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Original Article

Up-regulated AZGP1 promotes proliferation, migration and invasion in colorectal cancer

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Abstract: Zinc-α-2-glycoprotein (AZGP1) is a multi-function protein, which has been detected in different malignancies. But the expression and function of AZGP1 in colorectal cancer is not clear. In this study, we explored the expression and function of AZGP1 in colorectal cancer. We analyzed the Cancer Genome Atlas (TCGA) and found that AZGP1 gene in colorectal cancer was upregulated at the transcriptional level. Real-time quantitative PCR (qPCR) was performed to evaluate AZGP1 expression level in colorectal cancer specimens and normal mucosa specimens. The association between AZGP1 expression and clinicopathological factors was analyzed. We found that AZGP1 expression was positively associated with AJCC clinical stage, tumor invasion, and lymph node metastasis. In order to demonstrate the function of AZGP1, AZGP1-downregulation shRNA was constructed and subsequently stably transfected into HTC116 cells. We found that proliferation, migration and invasion were inhibited in AZGA1-downregulation HCT116 cell line, which is consistent with cell cycle blocked. Meanwhile, knockdown of AZGP1 promoted E-cadherin expression, which may induce invasion and migration. In conclusion, AZGP1 was an oncogene in colorectal cancer.

Keywords: Colorectal cancer, AZGP1, HCT116

Introduction

Colorectal cancer is one of the most common cancers diagnosed among human and the fourth most common cancer cause of death globally [1]. It is rapidly that previously low-risk countries in east Asia increase, and one of important reasons is western lifestyle [1]. Therefore, it is urgent to explore carcinogenesis of colorectal cancer and to explore good measures to diagnose new patients and predict prognosis.

Zinc-α2-glycoprotein (AZGP1) was first found in human serum and subsequently purified in 1961 [2]. AZGP1 is a 40-kDa secreted protein, whose coding gene is located at chromosome 7q22 [3]. It has been found that AZGP1 is expressed in many normal organs including liver, kidney, epidermal [4]. The expression of AZGP1 is significantly increased in carcinomas [5]. The exact mechanism by which AZGP1 anticipates in tumor progression is not clearly known. It has been reported that AZGP1 could regulate fertilization, immunoregulation, cell adhesion or RNase activity [4]. Besides some researcher found the AZGP1 can be a potential biomarker in different types of carcinomas. Brooks JD found that absent or weak expression of AZGP1 protein is associated with recurrence free survival in patients after radical prostatectomy [6]. And expression of AZGP1 affecting outcomes in patients was reported in gastric cancer, breast carcinoma ovarian serum carcinoma [7-9]. However, the relation between AZGP1 and colorectal cancer is not exactly clear.

In order to clarify the effect of AZGP1 in colorectal cancer, our study examined AZGP1 expression in colorectal cancer specimens and normal mucosa specimens by using qPCR. We also used TCGA database to verify our clinical specimen data. Besides, we characterize the function of AZGP1 in colorectal cancer cell line.

Materials and methods

Patients and colorectal tissue samples

All patient-derived specimens and patients' information were collected and archived under

protocols approved by the institutional review board of the First Affiliated Hospital of Wenzhou Medical University. A written informed consent was obtained from each subject before the trials. For qPCR and WB analysis, tissue samples were gathered from 89 patients who had recently undergone colectomy. There are 30 pair tissue specimens including tumor and normal tissue, the other 59 specimens are only tumor specimens. These 119 fresh tissues were subpackaged, immediately frozen in liquid nitrogen, and subsequently stored at -80°C.

Colorectal cancer mRNA expression data were downloaded from the TCGA data portal (https://tcga-data.nci.nih.gov/tcga/). Gene expression data were available for 288 colon cancer samples compared to 41 normal samples and 95 rectal cancer samples compared to 9 normal samples.

