and MKN45 cells by a siRNA-mediated knockdown experiment. After transfection for 24 hrs, AOC1 mRNA expression was examined via qRT-PCR assay, which suggested siRNAs targeting AOC1 could efficiently knockdown AOC1 in both the AGS and MKN45 cells (Figure 2A and B). siRNA1 was selected for the following experiments due to the most potent interference efficiency. Western blot assay validated the interference effect of si-AOC1 on AOC1 protein expression (Figure 2C). Next, we performed cell proliferation analysis in

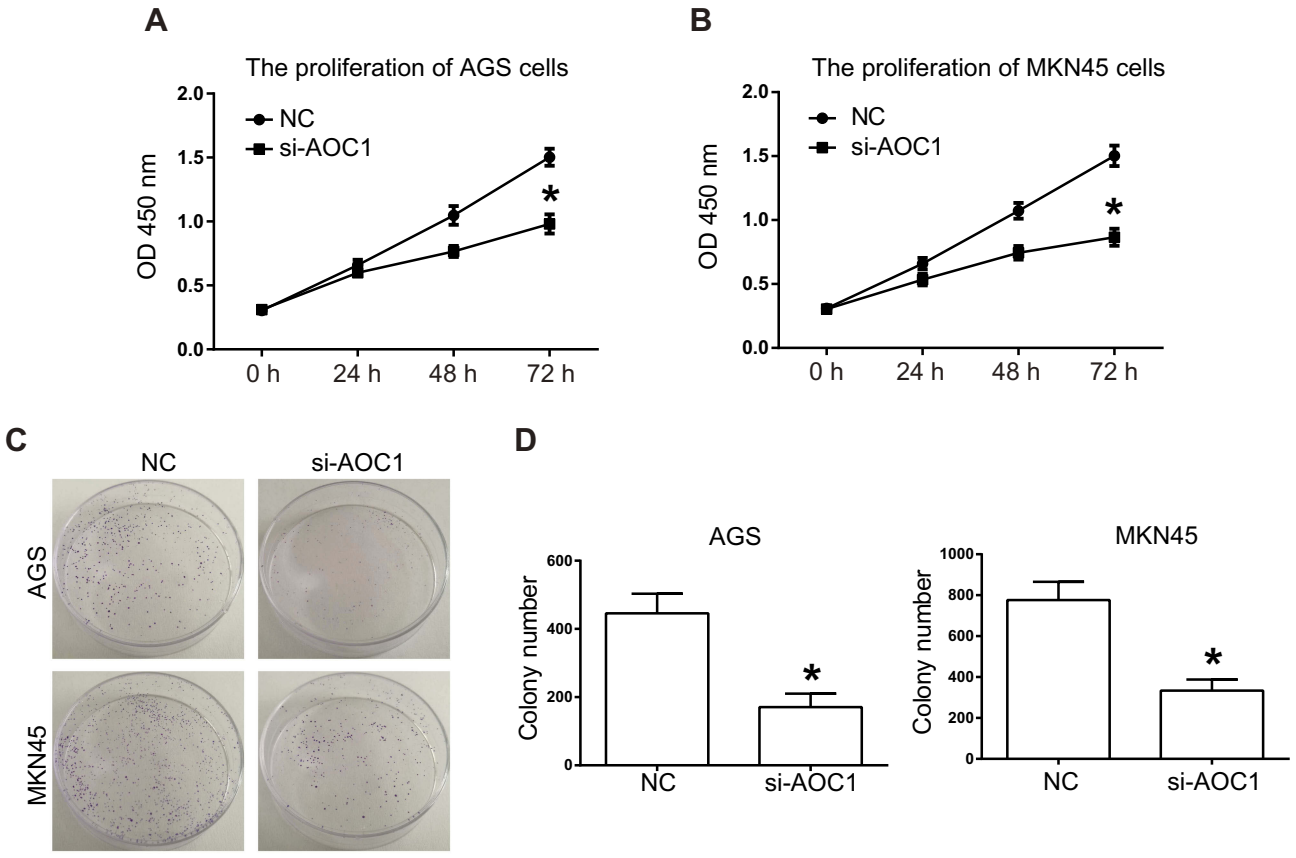

Figure 3 AOC1 knockdown induces growth inhibition in human gastric cancer cells. (**A**) and (**B**) After transfection with siNC or si-AOC1, the viability of AGS and MKN45 cells was detected by using CCK-8 assay. (**C**) and (**D**) Clone formation ability of AOC1 silenced gastric cancer cells was detected. All experiments were performed in triplicate. *P<0.05.

