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Original Article

Kelch-like protein 14 promotes proliferation and migration of ovarian cancer cells

Zheng Chen, Miaoxing Wu, Jiahua Liu

Department of Gynecology, Shengli Clinical Medical College of Fujian Medical University & Fujian Provincial Hospital, Fuzhou, Fujian, China

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Abstract: Kelch-like protein 14 (KLHL14) belongs to the Kelch gene family, which interacts with TorsinA and is associated with dystonia symptoms. However, the effect of KLHL14 on tumorigenesis remains unclear; thus, we aimed to explore the effects of KLHL14 on ovarian cancer cells. By analyzing information regarding ovarian cancer patients obtained from The Cancer Genome Atlas (TCGA)-Ovarian Cancer Database, we found that the KLHL14 gene is highly expressed in ovarian cancer, and patients with high KLHL14 expression had lower survival than those with low expression. qRT-PCR and western blot results revealed that the mRNA and protein levels of KLHL14 in ovarian cancer cells were higher in A-2780 cells than in KGN cells. After constructing cell lines with a knocked down KLHL14 gene, we used the MTT assay, flow cytometry with propidium iodide (PI), Annexin V-FITC/PI, and transwell assay and found that knockdown of KLHL14 gene inhibited proliferation of A-2780 cells, caused cell G0/G1 phase arrest, promoted apoptosis, and inhibited migration and invasiveness. In addition, Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis revealed that KLHL14 may promote development of ovarian cancer by regulating signaling pathways such as mTOR, WNT, and TGF-beta. In short, the KLHL14 gene plays an important role in ovarian cancer development and may be a target for ovarian cancer detection and treatment.

Keywords: Kelch-like protein 14, ovarian cancer, The Cancer Genome Atlas, cell migration, cell invasiveness

Introduction

Ovarian cancer is one of three types of malignant tumors of the female reproductive system [1, 2]. This cancer is characterized by high malignancy, difficult early detection, low survival rate, and high fatality rate [2]. Surgical excision and chemotherapy are commonly used methods of treating ovarian cancer, and with continuous research on the molecular mechanism underlying tumor pathogenesis, gene target therapy has gradually developed as an effective tumor treatment [3]. Currently, several drugs targeting specific molecules have been developed for ovarian cancer, such as the anti-angiogenic compound bevacizumab, the PARP inhibitor olaparib, and the mTOR inhibitor and the anti-α-FR monoclonal antibody farletuzumab [4]. These drugs, either alone or in combination with chemotherapy, serve as possible treatment strategies. In order to develop the optimal treatment strategy, understanding tumor molecular biology and identifying predictive biomarkers is necessary [4].

Kelch-like protein 14 (KLHL14), of the Kelch family, consisting of 42 human-origin genes, has a total of 628 amino acids and generally contains 1 BTB/POZ, 1 BACK, and 5-6 Kelch domains [5]. KLHL14 interacts with torsinA and is closely associated with dystonia symptoms caused by torsinA mutation [6]. In addition, previous reports indicate that the KLHL gene is related to cancer progression [7]. For instance, KLHL6 improves proliferation, migration, and invasiveness of stomach cancer cells, thereby promoting the occurrence and progression of stomach cancer [8]. KLHL20 promotes prostate cancer progression by degrading PML to potentiate HIF-1 [9], whereas downregulation of KLHL19 enhances the transcriptional activity of NRF2 to promote the proliferation of lung cancer cells [10]. KLHL14 is mainly expressed in the spleen and thyroid and is preferentially highly expressed in B cells. Moreover, studies

Table 1. Primer sequences of shKLHL14

Name	sequence (5'-3')
shKLHL14-F1	CCGGCAGCAGAATTCGCTCTAACAACTCGAGTTGTTAGAGCGAATTCTGCTGTTTTT
shKLHL14-R1	AATTAAAAACAGCAGAATTCGCTCTAACAACTCGAGTTGTTAGAGCGAATTCTGCTG
shKLHL14-F2	CCGGTGCTATAACCTAGAAACGAATCTCGAGATTCGTTTCTAGGTTATAGCATTTTT
shKLHL14-R2	AATTAAAAATGCTATAACCTAGAAACGAATCTCGAGATTCGTTTCTAGGTTATAGCA

have found that KLHL14 is methylated in endometrial cancer [11]. Recently, through bioinformatic analysis, researchers have found that KLHL14 is highly expressed in ovarian cancer and endometrial cancer, and that upregulation of KLHL14 is positively correlated with poor prognosis. As such, it is believed that KLHL14 may be a biomarker and treatment target for both ovarian cancer and endometrial cancer, but this idea currently lacks adequate experimental verification [7].

Through *in vitro* experiments and bioinformatics analysis, this study aimed to examine the expression of the KLHL14 gene in ovarian cancer, as well as its functions in ovarian cancer occurrence and development, to provide a theoretical basis for targeting KLHL14 for ovarian cancer treatment and for using KLHL14 as a new pathologic ovarian cancer diagnostic marker.

Materials and methods

Biological information analysis

Transcriptome information of 88 normal and 379 ovarian cancer patients was downloaded from The Cancer Genome Atlas (TCGA) (https://portal.gdc.cancer.gov/). KLHL14 expression data and patient prognoses were further analyzed using the limma software package (version 3.8) of R software (version 3.5.1). Signaling pathways associated with KLHL14 expression in patients with ovarian cancer were investigated through the Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis using GSEA software (http://software.broadinstitute.org/gsea/downloads.jsp), as previously described [12], and the graph was plotted using R software. Subsequently, the relationship between the mRNA levels of the functional molecules associated with signaling pathways in ovarian cancer from TCGA and the mRNA level of KLHL14 were analyzed using GraphPad Prism (version 8).