Quantitative real-time polymerase chain reaction

Total RNA was isolated according to the manufacturer's instructions (TRIzol, Invitrogen, Carlsbad, CA, USA). RNA samples were stored at -80°C, After they were resuspended in 40 mL of RNase free H_2O. The purity of the isolated RNA was measured at 260/280 nm by spectrophotometry (Thermo, San Jose, CA, USA). The first strand of complementary DNA (cDNA) was synthesized according to the manufacturer's instructions by the Hiscript 1st strand cDNA synthesis kit (Vazyme, Nanjing, China): a 20 μL reaction volume consisting of a 1 μg of extracted RNA, and 20 ml of RNase free ddH_2O, 10 mM 2×RT mix (containing dNTP), 2 μL (200 U) HiScipt enzyme mix (containing HiScipt reverse transcriptase and RNase inhibitor), 50 μM oligo (dT) 18 primers. These components were added Nuclease-free water to a 200 μL PCR tube. Amplifications of all transcripts were finished using PCR system (Eppendorf, Hamburg, Germany) under the following conditions from instructions: 50°C for 45 min and 85°C for 5 min. Subsequently, 5 μL complementary DNA (cDNA) was amplified in a reaction mixture which contains 25 μL 2× Phanta Master Mix [10 U of Taq DNA polymerase, 200 mM Tris-HCl (pH 8.3), 4 μL of 2.5 mM dNTP mix, 50 mM $MgCl_2$], and 4 μL of primer (AZGP1 Forward: GAGAATAACAGAAGCAGCGGAGCAT Reverse: ATTGTGAAGAACATCTCCCCGTAAC; E-cadherin Forward: AACGCATTGCCACATACA Reverse: CGGGCTTGTTGTCATTC; GADPH Forward: GTCTCCTCTGACTTCAACAGCG and Reverse: ACCACCCTGTTGCTGTAGCCAA.), and 17 μL H_2O for a final volume of 50 μL. qPCR was performed under the following cycling conditions: initial denaturation of 5 min at 94°C, followed by 40 cycles of denaturation at 94°C for 45 s, annealing at 60°C for 50 s, 90 s for elongation at 72°C, and a final extension at 72°C for 10 min. All qPCR products were resolved in a 2% (w/v) agarose gel with ethidium bromide (0.5 mg/mL). Then these products were subjected to electrophoresis, and the results were analyzed using a gel imaging and analysis system (Peiqing JS-780, Shanghai, China).

Cell culture and transfection

Four human colorectal cancer cell lines (HT-29, RKO, HCT116, and LoVo) were used in this study. These cells were obtained from Shanghai Cell Biology, Institute of the Chinese Academy of Sciences (Shanghai, China). HT-29, RKO, HCT116, and LoVo cells were cultured in RPMI-1640 medium supplemented with 100 units/mL penicillin, 100 mg/mL streptomycin, and 10% fetal bovine serum (Invitrogen, Gaithersburg, MD, USA). These cells were incubated in a standard cell culture incubator (Thermo, Waltham, MA, USA) at 37°C with 5% CO_2.

The expression of AZGP1 was detected by qPCR and Western blot analysis in order to choose target cells whose expression of AZGP1 was lower than that of other cells. A recombinant LV-AZGP1-shRNA Lentivirus was synthesized according to the manufacturer's instructions. The target cell was transiently transfected using the X-treme gene Hp DNA Transfection Reagent (Roche, Basel, Switzerland). In order to determine transfection efficiency, green fluorescence was detected using a fluorescence microscope (NIKON, TE2000, Tokyo, Japan), and Puromycin was used to select for stable clones. And we selected the highest transfection efficiency lentivirus by qPCR. In our study, cells were classified into three groups: wild HCT116 cells (mock group, mock), HCT116 cells were transfected by LV-control-shRNA lentivirus (negative control group, NC) and HCT116 cells were transfected by LV-AZGP1-shRNA lentivirus (shRNA group, shRNA1). Protein Isolation and Simon western blot analysis (ProteinSimple, California, USA) Target cells were concentrated and homogenized (IKA, R104, Staufen, Germa-

