Neoplasma 2022; 69(5): 1108–1118

doi:10.4149/neo_2022_220415N416

SPAG1 promotes the development of AML by activating the ERK/MAPK signaling pathway and affects the chemotherapy sensitivity of venetoclax

Lu LIU, Jing LIU, Xiao-Jun LIU, Meng-Han LIU, Xue-Feng FENG, Lin YANG*, Jian-Min LUO*

Department of Hematology, The Second Hospital of Hebei Medical University, Key Laboratory of Hematology, Shijiazhuang, Hebei, China

**Correspondence: luojm3155@163.com; ylhbsjz@163.com*

Received April 15, 2022 / Accepted August 5, 2022

Sperm-associated antigen 1 (SPAG1) is considered to be associated with infertility and tumorigenesis. However, its function in acute myeloid leukemia (AML) remains unclear. In this study, we evaluated the expression level of SPAG1 and explored its clinical prognostic value in patients with AML, as well as its biological function in AML cells. SPAG1 is widely expressed in AML patients, resulting in a poor prognosis. However, its expression was not associated with Fms-related receptor tyrosine kinase 3 (FLT3) mutations. Utilizing the RNA interference knockdown tests, we found that SPAG1 could promote the proliferation and survival of AML cells and regulate the expression of structural maintenance of chromosomes protein 3 (SMC3), activating the ERK/MAPK signaling pathway. Furthermore, we discovered that inhibiting SPAG1 impacted AML cell susceptibility to venetoclax. In conclusion, SPAG1 may serve as a potential therapeutic target in AML.

Key words: acute myeloid leukemia, sperm-associated antigen 1, MAPK, venetoclax, biomarker

Acute myeloid leukemia (AML) is the most common acute leukemia in adults, with a 5-year survival rate of only 15–30%. Chemotherapy and allogeneic stem-cell transplantation are the current treatment options for AML [1]. Although many AML patients achieve clinical remission after treatment, a significant portion of these patients, especially high-risk patients, relapse and die as a result of disease progression [2, 3]. Therefore, innovative and reliable biomarkers for AML diagnosis and prognosis are urgently needed.

SPAG1 is a sperm-associated antigen that is located mostly in the neck and midpiece of pachytene primary spermatocytes and has been linked to female infertility [4, 5]. It has been found to be upregulated in pancreatic cancer, and its participation in cellular motility has led to speculation that SPAG1 may be involved in early cancer dissemination [6]. Although bioinformatics studies have shown that high SPAG1 expression in AML is strongly associated with poor prognosis, high risk, and FLT3 and DNMT3A mutations [7], these bioinformatics findings must be validated experimentally to properly characterize the association between SPAG1 and AML. Therefore, our study verified the expression of SPAG1 in AML and explored its functional mechanism in AML.

In AML, the BCL-2 family plays a crucial role. In AML cells, BCL-2 is upregulated, especially in leukemia stem cells [8]. Venetoclax, a selective BCL-2 inhibitor, has been approved for the treatment of chronic lymphocytic leukemia and other hematological malignancies. The FDA approved venetoclax for the treatment of newly diagnosed elderly AML patients in combination with hypomethylating agents or low-dose cytarabine [9, 10]. However, the emergence of resistance following long-term treatment highlights the need for a greater understanding of the underlying mechanisms.

Patients and methods

GEPIA. Gene Expression Profiling Interactive Analysis (GEPIA) [11] is a widely used interactive web server that allows users to analyze gene expression profiles. The Cancer Genome Atlas (TCGA) and the GTEx database were used to create GEPIA, which contains 9,736 tumors and 8,587 normal tissues. We investigated the differential expression of the SPAG1 gene in AML and normal tissues by GEPIA.

GEO. The Gene Expression Omnibus (GEO) is a public data repository for functional genomics. Users can utilize the tools to search for and download experiments as well as curated gene expression profiles. In the GEO database, we looked for the expression of SPAG1 in several datasets.

cBioportal. The cBio Cancer Genomics Portal (cBioportal) is a user-friendly website that enables users to access and analyze cancer data from TCGA [12, 13]. We obtained the clinical data of AML patients via cBioportal.

UALCAN. UALCAN [14] is a cancer database that contains information on gene expression, gene methylation, disease survival, and gene association. In our research, we used UALCAN to look for genes that were co-expressed with SPAG1.

Cytoscape. Cytoscape [15] is a free and open software platform for viewing complex networks and combining them with any type of attribute data. We performed Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses of genes co-expressed with SPAG1 using the ClueGO plug-in.

Specimen collection. We extracted bone marrow mononuclear cells (BM-MNCs) from 79 newly diagnosed and untreated patients with AML from the Department of Hematology, Second Hospital of Hebei Medical University, Shijiazhuang, China. We used BM-MNCs from 26 healthy people as normal controls. Patients with AML were diagnosed by cell morphology, immunology, cytogenetics, and molecular biology. BM-MNCs were isolated using lymphocyte separation solution according to the directions. This study was approved by the Hospital Ethics Committee.

Cell lines. One human nontumor bone cell line, HS-5, and three human leukemia cell lines, Molm-13, K562, and U937, were maintained in our laboratory. MV-11 cells were purchased from the cell bank of the Chinese Academy of Sciences. Molm-13, K562, and U937 cells were cultured in Roswell Park Memorial Institute-1640 medium (RPMI-1640; Gibco), MV4-11 cells were cultured in Iscove's Modified Dulbecco Medium (IMDM), and HS-5 cells were cultured in Dulbecco modified Eagle medium (DMEM; Gibco) containing 10% fetal bovine serum (FBS; Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin. The temperature of the incubating environment was set at 37 °C, with humidified air containing 5% CO_2.

RNA extraction and RT-qPCR. TRIzol (Invitrogen, Carlsbad, CA, USA) was used to isolate total RNA from cells. A reverse transcriptase reaction was carried out using a reverse transcriptase reaction kit (Funeng, Guangzhou, China) according to the manufacturer's instructions. Real-time quantitative PCR was performed using a real-time quantitative PCR kit (Funeng, Guangzhou, China). The primer sets for RT-qPCR were SPAG1-specific primers (Hs-QRP-24686, Funeng, Guangzhou, China), SMC3-specific primers (sense, 5'-GATGAAGGAGAAGGGAGTGGT-3'; antisense, 5'-ATCAGAGCAAGGGCTACCAAG-3'), and GAPDH-specific primers (sense, 5'-CCTCTGACTTCAACA GCGACAC-3'; antisense, 5'-TGGTCCAGGGGTCTTAC-TCC-3').

Western blot analyses. A radioimmunoprecipitation assay (RIPA) buffer was used to extract total proteins from cells. The Bicinchoninic Acid Kit was used to determine protein concentration (Boster Biological Company, Ltd., Wuhan, China). A 10% SDS-PAGE analysis was performed, and 30 μg of protein from each sample was analyzed. The proteins were transferred to polyvinylidene difluoride membranes, which were then blocked with 5% defatted milk. The protein bands were then incubated overnight at 4 °C with the appropriate primary antibodies before being incubated for 1 h with the secondary antibodies. The primary antibodies were SPAG1 (1:600; Signalway Antibody Co Ltd, MD, USA; 31270), Bcl-2 (1:600; Boster Biological Company, Ltd., Wuhan, China; BM0200), Bax (1:600; Boster Biological Company, Ltd., Wuhan, China; A00183), cleaved-Caspase3 (1:1000; Abcam, CA, USA; ab32042), SMC3 (1:600, Boster Biological Company, Ltd., Wuhan, China; PB9746), p-MEK1/2 (1:1000; Cell Signaling Technology, USA; #9121S), MEK1/2 (1:1000; Cell Signaling Technology, USA; #9122S), p-ERK1/2 (1:1000; Cell Signaling Technology, USA; #8544S), ERK (1:1000; Cell Signaling Technology, USA; #9102S), β-actin (1:8,000; Abways Technology, New York, NY, USA; AB0035), and goat-anti-rabbit (1:10,000, Boster Biological Company, Ltd., Wuhan, China). The bands were visualized using the BioSpectrum Imaging System (UVP, LLC, Upland, CA, USA).

siRNA transfection. To mute the expression of SPAG1, small interfering RNAs (SPAG1-siRNA: 5'-GCUAUGAC-CAAAGAUUTT-3') were generated by Anhui General Biosystems Co., Ltd. (Anhui, China). SiRNA transfections were carried out using the Advanced DNA RNA Transfection Reagent (Zeta Life, USA, AD600025) according to the manufacturer's instructions.

