KLF5-mediated expression of CARD11 promotes the progression of gastric cancer

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Abstract. Caspase recruitment domain-containing protein 11 (CARD11) has been reported as functioning in multiple types of cancers. In the present study, the role and mechanism of CARD11 in gastric cancer was investigated. First, CARD11 expression in gastric cancer tissues and the association of CARD11 with overall survival were analyzed by the encyclopedia of RNA interactomes database. CARD11 expression in gastric cancer cells was detected by western blotting and reverse transcription-quantitative PCR analyses. After CARD11 silencing, cell proliferation was evaluated by Cell Counting Kit-8 assay, 5-ethynyl-2'-deoxyuridine staining and flow cytometry analysis. Wound healing and Transwell assays were used to measure the capacities of cell migration and invasion. Additionally, the expression levels of epithelial-mesenchymal transition (EMT)-related proteins and mTOR-related proteins were detected by western blot analysis. HumanTFDB predicted the binding of the transcription factor Krüppel-like factor 5 (KLF5) to the CARD11 promoter, which was confirmed by dual luciferase reporter and chromatin immunoprecipitation assays. To explore the regulatory effects between KLF5 and CARD11, KLF5 was overexpressed to perform the rescue experiments in gastric cancer cells with CARD11 silencing. Results revealed that CARD11 was highly expressed in gastric cancer and was associated with poor prognosis. CARD11 interference inhibited the proliferation of gastric cancer cells and induced cell cycle arrest. Additionally, CARD11 silencing suppressed the migration, invasion and EMT of gastric cancer cells, accompanied by upregulated E-cadherin expression and downregulated N-cadherin and vimentin expression. Moreover, the transcription factor KLF5 positively regulated the transcription of CARD11 in gastric cancer. KLF5 overexpression reversed the effects of interference of CARD11 expression in gastric cancer cells to promote their proliferation, migration, invasion and EMT. KLF5 overexpression also led to a reduction in cell cycle arrest. Finally, interference of CARD11 expression suppressed the mTOR pathway, which was activated by KLF5. In conclusion, KLF5-mediated CARD11 promoted the proliferation, migration and invasion of gastric cancer cells.

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Introduction

Gastric cancer is one of the commonest malignant tumors in the world and one of the four major causes of malignancy-related deaths (1). According to GLOBOCAN 2020 statistics, the incidence of gastric cancer in East Asia, including China, holds the first place. Nearly 1/3 of the patients with gastric cancer have advanced stage when diagnosed and this is usually associated with poor prognosis (2,3). The overall survival rate of patients with gastric cancer is still very low, although various clinical methods are available for the treatment of gastric cancer, such as surgery, chemoradiotherapy and immunotherapy (4,5). Therefore, the identification of new molecular targets of gastric cancer and the investigation of the potential mechanism of action of this disease have become the focus of the current research field in gastric cancer.

Caspase recruitment domain-containing protein (CARD) 11 is an important member of the CARD family of proteins and is mainly present in lymphocytes (6). Phosphorylation of CARD11 by protein kinase C family isoenzymes causes conformational change in the interior of the CARD11 molecule, which leads to recruitment of B-cell lymphoma/leukemia 10 (Bcl-10) to CARD11. Bcl-10 binds to muco-associated lymphoid tissue lymphoma translocation protein 1 (MALT1) to form an oligomeric CARMA/CARD-Bcl-10-MALT1 (CBM) complex (7). In activated B-cell-like diffuse large B cell lymphoma, mutation in the CARD11 gene can activate CARD11 activity and selectively enhance its binding activity towards Bcl-10, further leading to the activation of the NF-κB signaling pathway and the development of tumorigenicity (8). Patients with chronic lymphocytic leukemia and high CARD11 expression exhibit a poor survival rate (9). Furthermore, CTD-2020K17.1 regulates CARD11 expression to promote the migration, invasion and proliferation of ovarian cancer cells (10). In addition, CARD11 is mutated in human skin squamous cell carcinoma, leading to abnormal NF-κB regulation and increased CARD11 mRNA levels in skin squamous cell carcinoma (11). However, the role of CARD11 in gastric cancer has not been previously reported.

The encyclopedia of RNA interactomes (ENCORI) database (https://starbase.sysu.edu.cn/index.php, version 3.0) indicated that CARD11 was highly expressed in gastric cancer and that it was associated with poor disease prognosis.

HumanTFDB (http://bioinfo.life.hust.edu.cn/HumanTFDB#!/, version 3.0) predicted the binding of the transcription factor Krüppel-like factor 5 (KLF5) to the CARD11 promoter. Previous studies have shown that KLF5 activates long non-coding RNA DANCR to inhibit autophagy of cancer cells, thereby accelerating the progression of gastric cancer (12). KLF5 is activated by gene amplification in gastric cancer and promotes gastric cancer cell proliferation (13). Therefore, the present study aimed to detect the expression levels of KLF5 and CARD11 in gastric cancer cells and to investigate the underlying mechanism of action.

Materials and methods

Cell culture and cell transfection. Human normal gastric epithelial cells (GES-1) and human gastric cancer cells (MKN-45, KE-39 and AGS) were provided by BioVector NTCC Inc. The cells were cultured in RPMI-1640 medium (Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 μg/ml streptomycin and 100 units/ml penicillin at 37˚C in the presence of 5% CO_2.