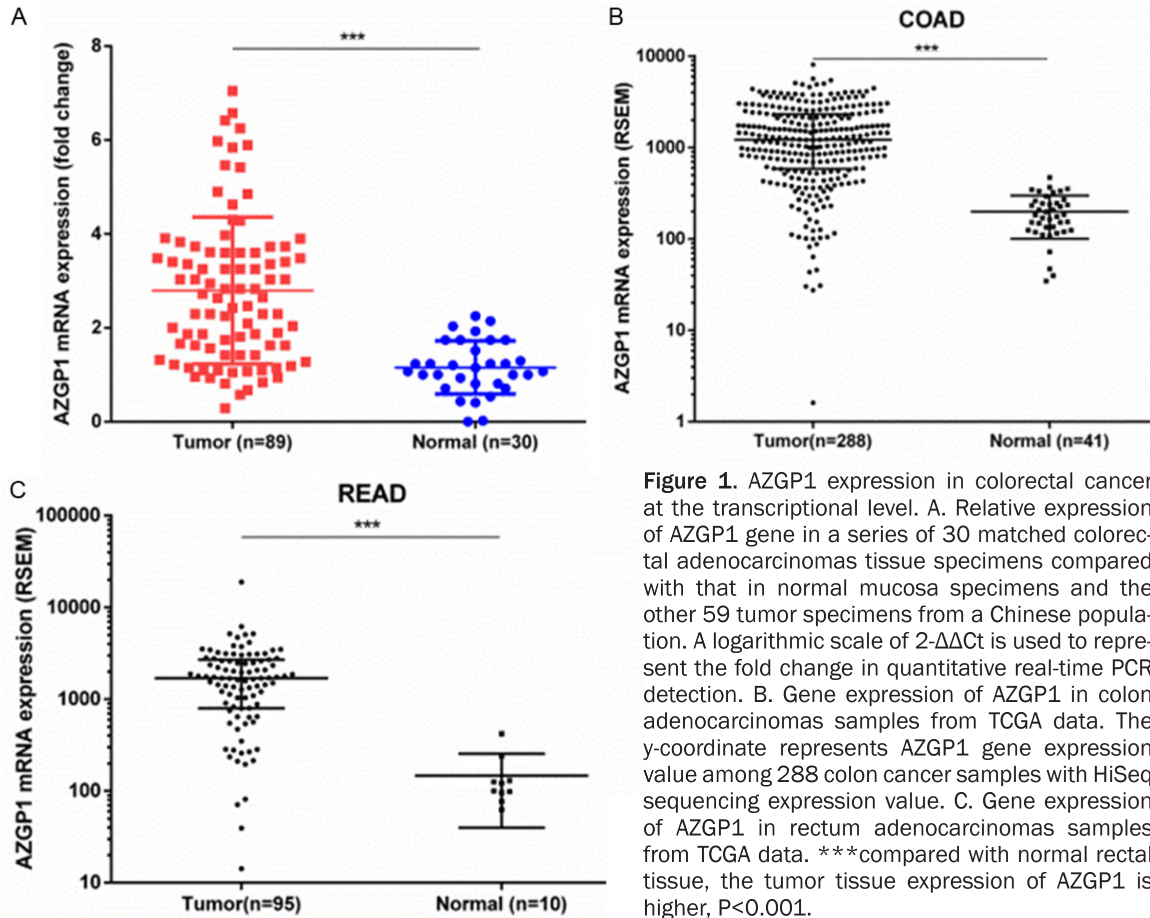

Figure 1. AZGP1 expression in colorectal cancer at the transcriptional level. A. Relative expression of AZGP1 gene in a series of 30 matched colorectal adenocarcinomas tissue specimens compared with that in normal mucosa specimens and the other 59 tumor specimens from a Chinese population. A logarithmic scale of 2-ΔΔCt is used to represent the fold change in quantitative real-time PCR detection. B. Gene expression of AZGP1 in colon adenocarcinomas samples from TCGA data. The y-coordinate represents AZGP1 gene expression value among 288 colon cancer samples with HiSeq sequencing expression value. C. Gene expression of AZGP1 in rectum adenocarcinomas samples from TCGA data. ***compared with normal rectal tissue, the tumor tissue expression of AZGP1 is higher, $P<0.001$.

ny) in 1 mL RIPA lysis buffer with 10 μL PMSF for cell protein extraction. The amount of cellular protein was measured using a bicinchoninic acid assay. In some cases, western blot analysis was performed using a capillary-based automated system (http://www.proteinsimple.com/simon.html). The western blot was performed using the standard manufacturer's protocol using a primary antibody dilution of 1:200 and the secondary antibodies from ProteinSimple were used neat.

Cell proliferation assay

The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to examine the proliferation of shRNA1 transfected HCT116 cells and untransfected HCT-116 cells. All cells were uniformly seeded into 96-well culture plates in a logarithmic growth phase at a concentration of 5 cells/μL in a volume of 150 μL per well. After 48 h, 30 μl of MTT (Kaiji, Nanjing, China) reagent (5 mg/mL) was added into each well, then the plates were incubated for 4 h at 37°C in 5% CO_2. After discarding the mixture, 200 μL dimethylsulfoxide (Sigma, St. Louis, USA) was added and shaken for 10 min to dissolve the MTT. Then the optical density (OD) value was measured by a spectrophotometric plate reader (Thermo, Waltham, MA, USA) at 492 nm. Each group was comprised of six duplicated wells, and the assay was performed three times independently.