Cell growth. Cells were seeded in 96-well plates at a density of 1×10^5 cells/well. They were cultured for 0, 24, 48, 72, and 96 h, in a certain order. Cells were cultured for 1–4 h at 37 °C in 100 μl of culture medium containing 10 μl of Cell Counting Kit-8 (Beibo Biological Reagent Co., Shanghai, China) reagent. At 450 nm, the absorbance was measured using a microplate reader (BioTek, Winooski, VT, USA).

Proliferation assays. For the colony formation assay, 3×10^3 cells were grown for 2 weeks in the methylcellulose medium at 37 °C with 5% CO_2 saturation in each well of 6-well plates. 2 g of methylcellulose, 50 ml of ultrapure water, and 50 ml of 2×RPMI-1640 (Gibco; 31800022) were used to make the methylcellulose medium, which was subsequently supplemented with 20% FBS and 1% antibiotics (100 U/ml streptomycin and 100 mg/ml penicillin). The number of colonies containing more than 50 cells was counted.

Flow cytometry. The cells were collected by centrifugation and washed in PBS. A FITC Annexin V/PI double staining test (BD Biosciences, Franklin Lakes, NJ, USA) was used to determine cell apoptosis. The cells were then incubated in the dark for 5 min. They were analyzed with an FC500 flow cytometer (Beckman Coulter).

Drug treatment. The CCK-8 assay is used to assess venetoclax chemosensitivity. Cells were plated in 96-well plates and treated with venetoclax at concentrations ranging from 0.0625–30 μmol/l for K562 and 0.009–20 μmol/l for U937. To each well was added 10 μl of CCK-8 solution after 48 h of incubation at 37 °C in a 5% CO_2 atmosphere. A nonlinear fit of log (inhibitor) vs. normalized response was performed in GraphPad Prism v8.0.2 to obtain the IC50

values. In addition, K562 cells were treated with venetoclax at a concentration of 7 μmol/l, and U937 cells were treated with venetoclax at a concentration of 1 μmol/l. The apoptosis experiments were conducted after 48 h.

Statistical analysis. For statistical analysis, SPSS version 26.0 software was utilized. To compare distinct groups, the Mann-Whitney test, Pearson's χ^2-test, and Fisher's exact test were utilized. The Kaplan-Meier technique with log-rank was used to estimate OS and DFS. A p-value <0.05 was considered statistically significant (*p<0.05, **p<0.01, ***p<0.001). The experiments were repeated at least three times. GraphPad Prism (version 8.0.2) was used to visualize the statistical results.

Results

SPAG1 is highly expressed in AML. To evaluate the expression differences of SPAG1 between AML and normal tissues, we applied the GEPIA web server to analyze the data of RNA sequencing expression. As illustrated in Figure 1A, SPAG1 is highly expressed in AML. Then, using the GEO database, we discovered that SPAG1 expression was higher in myeloid leukemia cell lines KG-1, THP-1, and U937 than in normal controls (Figure 1B) [16]. Since TCGA database contains a sufficient number of samples, we used cBioPortal to obtain SPAG1 expression data as well as clinical data from TCGA dataset to investigate the association between SPAG1 expression and AML clinical and laboratory features. Patients were separated into two groups based on their median SPAG1 expression: high expression (SPAG1 high, n=86) and low expression (SPAG1 low, n=87). We analyzed SPAG1 expression in different risk groups and discovered that it was significantly lower in the low-risk group than in the medium- and high-risk groups (Figure 1C, intermediate vs. favorable: p<0.001; adverse vs. favorable: p<0.001). As shown in Supplementary Table S1, we also discovered that patients with high SPAG1 expression had a considerably greater white blood cell count than individuals with low SPAG1 expression. No significant differences existed between the two groups based on the FAB (Supplementary Figure S1A) categorization and gender, age, blasts in peripheral blood, or blasts in bone marrow (Supplementary Table S1). We analyzed the patients' disease-free survival (DFS) time and overall survival (OS) time using clinical data downloaded from cBioPortal to determine whether SPAG1 influenced AML patient outcomes. The DFS and OS of the SPAG1 high-expression group were shorter than those of the SPAG1 low-expression group, as shown in Figures 1D–E (DFS-median: 7.6 vs. 16.6 months, p=0.016; OS-median: 8.8 vs. 26.8 months, p<0.0001). Furthermore, we discovered that patients with FLT3 or DNMT3A mutations had higher SPAG1 expression than patients without mutations (Supplementary Figure S1B, p<0.0001; Supplementary Figure S1C, p=0.015). No link existed between TP53, NRAS, IDH1, IDH2, NPM1, WT1, RUNX1, KIT, CEBPA, or TET2 mutations and SPAG1 expression (Supplementary Table S2). These results are consistent with those analyzed by Gu [7], which further leads us to believe that the SPAG1 gene may play a potential role in AML.

For further verification, we used 26 benign individuals and 79 newly diagnosed AML samples. To characterize SPAG1 mRNA and protein expression in AML, we performed qRT-PCR and western blot on AML patients and four myeloid leukemia cell lines (MV4-11, Molm-13, U937, and K562), as well as the non-bone marrow tumor cell line HS-5. As shown in Figures 1F, 1H, 1I, and 1J, SPAG1 showed a higher expression level in AML patients and myeloid leukemia cell lines than in non-leukemia samples. Seventy-nine patients were divided into groups based on whether they had the FLT3 mutation to confirm the link between SPAG1 expression and the FLT3 mutation. The FLT3 mutation was found in 31 of the 79 patients and not found in 48. However, there was no statistical difference in this outcome (Figure 1G). Because of the small sample size, we considered whether the test result was insignificant. On the other hand, MV4-11 and Molm-13 are well-known to be FLT3 mutant cell lines, and we discovered that SPAG1 expression in these cell lines was not higher than in the other two FLT3 mutation-negative cell lines, at either the mRNA or protein levels. On appeal, we were unable to establish a link between the SPAG1 and FLT3 mutations (Figures 1H, 1J). As a result, we conclude that SPAG1 is highly expressed in both AML patients and AML cell lines but is unrelated to FLT3 mutations.

SPAG1 promotes the proliferation and survival of AML cells. To further study the role of SPAG1 in the development of AML, we transfected specific siRNA into AML cells, which could significantly inhibit SPAG1 expression (Figure 2A). Using the CCK-8 assay and colony formation assay, we investigated the influence of SPAG1 on the proliferative potential of K562 and U937 cells and discovered that downregulation of SPAG1 dramatically reduced cell proliferation in AML compared to controls (Figures 2B, 2C). Furthermore, inhibiting SPAG1 expression increased the proportion of apoptotic cells (Figure 2D), and the anti-apoptotic protein BCL-2 was downregulated while apoptosis-related proteins were upregulated (Figure 2F). Furthermore, we performed cell cycle assays and discovered that knocking down SPAG1 arrested AML cells in the S phase of the cell cycle (Figure 2E). Collectively, our findings revealed that SPAG1 increases AML cell survival and regulates cell physiological processes *in vitro*.