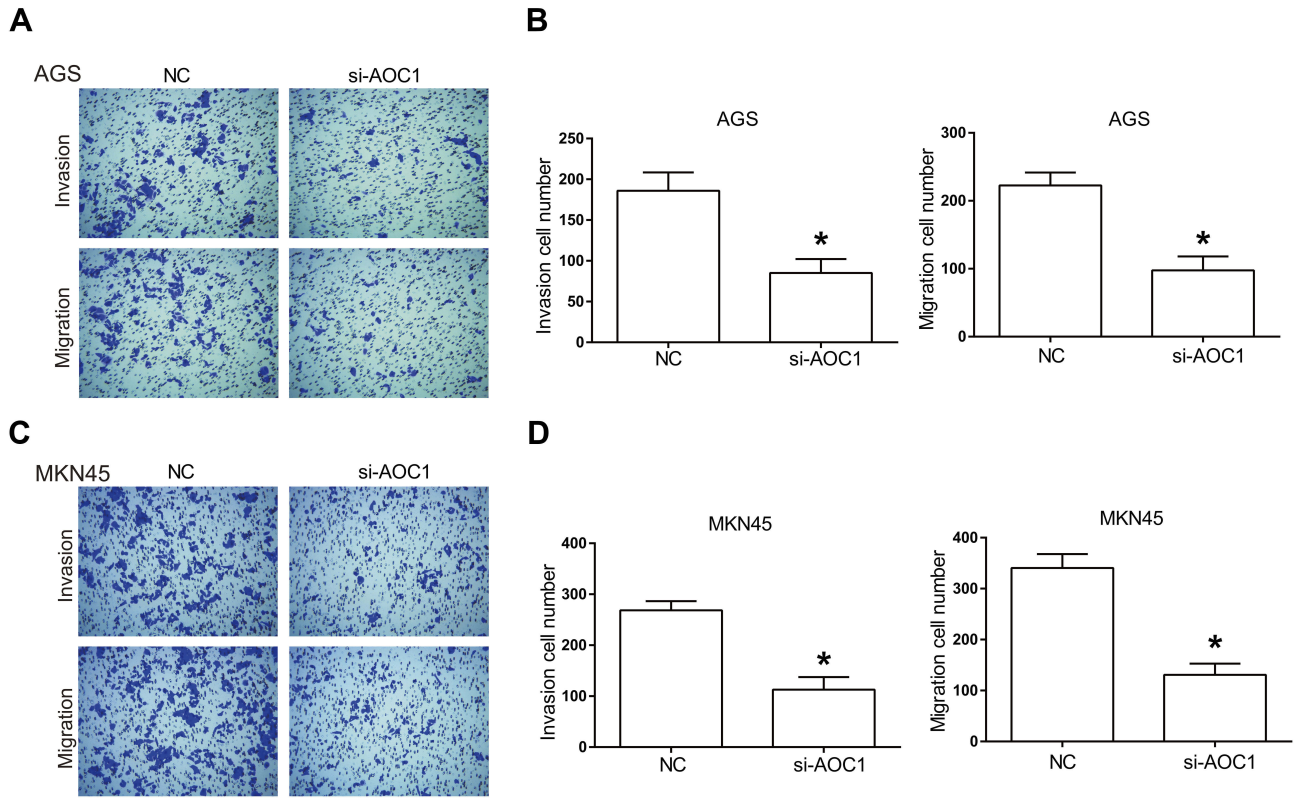

Figure 4 Knockdown of AOC1 inhibits cell invasion and migration in human gastric cancer cells. (**A**) and (**C**) Transwell assays detecting the invasion and migration of human AGS and MKN45 cells. (**B**) and (**D**) Invaded and migrated cells in si-AOC1 group or NC group were counted. All experiments were performed for three repeated times. *P<0.05.