Apoptosis detection and cell cycle analysis

An annexin-V-FITC apoptosis detection kit was used (Kaijibio, Nanjing, China) to detect apoptosis. Treated cells were harvested and centrifuged at 1300 rpm for 5 min and resuspended with precooled PBS. Then resuspended cells (5×10^5) were taken out and centrifuged at 1300 rpm for 5 min. After the supernatant was discarded, it was resuspended gently in 200 μL binding buffer, mixed with 10 μL Annexin V-FITC (Kaijibio, Nanjing, China). After staining, the cells were protected from the light for 10 min at room temperature (Annexin V-FITC: red). The

Table 1. Correlation between AZGP1 expression and clinicopathologic characteristics

Case		AZGP1 mRNA expression		P value
		Low expression	High expression	
Gender				
Male	46	23	23	0.913
Female	43	21	22	
Age				
<60	29	14	15	0.879
≥60	60	30	30	
Tumor invasion				
T1-T2	32	21	11	0.022
T3-T4	57	23	34	
Lymph node metastasis				
No	47	32	15	0.001
Yes	42	12	30	
Distant metastasis				
No	67	37	30	0.057
Yes	22	7	15	
Stage				
I-II	45	30	15	0.001
III-IV	44	14	30	
Lesion location				
Colon	62	32	30	0.534
Rectum	27	12	15	
CEA				
<5 ng/μL	32	17	15	0.602
≥5 ng/μL	57	27	30	

data was analyzed immediately by flow cytometer (Guava easyCyte HT, Millipore, USA).

Cell migration and invasion assay

Cells growing were trypsinized at the logarithmic phase, then resuspended in serum-free medium, and seeded into Boyden chambers (8 μm pore size with polycarbonate membrane). Then the chambers were inserted into transwell apparatus (Costar, Cambridge, MA, USA). When it comes to invasion assays, the chambers were needed to coat with Matrigel (BD Biosciences, San Jose, USA). Medium with 10% FBS (500 μl) was added to the lower chamber. After incubating for 48 hours, cells on the top surface of the insert were wiped using cotton swab. Cells that migrated to the bottom surface of the insert were stained by 0.3% crystal violet for 30 min. After staining, membranes were rinsed in PBS and subjected to microscopic inspection. Images of four random fields (10×) were captured from each membrane, and the number of migratory or invasive cells was counted. The migration and invasion results were normalized by cell proliferation under the same treatment conditions. Triplicate assays were used or each experiment.

Statistical analysis

The statistical significance of the differences was determined using Student's t tests. The relationship between AZGP1 expression in tissue specimen and clinicopathological variables was analyzed by chi-square test. $P<0.05$ was considered to be statistically significant. Analyses were performed using the SPSS statistical software program version 23.0 (SPSS Inc., Chicago, IL, USA).

Result

AZGP1 was up-regulated in colorectal cancer

To investigate the role of AZGP1 in colorectal cancer tumorigenesis, the expression levels of AZGP1 were detected in 89 colorectal cancer tissue and 30 adjacent normal tissues by qPCR. AZGP1 expression was significantly higher in colorectal cancer specimens compared to the adjacent normal tissues ($P<0.001$, **Figure 1A**). Further, The Cancer Genome Atlas (TCGA) also showed that AZGP1 was upregulated in colon adenocarcinoma as well as in rectal adenocarcinoma ($P<0.05$, **Figure 1B** and **1C**). Taken together, these results showed that the AZGP1 might be oncogene in colorectal adenocarcinomas. In order to find the correlation between AZGP1 expression and clinicopathologic characteristics, we divided the expression level into two groups and summarized clinical information in qPCR cohort. AZGP1 expression was significantly associated with AJCC clinical stage ($P<0.001$), tumor invasion ($P=0.022$), and lymph node metastasis ($P<0.001$, **Table 1**). However, in our study, AZGP1 expression level is not associated with gender, age, distance metastasis, lesion location and CEA.