Cell culture

Human ovarian-cancer cell lines HEY, A2780, SK-OV-3, and COC1, as well as the ovarian granulosa-like cell line KGN, were purchased from the Cell Resource Center of the Shanghai Academy of Biological Sciences, Chinese Academy of Sciences. Cells were cultured in DMEM (Gibco, Waltham, Massachusetts, USA) with 10% fetal bovine serum, 100 U/mL penicillin, and 100 U/mL streptomycin (Gibco) at 37°C.

Construction of stable KLHL14-knockdown cell lines

pLV-sh-puro vectors, which were used to prepare shRNA (shKLHL14) and control (shNC) slow viruses for KLHL14, were purchased from Biosettia. Two groups of small interference RNAs (siRNAs) were designed according to the human KLHL14 genes and were labeled as shKLHL14-1 and shKLHL14-2. The corresponding shKLHL14 primer sequences were designed according to the pLV-sh-puro vector specification (**Table 1**).

To prepare the lentivirus, Lipofectamine 2000 (Invitrogen, Waltham, Massachusetts, USA) was used to transfect 9 μg of shKLHL14 with the suitable packaging plasmids (3 μg of pMD2G and 6 μg of pspax2) into 293T cells. After 48 h of transfection, the medium containing the lentivirus was collected and inoculated into A-2780 and SK-OV-3 cells. After 48 h, the medium was replaced with a fresh one and puromycin was added at a final concentration of 1.0 μg/mL. After 72 h, the cells were collected for KLHL14 expression analysis. The A-2780 and SK-OV-3 cells with the highest knock-down efficiency were used in all studies.

Cell-proliferation experiment

Cells were inoculated in a 96-well plate with 10,000 cells per well, and then 100 μL of complete medium was added. After 24, 48, and 72

h, 20 μL of 5 mg/mL MTT reagent was added, and the cells were incubated at 37°C for 4 h. Afterwards, the culture fluid was carefully suctioned off, and then 150 μL of DMSO was added. The mixture was oscillated for 10 min until the crystals were fully dissolved, and an enzyme-linked immunosorbent monitor was used at 490 nm to analyze the absorbance of each sample.

Cell-cycle and apoptosis analyses

To examine the cell cycle, the cells were digested with trypsin, fixed overnight with 75% ethanol at 4°C, incubated in 0.2% Triton X-100 with 10 μg/mL RNase at 37°C for 30 min, dyed using propidium iodide (PI), and subjected to flow cytometry.

An Annexin V-FITC/PI kit (Vazyme, catalog No.: A211-02, Nanjing, China) was used for apoptosis detection. The cells were stained according to the manufacturer's instructions. Detection was performed using a flow cytometer, and data analysis was conducted using GraphPad Prism 8.

Cell-migration and invasiveness experiments

Transwell experiments were performed to detect cell migration and invasiveness. We inoculated 200 μL of serum-free culture fluid containing 1×10^5 cells into a transwell chamber (Corning, #3422, 8 μm aperture) with or without Matrigel for migration or invasiveness experiments. The chamber was placed in a 24-well plate with 600 μL of 10% fetal bovine serum per well. After 16-24 h of incubation, the cells in the upper chamber were carefully wiped with a cotton swab. The cells in the lower chamber were fixed with ethanol and stained with crystalline purple. The number of cells that migrated or invaded the field of view of six independent microscope images was counted.

RNA extraction and real-time fluorescent quantitation PCR (qRT-PCR)

According to the manufacturer's specifications, total RNA was extracted from the cells using RNA isolater Total RNA Extraction Reagent (Vazyme, catalog No.: R401-01), and HiScript II One Step RT-PCR Kit (Vazyme, catalog No.: P611-01) was used to synthesize the cDNA. qRT-PCR was performed using ChamQ Universal SYBR qPCR Master Mix (Vazyme, catalog No.: Q711-02) on the Bio-Rad CFX96 fluorescence quantitative PCR instrument, and KLHL14 and 18s rRNA primers were synthesized by Xiamen Borui Biotech with the following sequences: KLHL14 (GenBank accession No.: NM_020805; forward primer: 5'-TACTTCACAGACTCCATT-3', reverse primer: 5'-TATTACAAGAGCCAACATC-3'); 18s rRNA (GenBank accession No.: NR_003286; forward primer: 5'-CGACGACCCATTCGAACGTCT-3', reverse primer: 5'-CTCTCCGGAATCGAACCCTGA-3'); 18s rRNAs were used as internal references for KLHL14 analysis, and three replicates were created per sample.

Protein extraction and western blot analysis

The total protein of cultured cells was extracted using RIPA lysis solution containing protease and phosphatase inhibitors and was quantitated using a BCA protein concentration determination kit (Beyotime, Cat. No.: P0012S). Subsequently, western blotting was performed as previously described [13]. The primary antibodies were KLHL14 antibody (Proteintech Group, Cat. No.: 14849-1-AP, 1:2000) and GAPDH antibody (Proteintech Group, Cat. No.: 60004-1-Ig, 1:10000), and the secondary antibodies were HRP-conjugated Goat Anti-Mouse IgG (Proteintech Group, Cat. No.: SA00001-1, 1:10,000) and HRP-conjugated Goat Anti-Rabbit IgG (Proteintech Group, Cat. No.: SA00001-2, 1:10,000). GAPDH was used as an internal reference to correct the detected values.