AOC1 silenced human gastric cancer cells. CCK-8 assay indicated that cell viability was significantly decreased by si-AOC1 transfection in both AGS and MKN45 cells (Figure 3A and B). Clone formation assay suggested that knockdown of AOC1 led to a significant decrease in the number of colonies in both cell lines (Figure 3C and D). Taken together, these data suggest that knockdown of AOC1 results in inhibited growth of human gastric cancer AGS and MKN45 cells.

Downregulation of AOC1 Inhibits Cell Invasion and Migration in Human Gastric Cancer Cells

To explore whether AOC1 was involved in the invasion and migration of human gastric cancer cells, we performed transwell assays. As shown in Figure 4A and B, knockdown of AOC1 significantly inhibited the invasion and migration capacities of human AGS cells. Moreover, knockdown of AOC1 had the same effect on MKN45 cells (Figure 4C and D). Taken together, these data suggest that AOC1 knockdown is able to negatively regulate cell invasion and migration of human gastric cancer.

Downregulation of AOC1 Promotes Apoptosis of Human Gastric Cancer Cells by Regulating Apoptosis-Related Protein Expression

Induction of cell apoptosis is an important mechanism for inhibition of cell viability. Therefore, we analyzed cell apoptosis in AOC1 silencing human gastric cancer cells using flow cytometry. As shown in Figure 5, AOC1 knockdown is correlated with a significant increase in the percentage of apoptotic cells in AGS and MKN45. Next, we examined the expression of mitochondria apoptotic pathway components, including Bcl2, Bax, Caspase-9 and Caspase-3. As shown in Figure 6A and B, knockdown of AOC1 increased the expression of Bax, Caspase-9, and Caspase-3, while this decreased the expression of Bcl2. Taken together, knockdown of AOC1 increased the apoptosis of human AGS and MKN45 cells by activating the mitochondrial apoptosis pathway.