Predicted functions and pathway enrichment analysis of genes co-expressed with SPAG1 in AML patients. Genes co-expressed with SPAG1 were collected from the UALCAN database to investigate which genes SPAG1 was associated with. Further function enrichment analysis was performed on genes with an absolute value of the correlation coefficient with SPAG1 greater than or equal to 0.3. For the GO and KEGG analyses, Cytoscape software (two tools: ClueGO and CluePedia) was utilized, with only routes with a p-value of less than 0.05 being displayed. Figure 3 depicts the outcomes

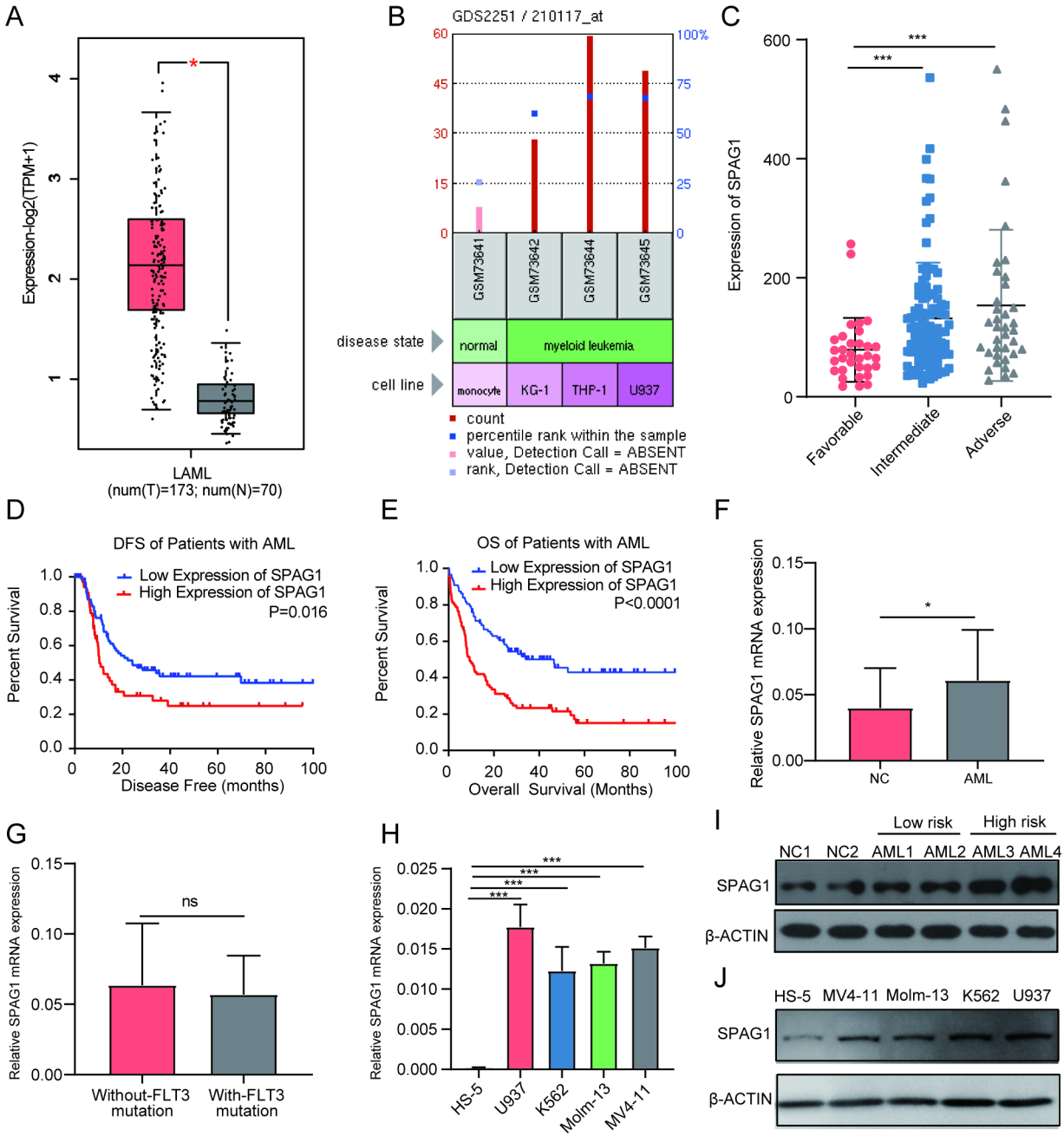


Figure 1. SPAG1 is highly expressed in AML. A) Expression of SPAG1 in AML compared with healthy control in the GEPIA database. B) Expression of SPAG1 in myeloid leukemia cell lines KG-1, THP-1, and U937 compared with normal controls in GDS2251. C) Expression of SPAG1 in AML based on risk status in TCGA database. D) The DFS of patients with AML categorized by SPAG1 median expression. E) The OS of patients with AML categorized by SPAG1 median expression. F) mRNA expression of SPAG1 in AML patients and normal control in our center. G) mRNA expression of SPAG1 in AML patients based on FLT3 mutation status in our center. H) mRNA expression of SPAG1 in myeloid leukemia cell lines. I) SPAG1 protein expression in AML patients and myeloid leukemia cell lines in our center. J) SPAG1 protein expression in myeloid leukemia cell lines.

of the analysis. We discovered that these genes influenced biological processes including the cell cycle process, cellular response to stress, mitotic cell cycle, chromosome organization, mitotic cell cycle process, DNA metabolic process, mitotic cell cycle process, cellular response to DNA damage stimulus, regulation of cell cycle, and DNA repair (Figure 3A). The KEGG pathways for these genes are associated with terms related to the cell cycle, cellular senescence, DNA replication, homologous recombination, non-homologous end-joining, Fanconi anemia pathway, nucleotide excision

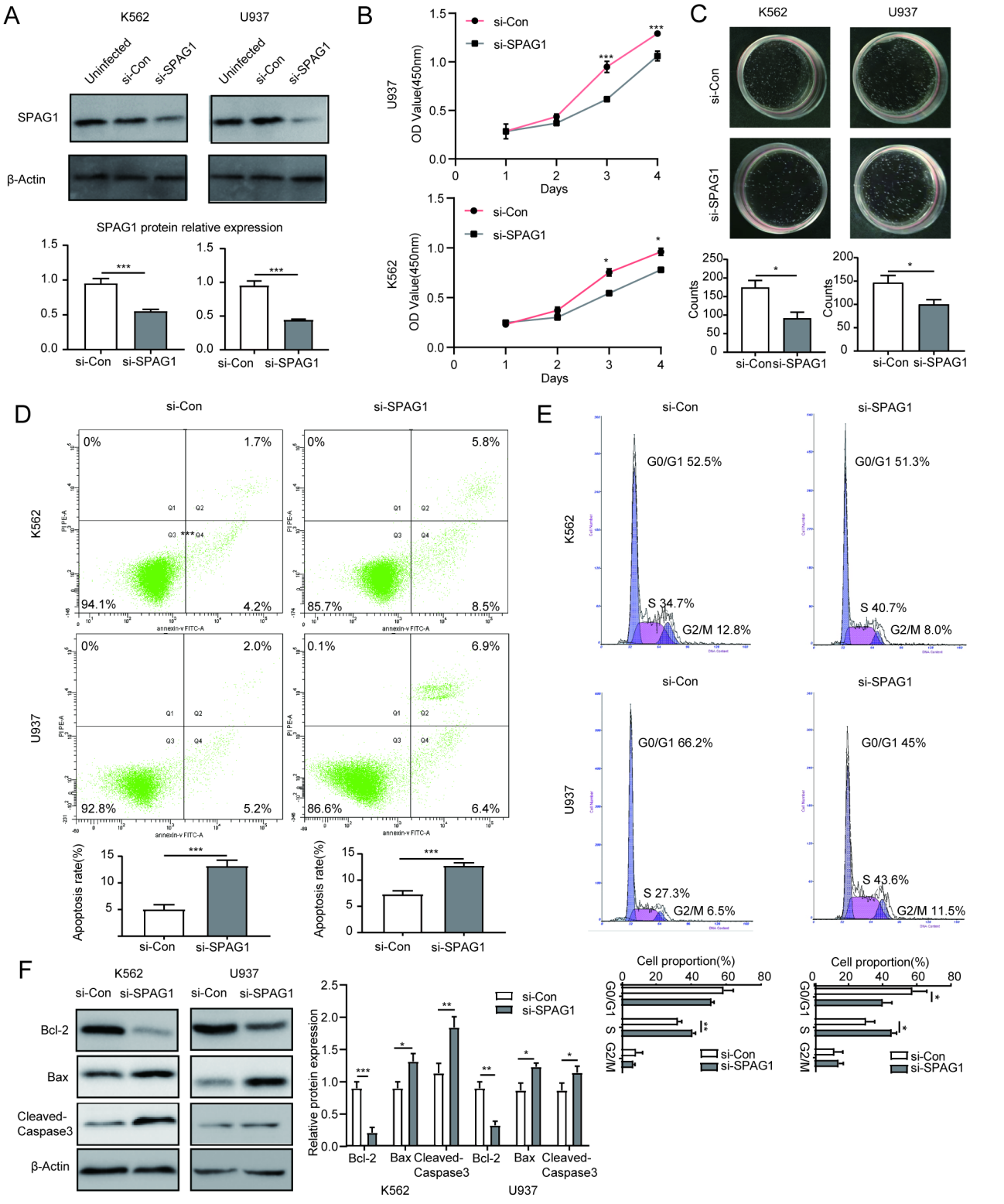


Figure 2. SPAG1 promotes the proliferation and survival of AML cells. A) SPAG1 protein expression in AML cell lines with or without silenced SPAG1. B) Cell proliferation of AML cells with or without SPAG1 knockdown was measured by the CCK-8 assay. C) Colony formation assay of AML cells transfected with siRNA targeting SPAG1 or control. D) The apoptosis levels of AML cells with or without SPAG1 knockdown were determined by flow cytometry. E) The cell cycle of AML cells with or without SPAG1 knockdown was detected by flow cytometry. F) Western blotting analysis was used to determine the expression of apoptosis-related proteins.

repair, and mismatch repair (Figure 3B). Figure 3C shows the network of KEGG-enriched terms colored by different clusters. According to these findings, a significant correlation exists between SPAG1 and genes such as proliferating cell nuclear antigen (PCNA) and chromosome structure maintenance protein 3 (SMC3), which supports our experimental finding that SPAG1 plays an essential role in the cell cycle and various important biological processes.