Statistical analysis

SPSS 19.0 software was used for statistical analysis (SPAA, Chicago, IL, USA). The difference between the two groups was analyzed using the Student's t test, and the difference between multiple groups was analyzed through analysis of variance (ANOVA). Data are presented as the mean ± standard deviation (SD). $P < 0.05$ was considered significant.

Results

KLHL14 gene in ovarian cancer was significantly highly expressed

To explore the relationship between KLHL14 and ovarian cancer, the TCGA-Ovarian Cancer database was analyzed. As shown in **Figure 1A**,

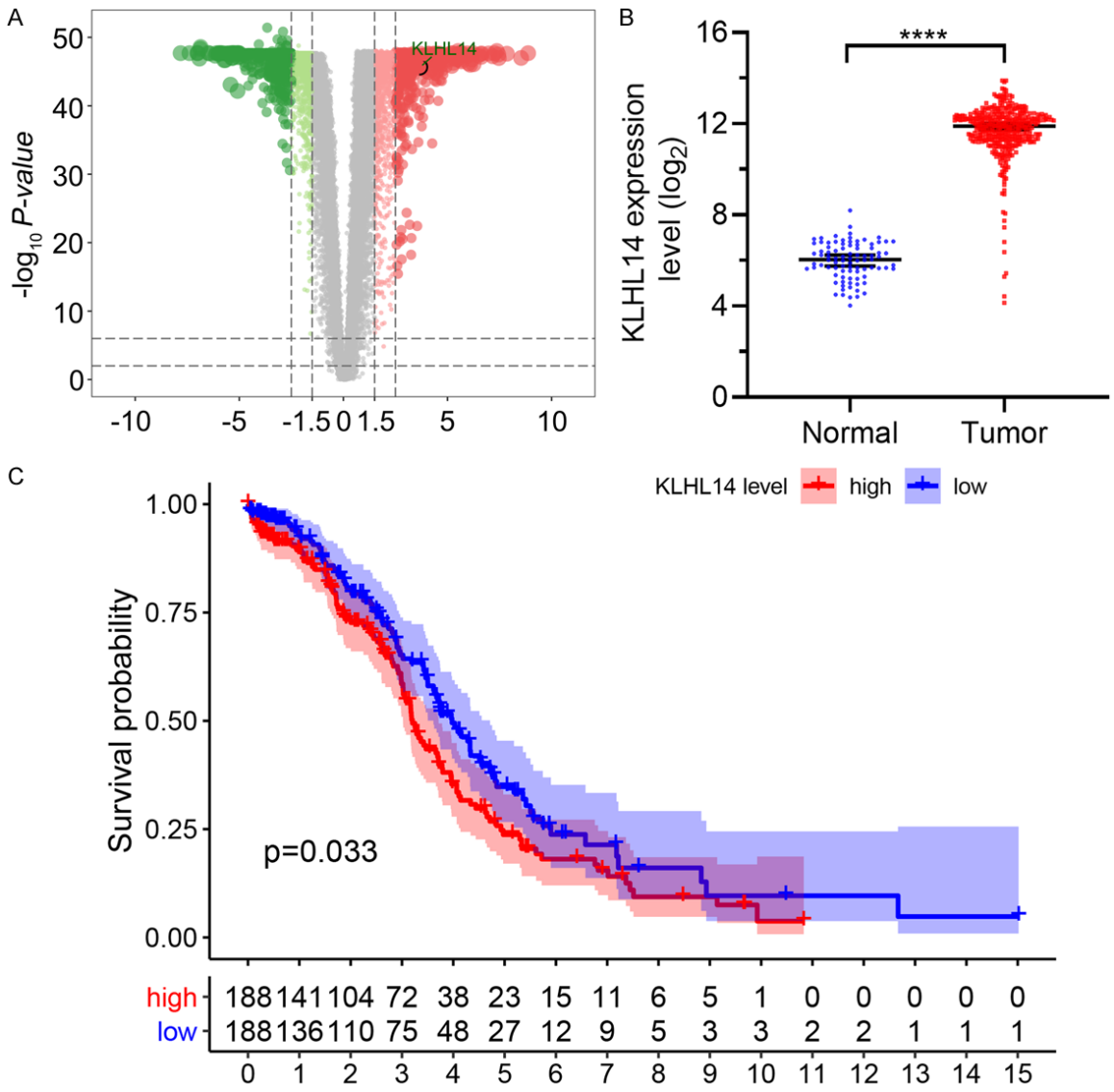

Figure 1. The mRNA level of KLHL14 gene is high in ovarian cancer, indicating a low survival rate. A. Volcanic maps about TCGA database of ovarian cancer tissue samples shows that KLHL14 mRNA was highly expressed in ovarian cancer. B. Scatter diagram shows that KLHL14 mRNA was highly expressed in ovarian cancer tissue. C. Survival curve shows that the overall survival rate of ovarian cancer patients with high expression of KLHL14 gene was lower than that of ovarian cancer patients with low expression of KLHL14 gene. ****: $P < 0.0001$.

the differential expression genes in ovarian cancer were analyzed in the form of a volcanic map. Results showed that KLHL14 was significantly highly expressed in ovarian cancer (**Figure 1A**). KLHL14 was more significantly highly expressed in ovarian cancer tissues than in normal tissues (**Figure 1B**). As shown in **Figure 1C**, after analyzing the survival and prognosis of the ovarian cancer patients, the total survival rate of ovarian cancer patients in the KLHL14 gene high-expression group was lower than that of the patients in the low expression group. These results suggested that KLHL14 may be a proto-oncogene that promotes ovarian cancer development and metastasis.