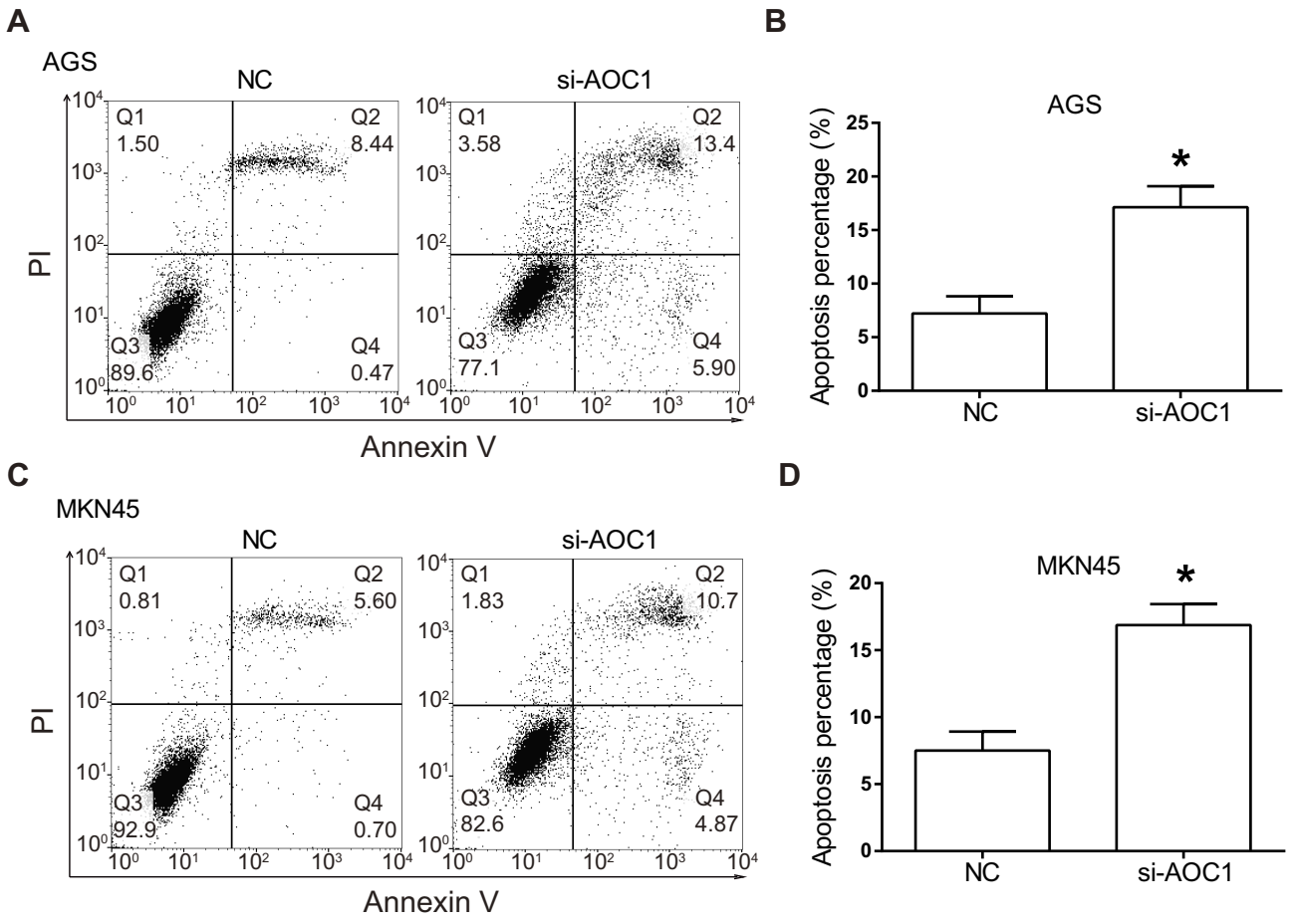

Figure 5 Knockdown of AOC1 induces apoptosis in human gastric cancer cells. (**A**) and (**B**) The effect of AOC1 knockdown on the apoptosis of AGS cells was detected using flow cytometry. (**C**) and (**D**) The effect of AOC1 knockdown on the apoptosis of MKN45 cells was detected using flow cytometry. All experiments were performed in triplicate. *P<0.05.

Downregulation of AOC1 Inhibits Activation of the AKT Signaling Pathway

In order to determine the specific mechanism of AOC1 involvement in human gastric cancer cells, we explored whether knockdown of AOC1 could affect the AKT signaling pathway. In this study, we found that knockdown of AOC1 decreased the phosphorylation levels of AKT and the expression of its downstream effectors, p70S6K and Cyclin D1, in both AGS and MKN45 cells (Figure 6C and D). These results indicate that the AKT signaling pathway was inactivated. To further confirm the effect of AOC1 on the AKT signaling pathway, we treated tAOC1 low expressing cells with IGF-1, an agonist of the AKT pathway. As shown in Figure 7, treatment with IGF-1, the inhibition of cell proliferation, migration, and invasion caused by AOC1 knockdown were arrested, suggesting that AOC1 acted by regulating AKT signaling pathway.

Next, we also investigated the expression of genes involved in epithelial–mesenchymal transition (EMT). Here we found that downregulation of AOC1 significantly increased the expression of E-cadherin (a mediator of epithelial cell-cell adhesions), but decreased the expression of mesenchymal marker N-cadherin (Figure 6E and F). Moreover, the upstream transcriptional factors, SNAIL and Slug, which promoted EMT by repressing the expression of E-cadherin, were also significantly downregulated when AOC1 silencing (Figure 6E and F).

Taken together, these data suggest that AOC1 knockdown inhibits the proliferation and movements of human gastric cancer cells by inducing inactivation of the AKT signaling pathway and regulating EMT-related protein expression.