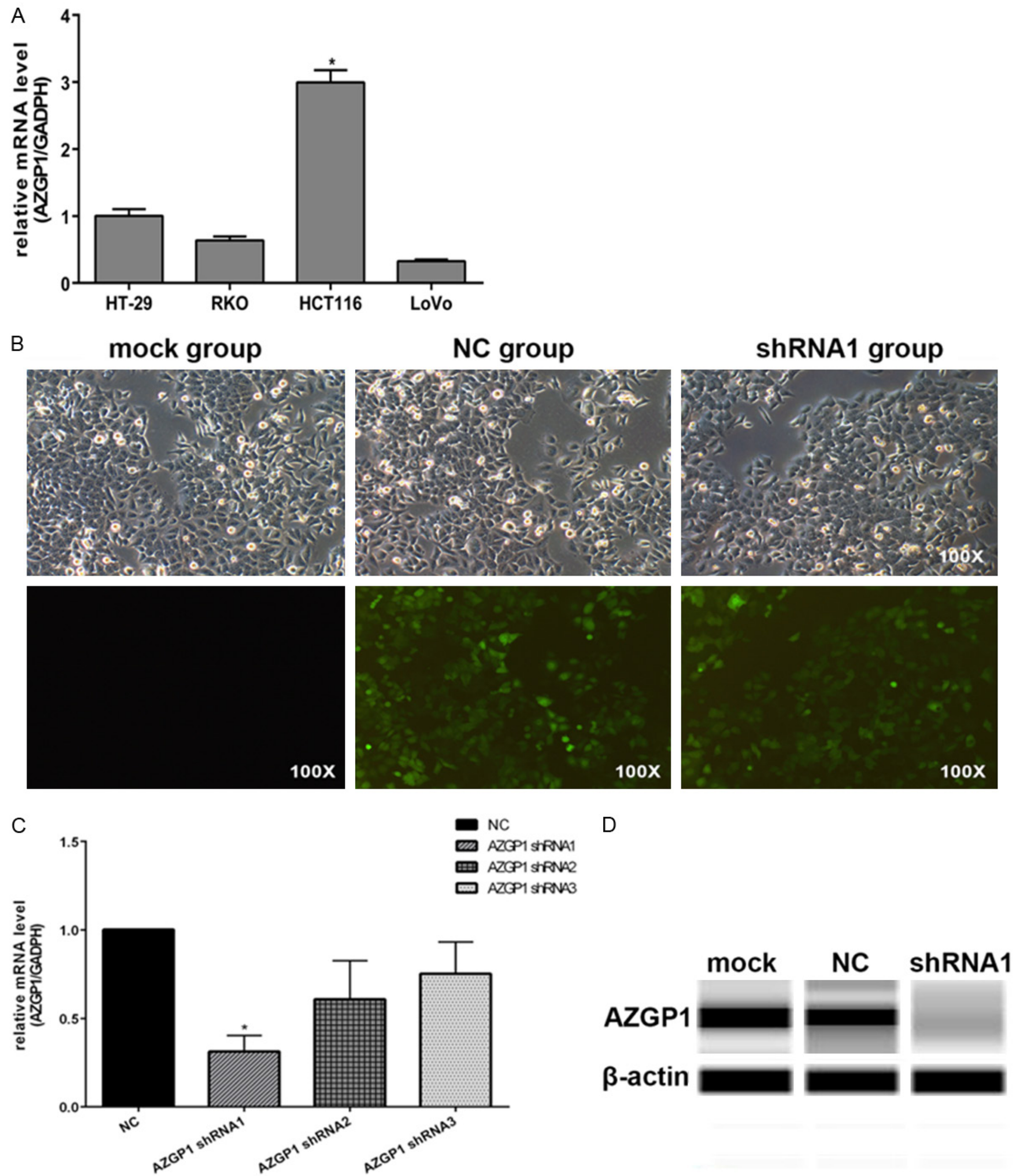

Figure 2. Target cell selection and lentivirus transfection efficiency. A. The relative expression of AZPG1 (compared with the GAPDH gene) using RT-PCR *compared to the other cell lines, HCT116 cells exhibited highest AZGP1 expression $P<0.05$. B. The transfection efficiency of three groups was detected by fluorescence microscope (6200). This picture depicts the mock group without the green fluorescent signal. The control group and the experimental group with have bright green fluorescence signals. C. The relative expression of target genes (compared with the GAPDH gene) in four groups. *compared with other groups. The expression of target genes in shRNA1 group was lower, compared with other groups; $P<0.05$. D. shRNA1-transfected HCT116 cells show lower expression of AZGP1. Blots were generated using a capillary-based western blot automated system (Simon, ProteinSimple).

Cell selection and lentivirus transfection

To evaluate the roles of AZGP1 in colorectal cancer, the expression level of AZGP1 in four human colorectal cancer cells (HT-29, RKO, HCT116, and LoVo) was detected (**Figure 2A**). Compared with HT-29, RKO and LoVo cells, HCT116 had the highest expression of AZGP1