KLHL14 gene was significantly highly expressed in ovarian cancer cells

Next, we further examined KLHL14 expression in ovarian cancer cell lines. We extracted the total RNA of the normal ovarian granule cell line KGN and those of the ovarian cancer cell strains HEY, A-2780, SK-OV-3, and COC1. KLHL14 mRNA was then detected by qRT-PCR, and results are shown in **Figure 2A**. It was

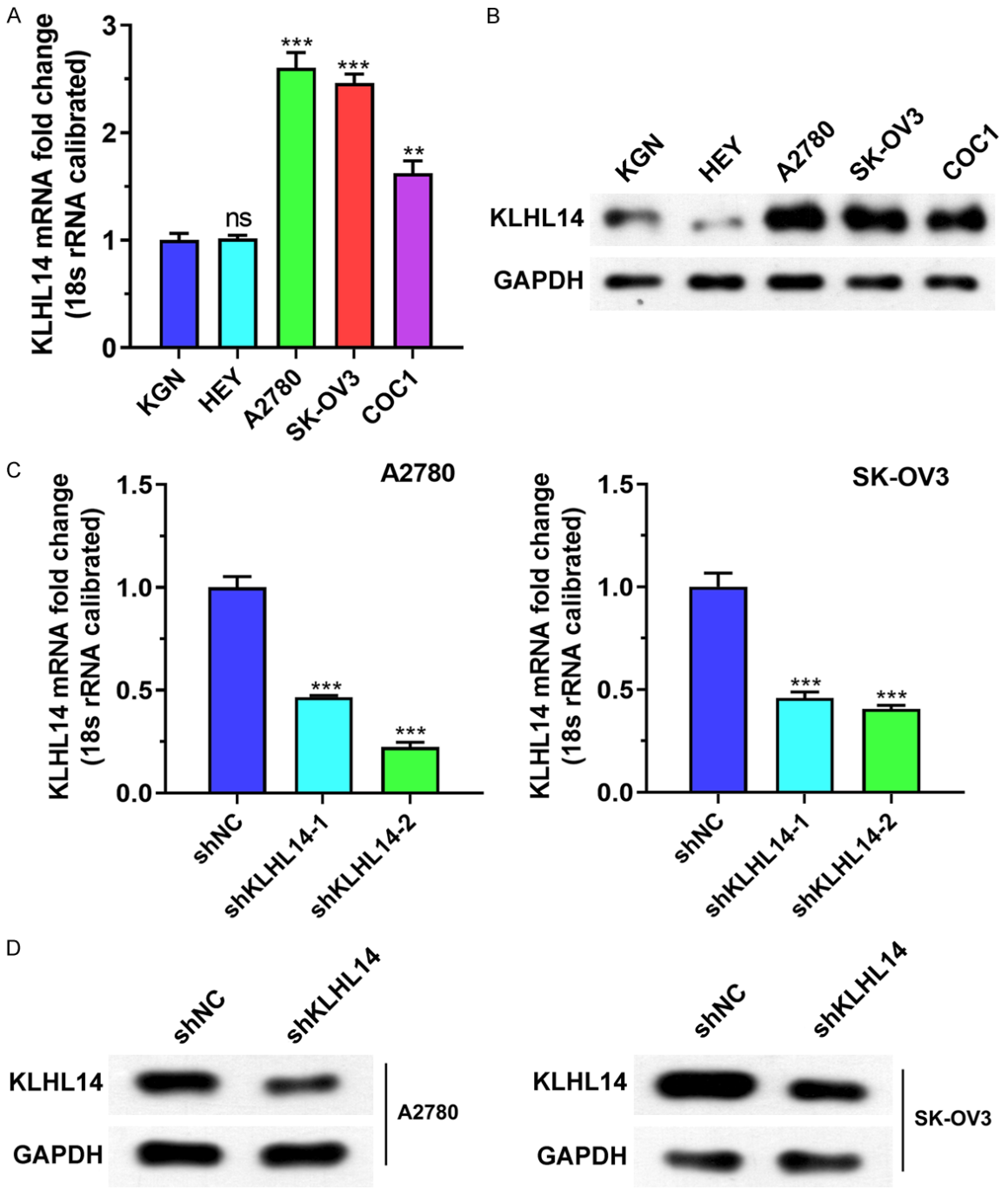

Figure 2. KLHL14 expression is significantly increased in ovarian cancer. (A) qRT-PCR results show KLHL14 mRNA levels in normal ovarian granulosa cell KGN and several ovarian cancer cell strains HEY, A2780, SK-OV-3, and COC1. (B) Western blot analysis results show protein levels of KLHL14 in KGN, HEY, A2780, SK-OV-3, and COC1. (C, D) Two shRNA vectors targeting KLHL14 were constructed, and qRT-PCR was used to detect the knock-down efficiency of shRNA vectors in A2780 and SK-OV-3 cells (C); the most efficient shKLHL14-2 was used for transfection of A2780 and SK-OV-3 cell strains, and western blot analysis was used to detect the knock-down efficiency of KLHL14 protein (D). ns: not significant; ***: $P < 0.001$; **: $P < 0.01$.

observed that KLHL14 mRNA expression levels in A-2780, SK-OV-3, and COC1 were higher than that in KGN, and the expression of KLHL-14 mRNA in HEY was not significantly different from that in KGN. We subsequently collected the total RNA of KGN, HEY, A-2780, SK-OV-3, and COC1 and detected the protein level of KLHL14 by western blot. As shown in **Figure**