Discussion

Human gastric cancer remains one of the leading causes of cancer-related deaths worldwide. Great efforts have been made to find potential diagnostic biomarkers or therapeutic targets for gastric cancer. AOC1 is a copper-containing amine oxidase, mainly functioning as a scavenger of

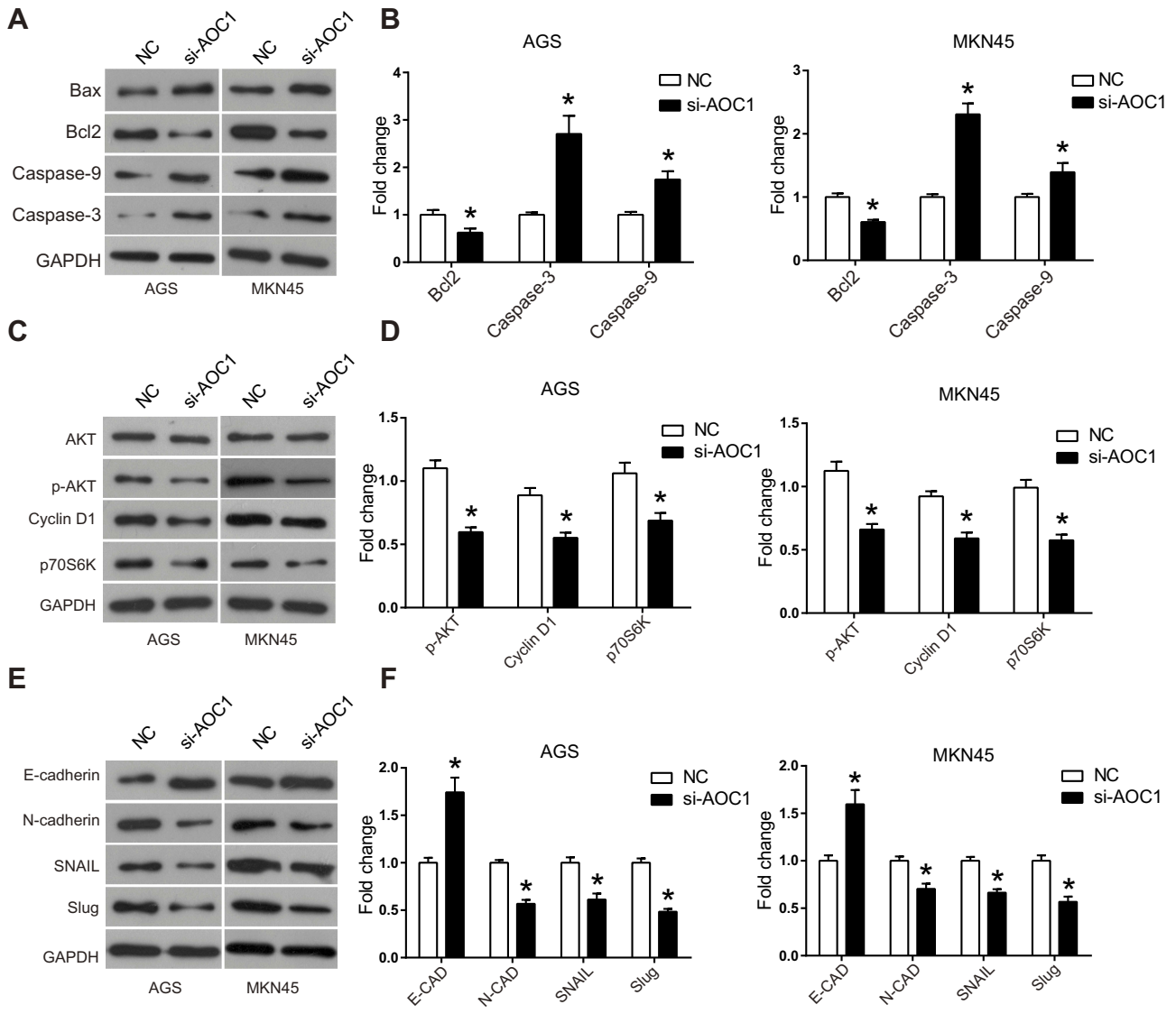

Figure 6 Knockdown of AOC1 induces activation of the mitochondrial apoptosis pathway, and also inhibits the AKT signaling pathway and EMT process. (**A** and **B**) The key members of the mitochondrial apoptosis pathway, including Bax, Bcl2, Caspase-9, and Caspase-3, were detected by Western blot. (**C** and **D**) AKT signaling pathway members, including AKT, p-AKT, Cyclin D1, and p70S6K, were detected by Western blot. (**E** and **F**) EMT-related proteins, including E-cadherin, N-cadherin, SNAIL and Slug, were detected by Western blot. All experiments were performed in triplicate. *P<0.05.

polyamines. The level of polyamines is controlled in a very narrow range in organisms, with levels outside this range causing very serious physiological consequences, such as growth inhibition and apoptosis.[14] Moreover, polyamine metabolism will generate reactive oxygen species, which is cytotoxic, mainly by destroying cell membrane, regulating the permeability of mitochondrial membrane and promoting apoptosis.[8] Therefore, AOC1 may be an important regulator in the physiological and pathological processes by modulating the homeostasis of polyamines. Here for the first time, we identified the function and mechanism of AOC1 in the progression of human gastric cancer.

Firstly, the data from GEPIA suggested that AOC1 was significantly upregulated in human gastric cancer tissues, which implicated that AOC1 might be involved in the tumorigenesis and development of human gastric cancer. Next, we demonstrated that knockdown of AOC1 inhibited cell proliferation, migration, and invasion in human AGS and MKN45 cells. In addition, AOC1 knockdown also induced cell apoptosis by upregulating the Bax/Bcl2 ratio and activating the caspase cascade. Bax is a pro-apoptotic member of the Bcl2 family, which increases the permeability of the mitochondrial outer membrane, inducing the release of cytochrome C and eventually leading to activation of the caspase cascade.[15,16] Bcl2 is an anti-apoptotic member of the Bcl2