SPAG1 regulates the expression of SMC3 and ERK MAPK pathway. Based on the enrichment results of genes co-expressed with SPAG1, we discovered that SPAG1 and SMC3 were co-expressed and enriched in the cell cycle, an important pathway. We retrieved the relationship between SPAG1 and SMC3 in GEPIA, as shown in Figure 4A. SPAG1 and SMC3 were discovered to have a positive association. Because SMC3 is a component of mucins, it plays a critical function in DNA recombination, heals DNA damage, and is involved in a variety of cancers [17–19]. As a result, we looked for SMC3 expression in AML by using RT-qPCR and WB and discovered that it was significantly expressed in AML patients (Figures 4B, 4D) and cell lines (Figures 4C, 4E). Next, to confirm the link between SPAG1 and SMC3, we measured SMC3 expression after interfering with SPAG1 expression and discovered that when SPAG1 was knocked down, the mRNA and protein expression of SMC3 were reduced compared to the control group (Figure 4F, 4G). These findings suggest that SPAG1 could influence SMC3 expression, but the particular mechanism needs to be investigated further.

The mitogen-activated protein kinase (MAPK) signaling pathway is an important intracellular signaling pathway with a three-stage kinase pattern at its core. These are widely used in clinical disease mechanism research because they partici-

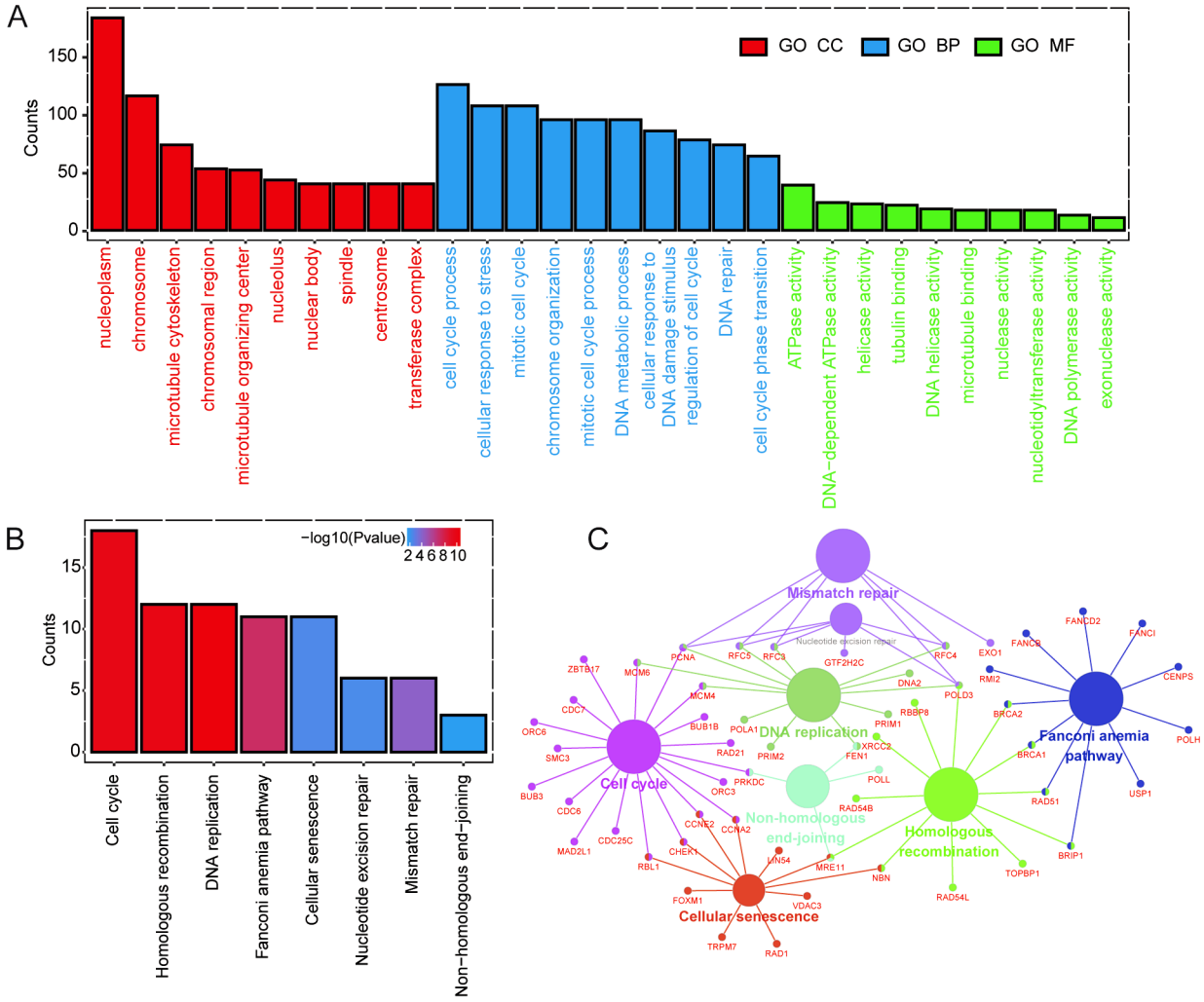


Figure 3. The functions of genes correlated with SPAG1. A) Gene Ontology–enriched terms of genes correlated with SPAG1 including BP, CC, and MF terms. (BP: Biological Process, MF: Molecular Function, CC: Cellular Component) B) KEGG-enriched terms of genes correlated with SPAG1. C) Network of KEGG-enriched terms of correlated with SPAG1 colored by different clusters.

pate in cell growth, differentiation, apoptotic metabolism, and environmental adaptation. However, previous research has shown that SPAG1 can play a role in meiosis execution by participating in the AMPK and MAPK signaling pathways [20]. We measured the phosphorylation levels of MEK and ERK in K562 and U937 cells to fully understand the effect of SPAG1 on the MAPK pathway of AML cells and discovered that knocking down the expression of SPAG1 could reduce the phosphorylation of MEK and ERK (Figure 4H). The findings suggest that SPAG1 plays a critical role in AML by regulating SMC3 expression and influencing the phosphorylation of the ERK/MAPK pathway.

SPAG1 knockdown increases the sensitivity of venetoclax in AML cells. We previously discovered that high SPAG1 expression was linked to a poor prognosis in AML and that knocking down SPAG1 expression in AML cells could influence proliferation, the cell cycle, and the production of the anti-apoptotic protein BCL-2. In recent years, a BCL-2 inhibitor has been approved for intravenous injection in patients with AML with a poor prognosis who are not candidates for aggressive induction chemotherapy [21, 22]. Our two selected cell lines, K562 and U937, were insensitive to the BCL-2 inhibitor venetoclax, so we attempted to observe whether the sensitivity of AML cells to venetoclax changed after interfering with SPAG1. We found that after interfering with SPAG1 expression, the IC50 of AML cells for venetoclax was reduced (Figure 5A). In addition, the proportion of apoptotic cells in the knockdown SPAG1 group was significantly higher than that in the control group when treated with the same concentration of venetoclax (Figure 5B). These results demonstrate a delicate relationship between the expression of SPAG1 and the BCL-2 inhibitor venetoclax, and SPAG1 can affect the drug response of AML cells to venetoclax.