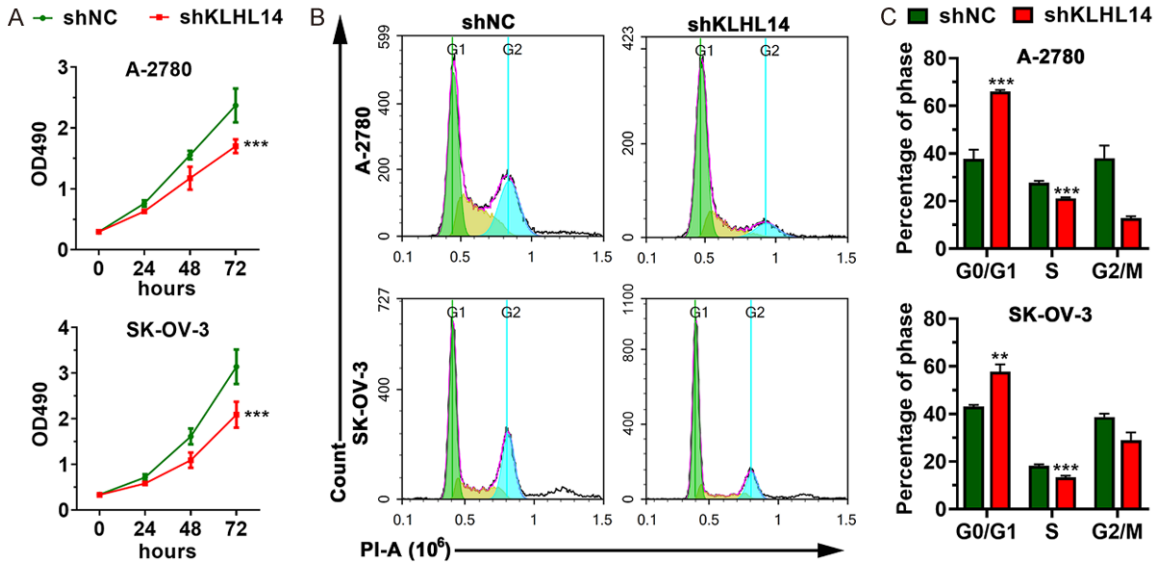

Figure 3. Knockdown of KLHL14 gene inhibits the proliferation of ovarian cancer cells and blocks cell cycle. A. After KLHL14 gene knock-down in A-2780 and SK-OV-3 cell strains, cell proliferation was detected through the MTT assay. B, C. PI staining and flow cytometry were used to examine cell cycles. ***: $P < 0.001$.

2B, KLHL14 protein expression in A-2780, SK-OV-3, and COC1 was higher than that in KGN, and the expression of KLHL14 protein in HEY was not significantly different from that in KGN. We then constructed two shRNAs targeting different regions of KLHL14, as well as strains of A-2780 and SK-OV-3 with KLHL14 knockdown. As shown in **Figure 2C**, the two shRNAs effectively knocked down the mRNA of KLHL14, and shKLHL14-2 was most effective at knocking down in A-2780 and SK-OV-3. Therefore, we further used shKLHL14-2 to reduce the A-2780 and SK-OV-3 cell strains of KLHL14 and performed western blot analysis to examine the effect of the KLHL14 protein knockdown. As shown by **Figure 2D**, shKLHL14-2 significantly reduced KLHL14 protein level; therefore, we used shKLHL14-2 to conduct cell function experiments.

Knockdown of KLHL14 gene inhibited proliferation of ovarian cancer cells and blocked the cell cycle

MTT analysis and cell cycle experiments were performed to examine KLHL14 regulation of the proliferation and cycle of ovarian cancer cells. As shown in **Figure 3A**, the A-2780 and SK-OV-3 cell strains with knock-down expression of KLHL14 were uniformly placed into 96-well plates, and the OD490 absorbance values were detected by MTT assay at 0, 24, 48, and 72 h, respectively. Through statistical analysis, we found that KLHL14 knock-down significantly inhibited ovarian cancer proliferation. As shown in **Figure 3B**, **3C**, decreasing the expression of KLHL14 in A-2780 and SK-OV-3 cells resulted in cell cycle arrest in the G0/G1 phase.

Knockdown of KLHL14 gene promoted apoptosis of ovarian cancer

The effect of KLHL14 gene on the apoptosis of ovarian cancer cells was also examined. As shown in **Figure 4**, the apoptosis ratio of A-2780 and SK-OV-3 cells significantly increased after KLHL14 gene knock-down. These data showed that KLHL14 suppressed apoptosis of ovarian cancer cell lines.