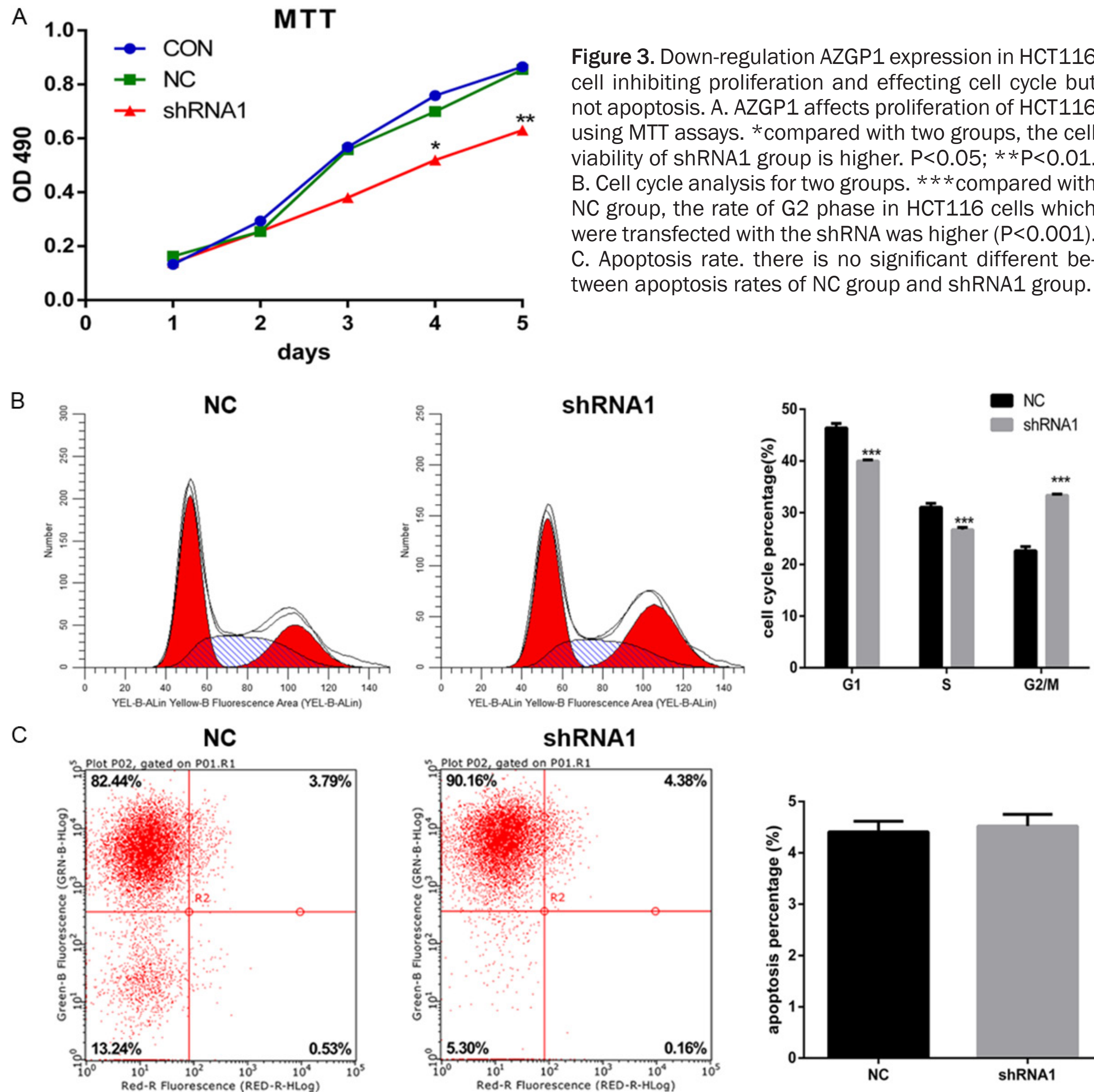

Figure 3. Down-regulation AZGP1 expression in HCT116 cell inhibiting proliferation and effecting cell cycle but not apoptosis. A. AZGP1 affects proliferation of HCT116 using MTT assays. *compared with two groups, the cell viability of shRNA1 group is higher. P<0.05; **P<0.01. B. Cell cycle analysis for two groups. ***compared with NC group, the rate of G2 phase in HCT116 cells which were transfected with the shRNA was higher (P<0.001). C. Apoptosis rate. there is no significant different between apoptosis rates of NC group and shRNA1 group.

(P<0.05). Subsequently, HCT116 cells were selected for further experiments because of being a highest AZGP1 protein expression. Three kinds of the LV-AZGP1-shRNA lentivirus and the LV-control-shRNA lentivirus were constructed in order to find a highest transfection efficiency. All of lentivirus was transfected into HCT116 cells separately. In order to detect the transfection efficiency of the lentivirus, green fluorescent signal was measured by fluorescence microscope after transfection (**Figure 2B**), the expression of AZGP1 gene in four groups was detected using qPCR (**Figure 2C**) and the expression of AZGP1 protein using western blot (**Figure 2D**). The rate of AZGP1 gene/GAPDH gene expression for the negative control (NC), AZGP1 shRNA1, AZGP1 shRNA2, AZGP1 shRNA3 groups were 0.3127±0.1561, 0.6083±0.3773, and 0.7530±0.3116, respectively (**Figure 1C**). Compared with NC groups, the highest transfection efficiency of three kinds of lentivirus is shRNA1 (P<0.05). So we chose the shRNA1 for this study.