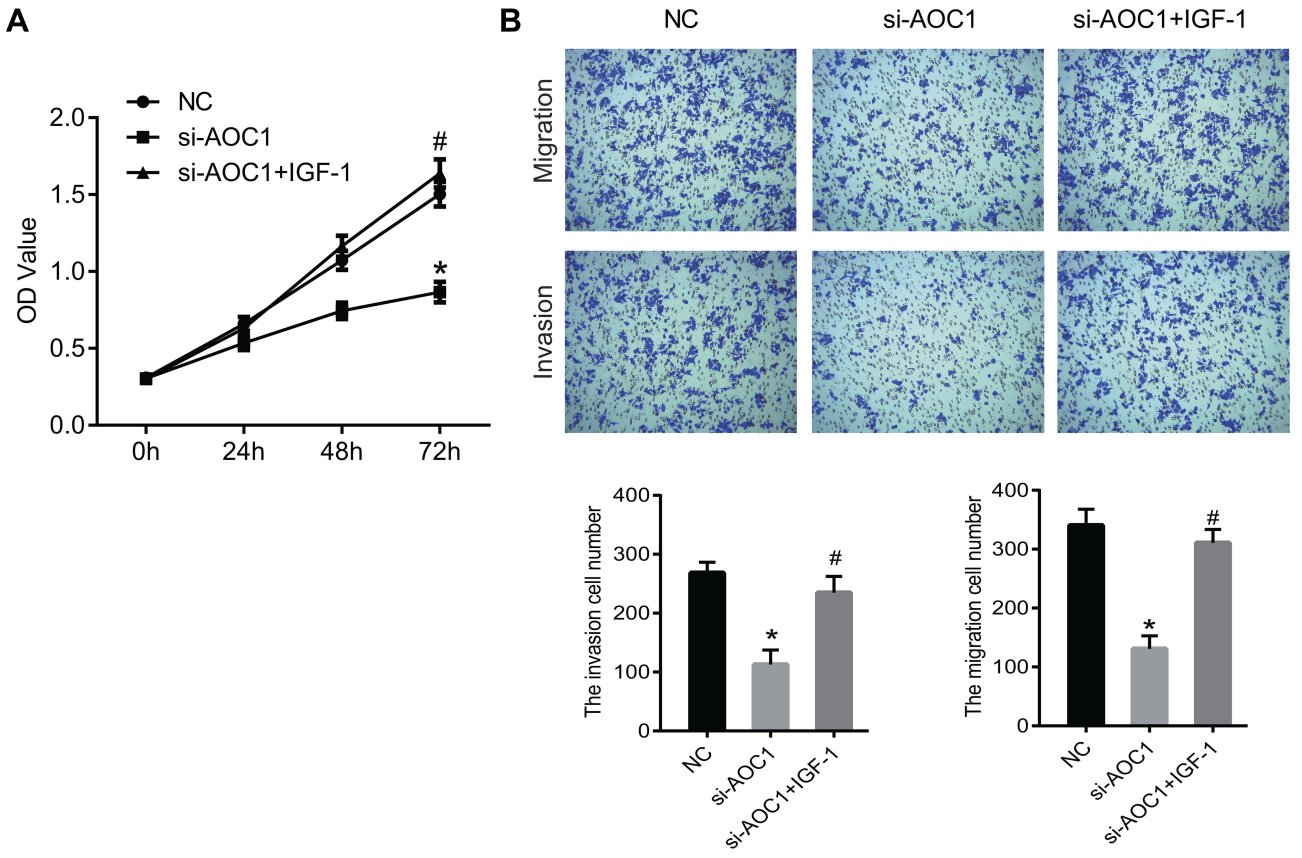

Figure 7 IGF-1 blocked the inhibition of cell proliferation, migration and invasion caused by AOC1 knockdown. AOC1 low expressing cells were treated with IGF-1, an agonist of the AKT pathway. (**A**) CCK8 was performed to detected the proliferation of each group cells. (**B**) Transwell was performed to detected the migration and invasion of each group cells. *P<0.05 vs. NC group; #P<0.05 vs. si-AOC1 group.

family, which prevents the release of cytochrome c by antagonizing the function of Bax.[17,18] These results suggest that AOC1 promotes tumorigenesis and development of human gastric cancer in vitro. Previous studies have found that polyamines are involved in promoting cell apoptosis by increasing mitochondrial permeability and promoting the release of cytochrome c[19] Therefore, we hypothesized that the pro-oncogenic role of AOC1 in human gastric cancer might be mediated by the degradation of polyamines.

Mechanism investigation suggested that knockdown of AOC1 inhibited the AKT signaling pathway, including decreased p-AKT and downstream effectors, p70S6K and Cyclin D1. The AKT signaling pathway is a key signaling pathway in the regulation of numerous cellular functions, such as promoting cellular proliferation, survival, and movement.[20,21] Moreover, phosphorylation-activated AKT will regulate a number of its downstream effectors important in cellular growth, such as p70S6 kinase (p70S6K). This results in enhanced translation of a subset of genes that are required for protein synthesis, as well as acceleration of cell proliferation, which finally lead to tumorigenesis.[22–24] Previous studies report that polyamines are involved in the inhibition of AKT phosphorylation in colorectal cancer cells.[25] In addition, knockdown of AOC1 suppressed the EMT process in human gastric cancer cells. EMT refers to the phenomenon in which epithelial cells lose cell-cell adhesion to acquire mesenchymal phenotypes.[26] EMT frequently initiates tumor metastasis in vivo.[26] Taken together, we identified for the first time the specific mechanism of AOC1 in human gastric cancer, which mainly occurs through regulation of the AKT pathway and EMT-related protein expression.

Conclusions

Our results suggest that downregulation of AOC1 inhibits the proliferation, invasion, and migration of human gastric cancer cells by suppressing the AKT signaling pathway and EMT process, which provides a potential therapeutic target for gastric cancer.

Data Sharing Statement

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

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Disclosure

The authors declare they have no competing interests in this work.

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