Knockdown of KLHL14 gene inhibited the ability of ovarian cancer cells to migrate and invade

Finally, the effects of KLHL14 gene on migration and invasiveness of ovarian cancer were further explored. As shown in **Figure 5A**, **5B**, after KLHL14 gene knockdown for 24 h, the number of migrating cells of the A-2780 and SK-OV-3 cells was significantly reduced. As shown in **Figure 5C**, **5D**, after KLHL14 gene knock-down for 24 h, the number of invasive

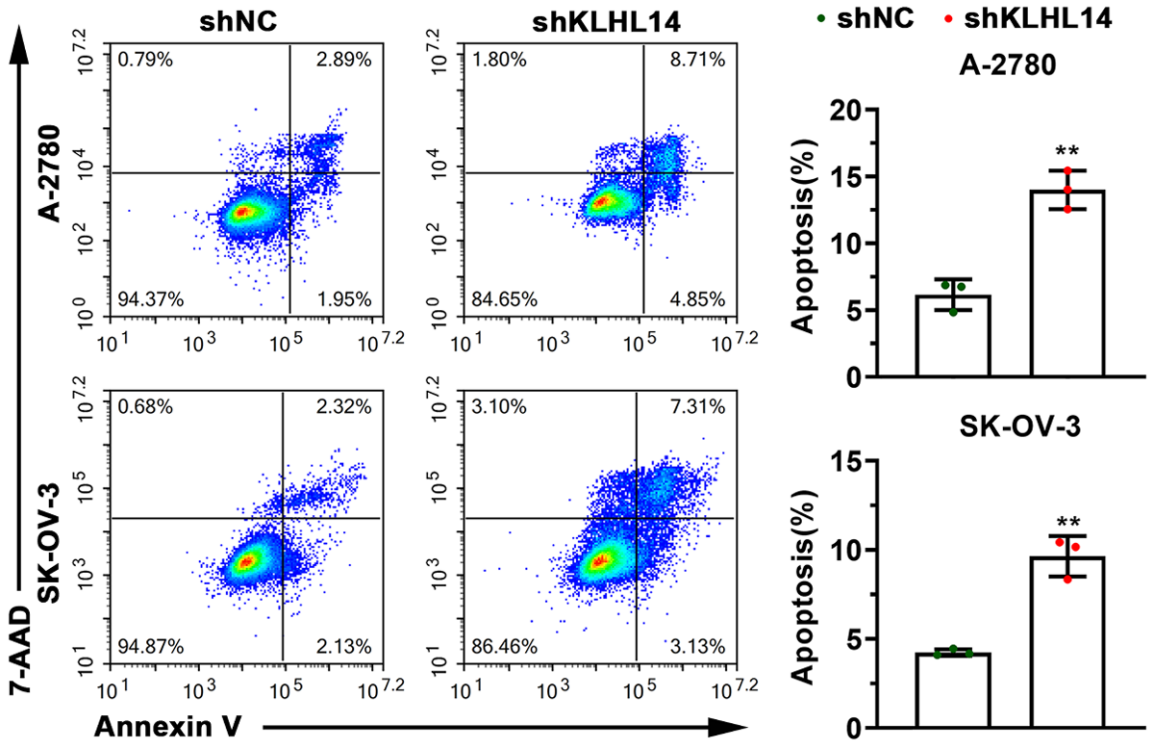

Figure 4. Knockdown of KLHL14 gene can promote the apoptosis of ovarian cancer cells. After knock-down of KLHL14 gene in A-2780 and SK-OV-3 cell lines, annexin V-FITC/PI double-staining method and flow cytometry were used to detect the proportion of apoptosis. **: $P < 0.01$.

cells of A-2780 and SK-OV-3 cells were significantly reduced. All these results indicated that knockdown of KLHL14 inhibited the migration and invasiveness of ovarian cancer cells.

KLHL14 gene may regulate mTOR, WNT, TGF-beta, and other tumor-related signaling pathways and genes

The mechanisms of the possible roles of KLHL14 were analyzed using TCGA-Ovarian Cancer Database. As shown in **Figure 6A**, the relationship between the KLHL14 level and signaling pathways was analyzed using KEGG enrichment. The enrichment curve showed that KLHL14 was positively correlated with tumor-related signaling pathways, such as the mTOR, WNT, and TGF-beta pathways (**Figure 6A**). Moreover, KLHL14 expression had a significant positive correlation with the cancer genes BMPR1B, FGFR2, PAX8, and TGFB2 (**Figure 6B**). These results suggested that KLHL14 may regulate its corresponding pathways by regulating the levels of BMPR1B, FGFR2, PAX8, and TGFB2, thereby promoting ovarian cancer development and metastasis.

Discussion

In 2012, ovarian cancer incidence was 91% in developed countries and 50% in developing countries [14]. In 2015, about 52,100 women in China were diagnosed with ovarian cancer, and about 22,500 women died from this disease [15]. In 2017, approximately 22,440 ovarian-cancer cases and 14,080 deaths were recorded in the United States [16]. Clinically, this cancer lacks typical symptoms, specific signs at the early stage, and effective early diagnostic markers for tumors; this indirectly leads to the low total 5-year survival rates of ovarian cancer patients. Timely treatment before ovarian cancer metastasis can effectively improve the patients' 5-year survival rates [3]. Tumor metastasis is also positively correlated with cell migration and invasion ability, but the detailed molecular mechanism remains unclear. Thus, studying the molecules that regulate the metastasis of ovarian cancer cells is necessary as these molecules may be important markers for predicting malignant progress [3]. By analyzing the data in TCGA, we found that KLHL14 was highly expressed in ovarian