Inhibition of AZGP1 blocks the proliferation and cell cycle

MTT assay, apoptosis detection and cell cycle analysis were used to show the proliferation and cell cycle of HCT116 cells. As shown in **Figure 3A**, the OD values at 492 nm were measured over five successive days. The prolifera-

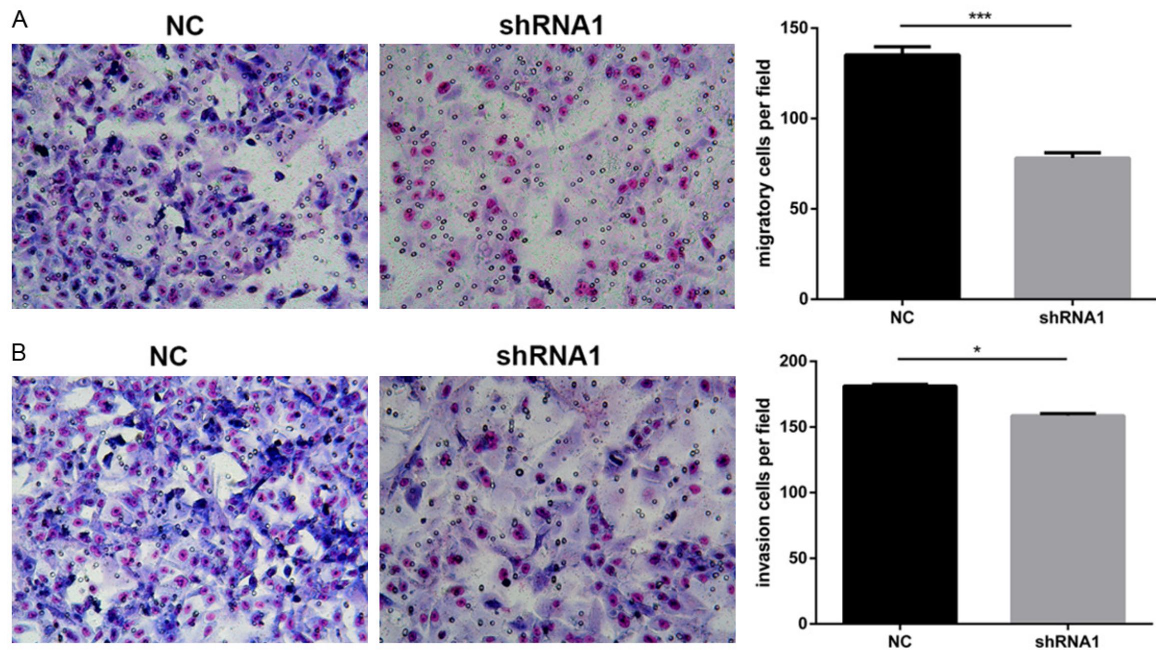

Figure 4. Down-regulation AZGP1 expression in HCT116 cell inhibiting migration and invasion. A, B. Transwell migration and Matrigel invasion assays in down-regulation HCT116 cells and their corresponding control cells. Quantitative results of migration and invasion assays. The stained cells were manually counted from 5 randomly selected fields and normalized with cell proliferation *P<0.05; ***P<0.001.

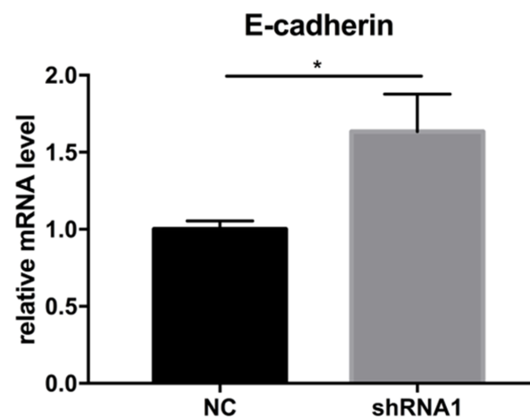

Figure 5. Gene expression of E-cadherin in HCT116 cell line. ***compared with negative control group, expression of E-cadherin in shRNA group is higher, P<0.001.

tion rate of the NC group was significantly lower than that in the other groups at 4th (P<0.05), 5th (P<0.01) day. Cells in which AZGP1 were knocked down showed an increased percentage of cells in the G2 phase (22.61%±0.8277%) when compared with the NC group (**Figure 3B**). However, there was no significant change in the apoptosis rate between the NC and shRNA1 (P>0.05, **Figure 3C**).