Discussion

AML is a type of hematopoietic malignancy with a high degree of heterogeneity, characterized by hematopoietic stem cell clone disorder, which results in abnormal blood

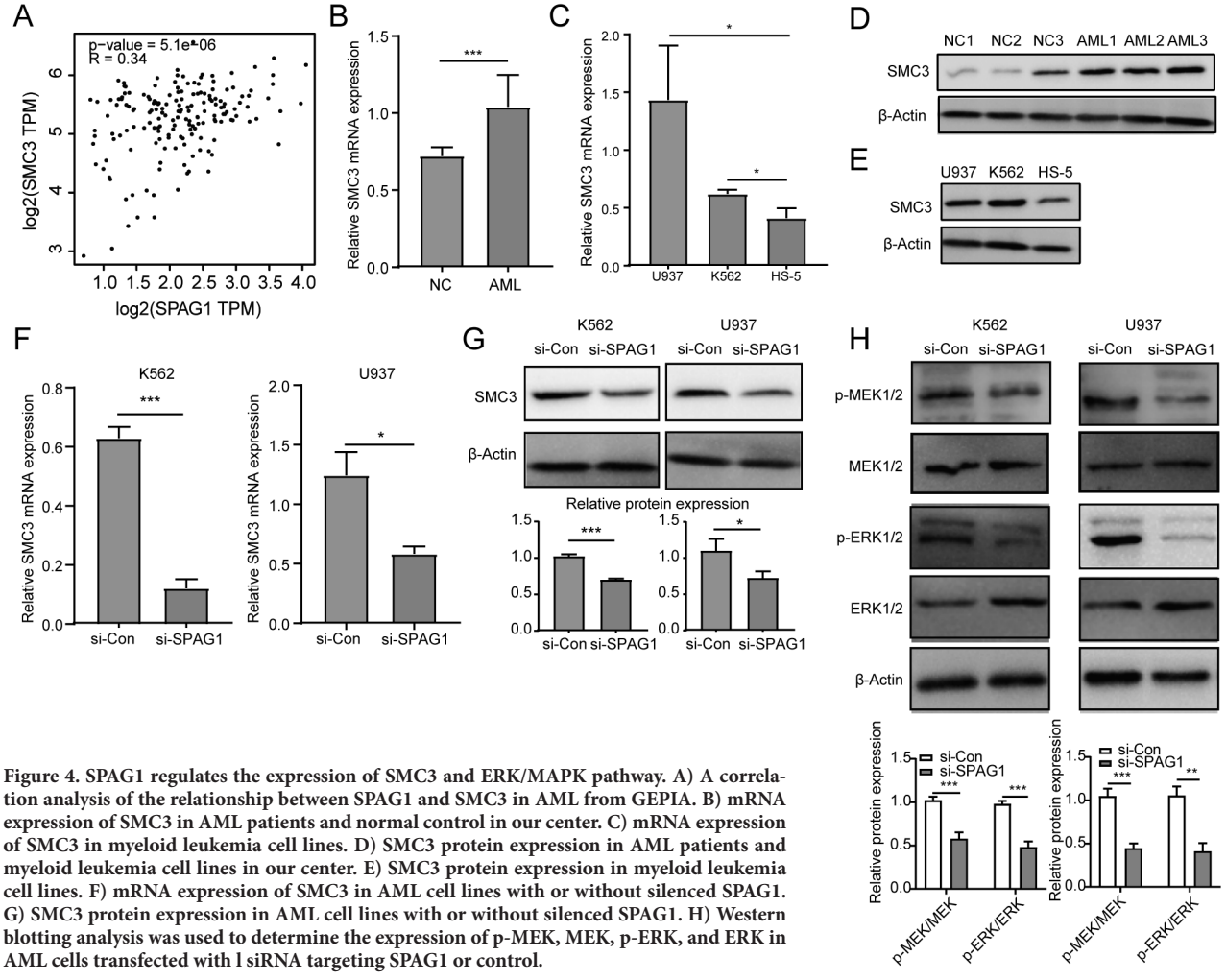


Figure 4. SPAG1 regulates the expression of SMC3 and ERK/MAPK pathway. A) A correlation analysis of the relationship between SPAG1 and SMC3 in AML from GEPIA. B) mRNA expression of SMC3 in AML patients and normal control in our center. C) mRNA expression of SMC3 in myeloid leukemia cell lines. D) SMC3 protein expression in AML patients and myeloid leukemia cell lines in our center. E) SMC3 protein expression in myeloid leukemia cell lines. F) mRNA expression of SMC3 in AML cell lines with or without silenced SPAG1. G) SMC3 protein expression in AML cell lines with or without silenced SPAG1. H) Western blotting analysis was used to determine the expression of p-MEK, MEK, p-ERK, and ERK in AML cells transfected with l siRNA targeting SPAG1 or control.

cell differentiation and maturation. Further research into the detailed molecular mechanisms involved in the development of AML is required.

SPAG1 is a 104-kDa protein on human chromosome 8q22.2 that is encoded by 21 exons. It is a multidomain protein that has a role in G protein-coupled receptor signaling during spermatogenesis and fertilization [4, 23], with mutations linked to primary cilia dyskinesia syndrome [24]. Furthermore, aberrant expression of SPAG1 has been detected in a range of human malignancies. For example, Silia et al. revealed that SPAG1 can be immunogenic and is upregulated notably in lung and breast tumors [25], comparable to prior research. High expression was also found in seminomas and embryonic carcinomas [26]. SPAG1 has previously been identified as a pancreatic cancer progression marker as well as a cell motility factor [27–29]. Previous bioinformatics analyses revealed that SPAG1 was highly expressed in AML, had an effect on AML patients' prognosis, and was associated with FLT3 mutation [7]. Therefore, we not only analyzed the RNA-seq data in TCGA database, but also validated the results using real-time quantitative PCR, WB analysis of AML bone marrow samples, and AML cell lines from our clinical center in this study. We discovered that, as previously reported, SPAG1 was highly expressed in AML; however, our findings did not reveal a link between SPAG1 expression and the FLT3 mutation. This may be because, among AML patients, some had only FLT3 mutations with low allelic ratios, so we performed AML cell line verification. Due to the stable state of the cell line and the mutation rate of FLT3 being 100%, if the expression of SPAG1 is not significantly increased in the FLT3-mutated cell line compared with the FLT3-mutation-negative cell line, we speculate that the expression of SPAG1 may be unrelated to the FLT3 mutation. Therefore, in combination with the previous reports, we have reason to believe that SPAG1 acts as an oncogene in AML cells, but its relationship with the FLT3 mutation deserves further consideration.

According to previous studies, cell motility indicates common biological functions between sperm and cancer cells, and it is a key event in the invasion process, requiring a complex and dynamic interaction between actin and the cytoskeleton. The cell migration ability was decreased after SPAG1 was knocked down. Shamsara observed that the amplification of SPAG1 was associated with decreased survival in patients with prostate cancer [30]. Another finding published by Vadlamudi et al. implicated that SPAG1 is an ESRP1-mediated target gene that can lead to cancer progression through selective splicing [31]. Dermouche et al. reported that SPAG1 can interact with chaperones Hsp70, Hsp90, and ruvbl1/2 [32, 33]. However, few studies exist on the biological function of SPAG1 in AML. In this study, we interfered with SPAG1 expression in AML cell lines and discovered that SPAG1 silencing repressed cell proliferation, accelerated cell apoptosis, and blocked the cell cycle of AML cells. In conjunction with the previous report, SPAG1 regulates the occurrence of spindle morphology, which may also explain the cell cycle arrest caused by SPAG1 knockdown. These findings support our hypothesis that SPAG1