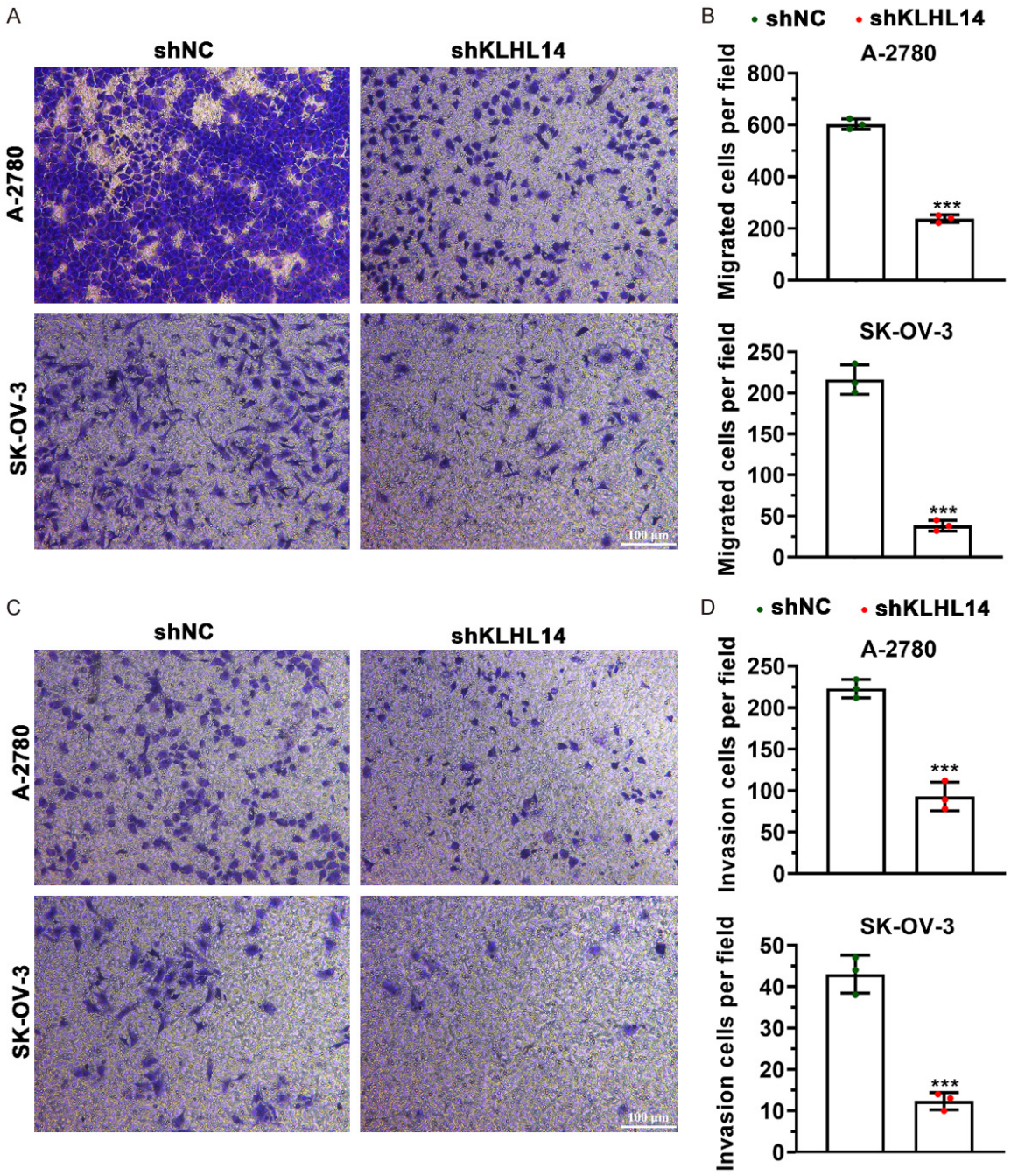

Figure 5. Knockdown of KLHL14 gene inhibits the migration and invasiveness of ovarian cancer cells. A, B. After knockdown of KLHL14 gene in A-2780 and SK-OV-3 cell lines, Transwell without Matrigel was used to examine cell migration. C, D. After knock-down of KLHL14 gene, Transwell with Matrigel was used to detect cell invasiveness. ***: $P < 0.001$.

cancer and that ovarian cancer patients with high KLHL14 expression showed poor prognosis. KLHL14 knockdown can also inhibit cancer cell proliferation, migration, and invasiveness and promote cancer cell apoptosis. These results showed that high expression of KLHL14 can promote migration and invasiveness of cancer cells, which may promote tumor metastasis. Thus, KLHL14 can be used as a marker of ovarian cancer to monitor tumor progression.

Furthermore, KEGG enrichment analysis revealed that KLHL14 may regulate mTOR, WNT,