Inhibition of AZGP1 inhibits migration and invasion of HCT116

Transwell assays were used to show the function of AZGP1 in HCT116 cells. In the migration assay, cells in which AZGP1 was knocked down migrated less efficiently than the other, its number (78.04±2.959) was also was lower than the NC group (135.1±4.506, **Figure 4A**). In the invasion assay, cells in which AZGP1 was knocked down invaded less efficiently than the other, its number (158.7±1.528) was also was lower than the NC group (181.3±1.264, **Figure 4B**).

AZGP1 inhibits migration and invasion via E-cadherin

Previous study showed that AZGP1 exerted biological function via regulating EMT-related protein expression [10]. E-cadherin is important in tumor progressive and epithelial-mesenchymal transition [11, 12]. In order to explore the potential mechanism of AZGP1 in colorectal adenocarcinomas, we measured the expression level of E-cadherin in HCT116 cell using qPCR. The result showed that cell transfected with shRNA1 had significantly higher E-cadherin ex-

pression (P<0.05, **Figure 5**). Our result demonstrated that AZGP1 could inhibit E-cadherin.

Discussion

In this study, we found the AZGP1 is significant associated with AJCC clinical stage, tumor invasion and lymph node metastasis. We demonstrated that the expression of AZGP1 in patients with colorectal adenocarcinomas is significantly higher than normal tissue. High AZGP1 expression patients were more inclined to have tumor progression and metastasis. Down-regulation AZGP1 inhibits proliferation, migration and invasion in HCT116 cell.

AZGP1 has complicated function, such as lipid metabolism, cell cycle, and cancer progression. In kidney and heart, some researchers demonstrated that AZGP1 is a negative regulator of fibrosis progression, and may have translational effect for treating fibrotic disease [13]. Although AZGP1 plays an important role in carcinogenesis [14], including gastric cancer, prostate cancer, hepatocellular carcinoma and so on [7, 15]. The expression level, even biological function is significantly different in various cancers. For example, in prostate cancer, loss of AZGP1 expression is associated with increased risk of biochemical relapse and prostate cancer-specific death [16]. Besides, the expression AZGP1 in lung tissue was associated with lung cancer status and may be a predictive of genetic predisposition to lung carcinoma in humans [17]. In hepatocellular carcinoma, loss of AZGP1 could reduce cell proliferation and apoptosis, activate survival signals and even promoting invasion [18]. Another study on gastric cancer showed that AZGP1 might act as a novel tumor suppressor and its expression may be a potential prognostic biomarker in gastric carcinogenesis [7].

Although, some researcher confirmed the serum concentration of AZGP1 and found the AZGP1 may be a potential biomarker [19, 20], the potential mechanism of AZGP1 remain unclear in colorectal cancer. In cell line experiment, Knock-down AZGP1 inhibited proliferation, migration and invasion of HCT116. The results show that AZGP1 may act as an important role in carcinogenesis. And the cell behavior of HCT116 is consistent with expression in tumor specimens and clinicopathologic characteristics.

In despite of some progression in therapeutic strategies, metastasis is still the major challenges in the management of CRC [21-23]. The epithelial-mesenchymal transition (EMT) is crucial to the acquisition of metastatic potential in cancer cells. The initial stage of metastatic progression is essentially dependent on EMT, which is characterized by the loss of epithelial marker and the gain of mesenchymal marker as well as a fundamental change in cellular morphology and phenotype with increased ability to migrate [24, 25]. E-cadherin, a cell surface glycoprotein is one the main drivers of morphology and differentiation at the cellular and tissue level [26]. Besides it is crucial protein mediated cell adhesion and tumor migration [27]. Loss of E-cadherin decreases the cellular adhesion, resulting in an increase in cellular motility [28]. It has been reported that down-regulation of E-cadherin in esophageal carcinoma was associated with an increased invasive and metastatic potential [29]. In our study, we observe that knock-down AZGP1 increases E-cadherin expression, which may reduce tumor invasion and metastasis.

Taken together, our study demonstrated that AZGP1 is a potential biomarker to predict patients' prognosis. Our results might provide a better understanding for exploring the exact mechanism of AZGP1 and developing novel biomarkers for the clinical diagnosis of colorectal cancer.

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Disclosure of conflict of interest

None.

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