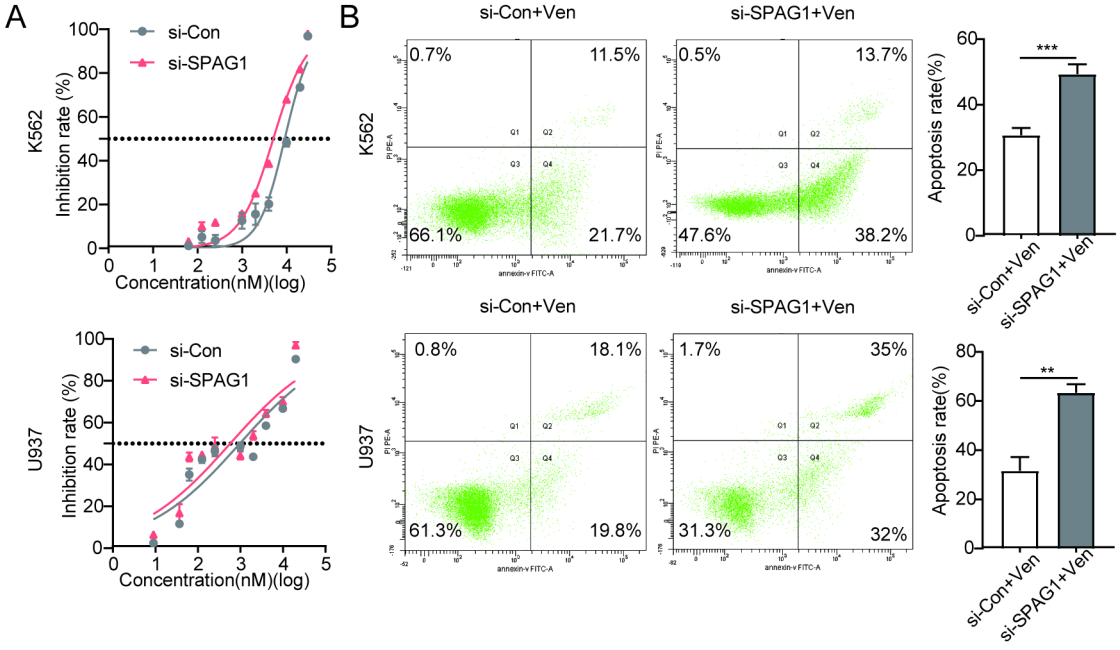


Figure 5. SPAG1 knockdown increases the sensitivity of venetoclax in AML cells. A) IC50 curves of venetoclax in AML cells transfected with siRNA targeting SPAG1 or control. B) Cell apoptosis rate of AML cells transfected with siRNA targeting SPAG1 or control after venetoclax drug treatment. (Ven: Venetoclax.)

regulates the pathophysiological processes of cells in a variety of cancers.

In addition to the possibility of SPAG1 being used as a biomarker for AML, we discovered a potential mechanism by which SPAG1 may play a role in AML. Through dimerization of their hinge domains, the subunits of SMC3 and SMC1A form a V shape. This V is connected with the subunit of double strand-break repair protein Rad21 homolog at the bottom to form a closed three-sided ring structure, which can surround sister chromosomes, mediate the adhesion of sister chromosome adhesion proteins, and play a key role in gene regulation [34, 35]. SMC3 can regulate chromosome structure and thus participate in important biological functions of cancer cells, as well as play an important role in the occurrence and progression of cancer [19, 36]. SMC3 can participate in important biological functions of cancer cells by regulating chromosomal structure, thereby contributing to the occurrence and progression of cancer. Previous studies have found that the expression of SMC3 is increased in AML [37], and we have also verified this result. After discovering a favorable association between SPAG1 and SMC3 by analyzing SPAG1 co-expressed genes, we evaluated whether SPAG1 could affect SMC3 expression by detecting SMC3 expression after knocking down SPAG1. We discovered that SPAG1 could definitely affect SMC3 expression. However, our result does not fully address the mechanism by which SPAG1 regulates SMC3 expression, which requires additional investigation.

The MAPK signaling pathway is linked to the occurrence and progression of leukemia and is involved in functional activities such as apoptosis, drug resistance, autophagy, and leukemia cell differentiation [38, 39]. The abnormal activation or blockade of these pathways has been linked to the development of tumor resistance. AML cells undergo apoptosis when ERK phosphorylation is inhibited [40, 41]. Early research suggested that SPAG1 plays a role in oocyte meiotic execution through its involvement in the AMPK and MAPK signaling pathways [20] and zygote sperm mtDNA degradation [27, 28]. Therefore, we hypothesize that SPAG1 may influence cell proliferation and survival in AML by affecting the MAPK pathway. Our study found SPAG1 to be involved in the phosphorylation of ERK in AML. As a result, our findings confirmed our hypothesis that SPAG1 mediates the activation of the MAPK signaling pathway and thus plays an important role in AML.

Venetoclax, a BCL-2 inhibitor, achieved high response rates when combined with low-dose chemotherapy or hypomethylating agents, even in patients who were previously thought to be unsuitable for standard chemotherapy [42]. In addition, we discovered that knocking out SPAG1 increases venetoclax sensitivity in AML cells. Chen et al. conducted a genome-wide CRISPR/Cas9 loss-of-function screen in the FLT3 gene mutation cell line molm-13 that was cultured in the presence of venetoclax for 8 and 16 days [43]. Since our analysis showed increased expression of SPAG1 in patients with FLT3 gene mutations, this difference was not verified in our small clinical sample. However, when we downloaded its published supplementary file, we discovered that SPAG1 was a negatively selected gene, indicating that it was sensitive to the BCL-2 inhibitor venetoclax treatment, which may be indirectly complementary to our study. More specifically, a subtle relationship exists between SPAG1 expression and the BCL-2 inhibitor venetoclax, and SPAG1 can influence AML cell responses to venetoclax. As a result, these findings may imply that SPAG1 is an important regulator of cell proliferation, which may affect the efficacy of venetoclax chemotherapy.

Our study has limitations. First, more validations from multicenter and high sample size cohorts are required. Second, because our study lacks *in vivo* validation results, this experiment may require additional research into the role and mechanism of SPAG1 in a mouse model of leukemia to better understand the role of SPAG1 in AML.

In conclusion, we have demonstrated the clinical significance of SPAG1 in human AML, indicating that SPAG1 promotes AML progression by regulating the expression of SMC3 and activating the ERK/MAPK signaling pathway. This provides a good idea for further investigation of SPAG1's role in the regulation of leukemia immune mechanisms, as well as making SPAG1 a new target for relevant biomarkers for AML diagnosis and treatment.

Supplementary information is available in the online version of the paper.

Acknowledgments: This work was supported by The Natural Science Foundation of Beijing-Tianjin-Hebei Cooperation Project, China (No.H2019206713).

References

[1] SHORT NJ, RYTTING ME, CORTES JE. Acute myeloid leukaemia. Lancet. 2018; 392: 593–606. https://doi.org/10.1016/S0140-6736(18)31041-9

[2] SHORT NJ, KONOPLEVA M, KADIA TM, BORTHAKUR G, RAVANDI F et al. Advances in the Treatment of Acute Myeloid Leukemia: New Drugs and New Challenges. Cancer Discov 2020; 10: 506–525. https://doi.org/10.1158/2159-8290.CD-19-1011

[3] BULLINGER L, DÖHNER K, DÖHNER H. Genomics of Acute Myeloid Leukemia Diagnosis and Pathways. J Clin Oncol 2017; 35: 934–946. https://doi.org/10.1200/JCO.2016.71.2208

[4] LIN W, ZHOU X, ZHANG M, LI Y, MIAO S et al. Expression and function of the HSD-3.8 gene encoding a testis-specific protein. Mol Hum Reprod 2001; 7: 811–818. https://doi.org/10.1093/molehr/7.9.811

[5] ZHANG ML, WANG LF, MIAO SY, KOIDE SS. Isolation and sequencing of the cDNA encoding the 75-kD human sperm protein related to infertility. Chin Med J (Engl) 1992; 105: 998–1003.