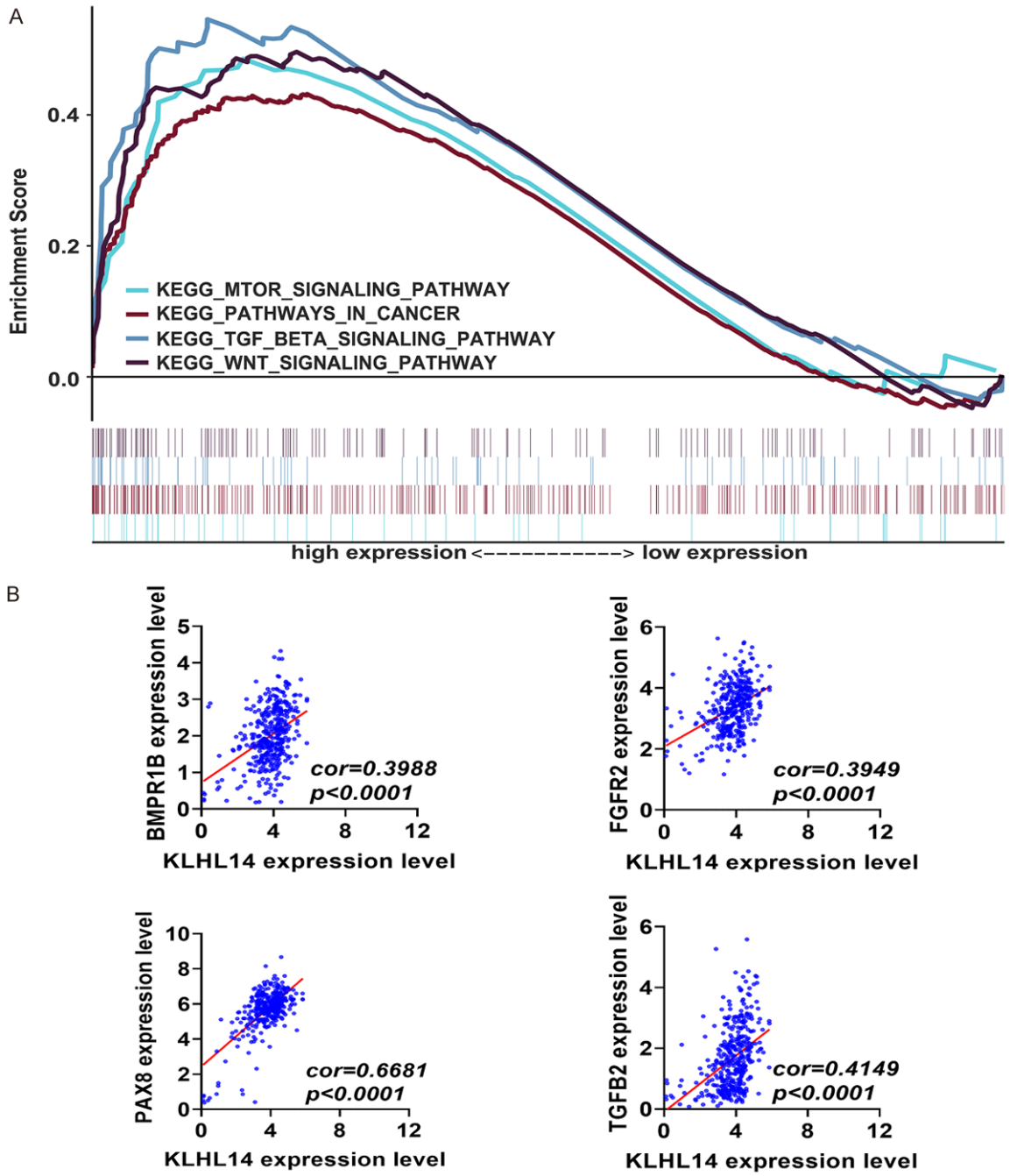

Figure 6. KEGG analysis was used to predict the signaling pathways that KLHL14 gene may regulate. A. KEGG enrichment analysis shows that KLHL14 may regulate mTOR, WNT, TGF-beta, and other tumor-related signaling pathways. B. Genetic correlation analysis shows that KLHL14 level is positively associated with many genes' expression levels in these tumor-related signaling pathways (BMPR1B, FGFR2, PAX8, and TGFB2). KEGG: Kyoto Encyclopedia of Genes and Genomes.

TGF-beta, and other tumor-related signaling pathways. Many studies have also shown that mTOR, WNT, and TGF-beta are involved in the development of breast cancer, non-small cell lung cancer, stomach cancer, hemangioma, tongue squamous cell carcinoma, ovarian cancer, liver cancer, esophageal cancer, and other cancers [17-26]. Through genetic correlation analysis, we found that KLHL14 was positively correlated with many genes in these tumor-related signaling pathways including BMPR1B, FGFR2, PAX8, and TGFB2. Moreover, numerous

studies have confirmed that these genes are involved in the development or metastasis of cancer and may also be targets for treating or diagnosing cancer. For example, BMPR1B can inhibit the proliferation of lung cancer NCI-H460 cells and induce the apoptosis of renal carcinoma cells [27, 28], and FGFR2 can participate in the process of diffuse gastric cancer, hepatic bile duct cancer, and throat squamous cancer and can inhibits the progression of esophageal squamous cancer [29-32]. Moreover, PAX8 can promote the proliferation of stomach cancer cells and participate in the progression of ovarian cancer [33-35]. Certain variations of PAX8 expression exist among ovarian plasma cancers of different levels, different FIGO stages, and different pathologic classifications, which can then be used for the clinical differentiation of pathologic classification, staging, and category [36-38]. Furthermore, the PAX8/NOTCH1 signaling pathways can be activated to promote the progression of pancreatic and hepatocellular carcinomas [39, 40]. Finally, inhibition of TGFB2 expression can promote cell proliferation in squamous cell carcinoma [41], and targeting TGFB2 can inhibit the occurrence and development of renal cell carcinoma [42].

In summary, bioinformatics analysis revealed that KLHL14 was highly expressed in ovarian cancer and that ovarian cancer patients with higher KLHL14 expression had poor prognosis. Through MTT assay, cell cycle detection, apoptosis analysis, and transwell experiments, we further verified that KLHL14 promoted ovarian cancer cell proliferation, migration, and invasiveness, and it also inhibited apoptosis. These findings indicated that KLHL14 may be a new target for the diagnosis and treatment of ovarian cancer and as a new indicator for prognostic monitoring of patients with this disease.

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Disclosure of conflict of interest

None.

Address correspondence to: Jiahua Liu, Department of Gynecology, Shengli Clinical Medical College of Fujian Medical University & Fujian Provincial Hospital, No. 134, East Street, Gulou District, Fuzhou 350001, Fujian, China. Tel: +86-0591-88217090; E-mail: liujiahua1962@163.com

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