[6] NEESSE A, GANGESWARAN R, LUETTGES J, FEAKINS R, WEEKS ME et al. Sperm-associated antigen 1 is expressed early in pancreatic tumorigenesis and promotes motility of cancer cells. Oncogene 2007; 26: 1533–1545. https://doi.org/10.1038/sj.onc.1209961

[7] GU Y, CHU MQ, XU ZJ, YUAN Q, ZHANG TJ et al. Comprehensive analysis of SPAG1 expression as a prognostic and predictive biomarker in acute myeloid leukemia by integrative bioinformatics and clinical validation. Bmc Med Genomics 2022; 15: 38. https://doi.org/10.1186/s12920-022-01193-0

[8] TIRIBELLI M, MICHELUTTI A, CAVALLIN M, DI GIUSTO S, SIMEONE E et al. BCL-2 Expression in AML Patients over 65 Years: Impact on Outcomes across Different Therapeutic Strategies. J Clin Med 2021; 10: 5096. https://doi.org/10.3390/jcm10215096

[9] POTLURI J, XU T, HONG WJ, MABRY MH. Phase 3, randomized, double-blind, placebo-controlled study of venetoclax combined with azacitidine versus azacitidine in treatment-naïve patients with acute myeloid leukemia. J Clin Oncol 2017; 35: TPS7069–TPS7069. https://doi.org/10.1200/JCO.2017.35.15_suppl.TPS3099

[10] PHAM HA, MILEV S, LI S, ZOU D, HU Y et al. Budget impact of glasdegib in combination with low-dose cytarabine for the treatment of first-line acute myeloid leukemia in the United States. Blood 2019; 134: 5852. https://doi.org/10.1182/blood-2019-122709

[11] TANG Z, LI C, KANG B, GAO G, LI C et al. GEPIA: a web server for cancer and normal gene expression profiling and interactive analyses. Nucleic Acids Res 2017; 45: W98–W102. https://doi.org/10.1093/nar/gkx247

[12] CERAMI E, GAO J, DOGRUSOZ U, GROSS BE, SUMER SO et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discov 2012; 2: 401–404. https://doi.org/10.1158/2159-8290.CD-12-0095

[13] GAO J, AKSOY BA, DOGRUSOZ U, DRESDNER G, GROSS B et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Sci Signal 2013; 6: l1. https://doi.org/10.1126/scisignal.2004088

[14] CHANDRASHEKAR DS, BASHEL B, BALASUBRAMANYA S, CREIGHTON CJ, PONCE-RODRIGUEZ I et al. UALCAN: A Portal for Facilitating Tumor Subgroup Gene Expression and Survival Analyses. Neoplasia 2017; 19: 649–658. https://doi.org/10.1016/j.neo.2017.05.002

[15] SHANNON P, MARKIEL A, OZIER O, BALIGA NS, WANG JT et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res 2003; 13: 2498–2504. https://doi.org/10.1101/gr.1239303

[16] GEBHARD C, SCHWARZFISCHER L, PHAM TH, SCHILLING E, KLUG M et al. Genome-wide profiling of CpG methylation identifies novel targets of aberrant hypermethylation in myeloid leukemia. Cancer Res 2006; 66: 6118–6128. https://doi.org/10.1158/0008-5472.CAN-06-0376

[17] CHAVDA AP, ANG K, IVANOV D. The torments of the cohesin ring. Nucleus-Phila 2017; 8: 261–267. https://doi.org/10.1080/19491034.2017.1295200

[18] WANG D, WANG L, ZHANG Y, ZHAO Y, CHEN G. Hydrogen gas inhibits lung cancer progression through targeting SMC3. Biomed Pharmacother 2018; 104: 788–797. https://doi.org/10.1016/j.biopha.2018.05.055

[19] GHISELLI G. SMC3 knockdown triggers genomic instability and p53-dependent apoptosis in human and zebrafish cells. Mol Cancer 2006; 5: 52. https://doi.org/10.1186/1476-4598-5-52

[20] HUANG C, WU D, KHAN FA, JIAO X, GUAN K et al. The GTPase SPAG-1 orchestrates meiotic program by dictating meiotic resumption and cytoskeleton architecture in mouse oocytes. Mol Biol Cell 2016; 27: 1776–1785. https://doi.org/10.1091/mbc.E 16-02-0132

[21] POLLYEA DA, PRATZ K, LETAI A, JONAS BA, WEI AH et al. Venetoclax with azacitidine or decitabine in patients with newly diagnosed acute myeloid leukemia: Long term follow-up from a phase 1b study. Am J Hematol 2020; 96: 208–217. https://doi.org/10.1002/ajh.26039

[22] DINARDO CD, MAITI A, RAUSCH CR, PEMMARAJU N, NAQVI K et al. 10-day decitabine with venetoclax for newly diagnosed intensive chemotherapy ineligible, and relapsed or refractory acute myeloid leukaemia: a single-centre, phase 2 trial. Lancet Haematol 2020; 7: E724–E736. https://doi.org/10.1016/S2352-3026(20)30210-6

[23] LIN W, MIAO SY, ZHANG L, WANG LF. [Study on the function of HSD-3.8 gene encoding a testis-specific protein with yeast two-hybrid system]. Zhongguo Yi Xue Ke Xue Yuan Xue Bao 2002; 24: 582–587.

[24] GUAN Y, YANG H, YAO X, XU H, LIU H et al. Clinical and Genetic Spectrum of Children with Primary Ciliary Dyskinesia in China. Chest 2021; 159: 1768–1781. https://doi.org/10.1016/j.chest.2021.02.006

[25] SILIŅA K, ZAYAKIN P, KALNIŅA Z, IVANOVA L, MEISTERE I et al. Sperm-associated antigens as targets for cancer immunotherapy: expression pattern and humoral immune response in cancer patients. J Immunother 2011; 34: 28–44. https://doi.org/10.1097/CJI.0b013e3181fb64fa

[26] BIERMANN K, HEUKAMP LC, STEGER K, ZHOU H, FRANKE FE et al. Genome-wide expression profiling reveals new insights into pathogenesis and progression of testicular germ cell tumors. Cancer Genomics Proteomics 2007; 4: 359–367.

[27] HAYASHIDA K, OMAGARI K, MASUDA J, HAZAMA H, KADOKAWA Y et al. The sperm mitochondria-specific translocator has a key role in maternal mitochondrial inheritance. Cell Biol Int 2005; 29: 472–481. https://doi.org/10.1016/j.cellbi.2004.09.016

[28] HAYASHIDA K, OMAGARI K, MASUDA J, KOHNO S. An integrase of endogenous retrovirus is involved in maternal mitochondrial DNA inheritance of the mouse. Biochem Biophys Res Commun 2008; 366: 206–211. https://doi.org/10.1016/j.bbrc.2007.11.127

[29] SMITH AJ, BUSTAMANTE-MARIN XM, YIN W, SEARS PR, HERRING LE et al. The role of SPAG1 in the assembly of axonemal dyneins in human airway epithelia. J Cell Sci 2022; 135: jcs259512. https://doi.org/10.1242/jcs.259512

[30] SHAMSARA E, SHAMSARA J. Bioinformatics analysis of the genes involved in the extension of prostate cancer to adjacent lymph nodes by supervised and unsupervised machine learning methods: The role of SPAG1 and PLEKHF2. Genomics 2020; 112: 3871–3882. https://doi.org/10.1016/j.ygeno.2020.06.035

[31] VADLAMUDI Y, DEY DK, KANG SC. Emerging Multicancer Regulatory Role of ESRP1: Orchestration of Alternative Splicing to Control EMT. Curr Cancer Drug Targets 2020; 20: 654–665. https://doi.org/10.2174/1568009620666200621153831

[32] DERMOUCHE S, CHAGOT ME, MANIVAL X, QUINTERNET M. Optimizing the First TPR Domain of the Human SPAG1 Protein Provides Insight into the HSP70 and HSP90 Binding Properties. Biochemistry 2021; 60: 2349–2363. https://doi.org/10.1021/acs.biochem.1c00052

[33] CHAGOT ME, DOS SMR, DERMOUCHE S, LEFEBVRE D, MANIVAL X et al. Binding properties of the quaternary assembly protein SPAG1. Biochem J 2019; 476: 1679–1694. https://doi.org/10.1042/BCJ20190198

[34] GLIGORIS TG, SCHEINOST JC, BÜRMANN F, PETELA N, CHAN KL et al. Closing the cohesin ring: structure and function of its Smc3-kleisin interface. Science 2014; 346: 963–967. https://doi.org/10.1126/science.1256917

[35] BECKOUËT F, HU B, ROIG MB, SUTANI T, KOMATA M et al. An Smc3 acetylation cycle is essential for establishment of sister chromatid cohesion. Mol Cell 2010; 39: 689–699. https://doi.org/10.1016/j.molcel.2010.08.008

[36] SHIMIZU K, SHIRATAKI H, HONDA T, MINAMI S, TAKAI Y. Complex formation of SMAP/KAP3, a KIF3A/B ATPase motor-associated protein, with a human chromosome-associated polypeptide. J Biol Chem 1998; 273: 6591–6594. https://doi.org/10.1074/jbc.273.12.6591

[37] KRAFT B, LOMBARD J, KIRSCH M, WUCHTER P, BUGERT P et al. SMC3 protein levels impact on karyotype and outcome in acute myeloid leukemia. Leukemia 2019; 33: 795–799. https://doi.org/10.1038/s41375-018-0287-6

[38] VAN DER ZWET J, BUIJS-GLADDINES J, CORDO' V, DEBETS DO, SMITS WK et al. MAPK-ERK is a central pathway in T-cell acute lymphoblastic leukemia that drives steroid resistance. Leukemia 2021; 35: 3394–3405. https://doi.org/10.1038/s41375-021-01291-5

[39] HAN L, ZHANG Q, DAIL M, SHI C, CAVAZOS A et al. Concomitant targeting of BCL2 with venetoclax and MAPK signaling with cobimetinib in acute myeloid leukemia models. Haematologica 2020; 105: 697–707. https://doi.org/10.3324/haematol.2018.205534

[40] LU J, CHEN S, TAN H, HUANG Z, LI B et al. Eukaryotic initiation factor-2, gamma subunit, suppresses proliferation and regulates the cell cycle via the MAPK/ERK signaling pathway in acute myeloid leukemia. J Cancer Res Clin Oncol 2021; 147: 3157–3168. https://doi.org/10.1007/s00432-021-03712-5

[41] STEELMAN LS, FRANKLIN RA, ABRAMS SL, CHAPPELL W, KEMPF CR et al. Roles of the Ras/Raf/MEK/ERK pathway in leukemia therapy. Leukemia 2011; 25: 1080–1094. https://doi.org/10.1038/leu.2011.66

[42] DINARDO CD, JONAS BA, PULLARKAT V, THIRMAN MJ, GARCIA JS et al. Azacitidine and Venetoclax in Previously Untreated Acute Myeloid Leukemia. New Engl J Med 2020; 383: 617–629. https://doi.org/10.1056/NEJMoa2012971

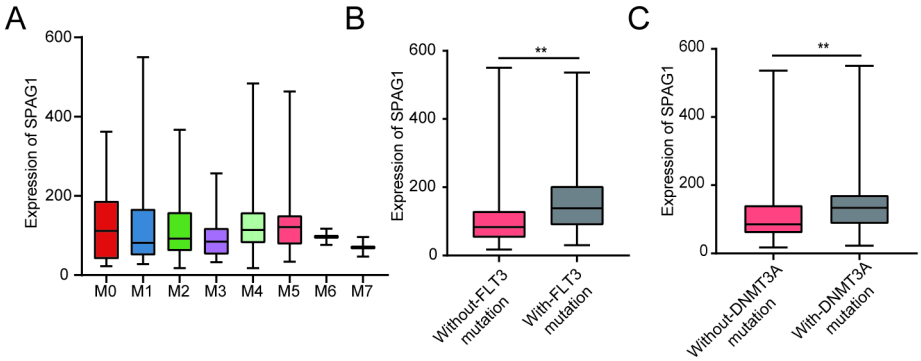
[43] CHEN X, GLYTSOU C, ZHOU H, NARANG S, REYNA DE et al. Targeting Mitochondrial Structure Sensitizes Acute Myeloid Leukemia to Venetoclax Treatment. Cancer Discov 2019; 9: 890–909. https://doi.org/10.1158/2159-8290.CD-19-0117

https://doi.org/10.4149/neo_2022_220415N416

SPAG1 promotes the development of AML by activating the ERK/MAPK signaling pathway and affects the chemotherapy sensitivity of venetoclax

Lu LIU, Jing LIU, Xiao-Jun LIU, Meng-Han LIU, Xue-Feng FENG, Lin YANG*, Jian-Min LUO*

Supplementary Information



Supplementary Figure S1. A) Expression of SPAG1 in AML based on FAB classification in TCGA database. B) Expression of SPAG1 in AML based on FLT3 mutation status in TCGA database. C) Expression of SPAG1 in AML based on DNMT3A mutation status in TCGA database.

Supplementary Table S1. Relationships between SPAG1 mRNA expression and baseline characteristics of patients with AML from cBioportal.

Clinical Characteristics	Total	SPAG1 low (n=87)	SPAG1 high (n=86)	p-value
Age				
<60	91	50 [54.9%]	41 [45.1%]	0.197[a]
>60	82	37 [45.1%]	45 [54.9%]	
Gender				
Male	92	46 [50%]	46 [50%]	0.935[a]
Female	81	41 [50.6%]	40 [49.4%]	
Risk				
Favorable	32	25 [78.1%]	7 [21.9%]	0.002[a]
Intermediate	101	44 [43.6%]	57 [56.4%]	
Adverse	37	16 [43.2%]	21 [56.8%]	
Missing	3	2 [66.7%]	1 [33.3%]	
FAB				
M0	16	7 [43.8%]	9 [56.3%]	0.031[b]
M1	44	27 [61.4%]	17 [38.6%]	
M2	38	21 [55.3%]	17 [44.7%]	
M3	16	11 [68.8%]	5 [31.3%]	
M4	34	12 [35.3%]	22 [64.7%]	
M5	18	5 [27.8%]	13 [72.2%]	
M6	2	1 [50%]	1 [50%]	
M7	3	3 [100%]	0 [0%]	
Missing	2	0 [100%]	2 [100%]	
Lab examinations				
WBC		11 [2.6–45]	27.65 [5.55–68.95]	0.014[c]
PB		41 [8–66.25]	36 [7–67.75]	0.543[c]
BM		71 [49.75–86]	75 [57.35–85.75]	0.375[c]
Disease Free (Months)		16.6 [6.7–40.3]	7.6 [3.575–12.45]	<0.001[c]
Overall Survival (Months)		26.8 [10.5–46.5]	8.8 [4.375–27.025]	<0.001[c]

Notes: [a]Pearson χ^2-test; [b]Fisher exact test; [c]Mann-Whitney test

Supplementary Table S2. The relation of SPAG1 expression and common mutation in AML.

Gene Mutation		Total	SPAG1 low (n=87)	SPAG1 high (n=86)	p-value
FLT3	Mutated	49	14 [28.6%]	35 [71.4%]	<0.001[a]
	Wild-type	124	73 [58.9%]	51 [41.4%]	
TP53	Mutated	14	6 [42.9%]	8 [57.1%]	0.562[a]
	Wild-type	159	81 [50.9%]	78 [49.1%]	
NRAS	Mutated	12	6 [50%]	6 [50%]	0.983[a]
	Wild-type	161	81 [50.3%]	80 [49.7%]	
IDH1	Mutated	16	9 [56.3%]	7 [43.8%]	0.617[a]
	Wild-type	157	78 [49.7%]	79 [50.3%]	
IDH2	Mutated	17	10 [58.8%]	7 [41.2%]	0.459[a]
	Wild-type	156	77 [49.4%]	79 [50.6%]	
NPM1	Mutated	48	20 [41.7%]	28 [58.3%]	0.160[a]
	Wild-type	125	67 [53.6%]	58 [46.4%]	
WT1	Mutated	10	2 [20%]	8 [80%]	0.057[a]
	Wild-type	163	85 [52.1%]	78 [47.9%]	
RUNX1	Mutated	15	8 [53.3%]	7 [46.7%]	0.805[a]
	Wild-type	158	79 [50%]	79 [50%]	
KIT	Mutated	7	6 [85.7%]	1 [14.3%]	0.117[b]
	Wild-type	166	81 [48.8%]	85 [51.2%]	
DNMT3A	Mutated	42	12 [28.6%]	30 [71.4%]	0.001[a]
	Wild-type	131	75 [57.3%]	56 [42.7%]	
CEBPA	Mutated	13	6 [46.2%]	7 [53.8%]	0.757[a]
	Wild-type	160	81 [50.6%]	79 [49.4%]	
TET2	Mutated	15	9 [60%]	6 [40%]	0.431[a]
	Wild-type	158	78 [49.4%]	80 [50.6%]	

Notes: [a]Pearson χ^2-test; [b]Fisher exact test