Small interfering (si) RNA of CARD11 (si-CARD11#1, AGCTAAAGCACCGGTTGAATAAG; si-CARD11#2, CAG TCTCTAAAACTGAAGAATGA) or siRNA negative control (NC, AAGACAUUGUGUGUCCGCCTT) were synthesized by Guangzhou RiboBio Co., Ltd. The KLF5 overexpression vector pcDNA3.0-KLF5 (oe-KLF5) and the empty plasmid pcDNA3.0 (oe-NC) were obtained from MiaolingBio. AGS cells were seeded into 6-well plates at a density of $2x10^5$ cells/well and cultured until the cell confluence reached 80%. Subsequently, a total of 100 nM plasmids were transfected into AGS cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C for 48 h. Following 48 h of culture at 37˚C, the cells were harvested and used for further experiments.

Reverse transcription-quantitative (RT-q) PCR. Following transfection, $1x10^4$ AGS cells were collected and treated with TRIzol® (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions to obtain the total RNA. The first-strand cDNA was reverse transcribed from RNA using the PrimeScript RT Reagent kit (Takara Bio, Inc.) following the manufacturer's instructions and qPCR was carried out by SYBR Real-time PCR kit (Takara Bio, Inc.) on an ABI 7500 system (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The thermocycling conditions were: Initial denaturation at 95˚C for 3 min; followed by 40 cycles of denaturation at 95˚C for 30 sec, annealing at 60˚C for 30 sec and extension at 72˚C for 30 sec. The following were the sequences of primers: CARD11 forward primer: 5'-GCTCACAACCGCATCCCAA-3', reverse primer: 5'-CTCCTCATGACCGCCATGTT-3'; KLF5 forward primer: 5'-AGCTACAATACGCTTGGCCT-3', reverse primer: 5'-ATGTGTGTTACGCACGGTCT-3'; GAPDH forward primer: 5'-GGAGCGAGATCCCTCCAAAAT-3', reverse primer: 5'-GGCTGTTGTCATACTTCTCATGG-3'. The mRNA expression levels of CARD11 and KLF5 were quantified using the $2^{-\Delta\Delta Cq}$ method and the internal control used was GAPDH (14). All experiments were replicated three times.

Western blot analysis. Following transfection, total proteins were extracted from AGS cells using RIPA lysis buffer (Beyotime Institute of Biotechnology) and protein concentration was determined using bicinchoninic protein quantification kit (Beyotime Institute of Biotechnology). Equal amounts of protein samples (60 μg/lane) separated by 10% SDS-PAGE were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore). Subsequently, the membranes were blocked with 5% non-fat dry milk at room temperature for 2 h and incubated with primary antibodies, including those against CARD11 (1:1,000; cat. no. ab124730; Abcam), E-cadherin (1:1,000; cat. no. ab40772; Abcam), N-cadherin (1:5,000; cat. no. ab76011; Abcam), vimentin (1:1,000; cat. no. ab92547; Abcam), KLF5 (1:1,000; cat. no. ab137676; Abcam), phosphorylated (p)-mTOR (1:1,000; cat. no. ab109268; Abcam), p-P70S6K (1:1,000; cat. no. ab59208; Abcam), mTOR (1:10,000; cat. no. ab134903; Abcam), P70S6K (1:5,000; cat. no. ab32529; Abcam) and GAPDH (1:2,500; cat. no. ab9485; Abcam) at 4˚C overnight; the following day, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:2,000; cat. no. ab6721; Abcam) at room temperature for 1 h. The protein bands were presented using ECL Plus (Thermo Fisher Scientific Inc.) and quantified by ImageJ 1.51 software (National Institutes of Health).

Cell Counting Kit-8 (CCK-8) assay. The cell viability was detected using CCK-8 assay (Beyotime Institute of Biotechnology). Following transfection, AGS cells were cultured for 48 h, seeded into a 96-well plate ($3x10^3$ cells/well) and further cultured at 37˚C, in the presence of 5% CO_2. Subsequently, 10 μl CCK-8 was added to the cultured cells at 24, 48 and 72 h and further cultured for an additional 1 h. The absorbance value (OD value) of the cells at 490 nm was detected by a microplate reader.

5-ethynyl-2'-deoxyuridine (EdU) staining. An EdU kit (BeyoClick™ EdU Cell Proliferation Kit with Alexa Fluor 488; Beyotime Institute of Biotechnology) was used to detect cell proliferation. Following culture for three days at 37˚C in 24-well plates, AGS cells were incubated with EdU, fixed with 4% paraformaldehyde for 15 min at room temperature and stained with Hoechst 33342 for 2, 0.5 h and 10 min. Finally, the proliferation of the cells was observed and images captured by an inverted fluorescence microscope.

Flow cytometry analysis. Following transfection, AGS cells were incubated into 24-well plates and cultured for 24 h. The cells were centrifuged at 300 x g for 5 min and the medium was discarded. Subsequently, they were washed with pre-chilled PBS twice and fixed with 75% ethanol at 4˚C overnight. Following centrifugation, the cells were fixed with 100 μg/ml RNase A at 37˚C for 30 min and subsequently stained with 50 μg/ml PI (Thermo Fisher Scientific, Inc.) for 5 min in the dark. Finally, the cell cycle was analyzed by flow cytometry (NovoCyte 2060R; ACEA Biosciences, Inc.) with Software NovoExpress 1.4.0 (ACEA Biosciences, Inc.).

Wound healing assay. Following transfection, $2x10^5$ AGS cells were added to the each well of the 6-well plate and was cultured to form a cell monolayer. The cell monolayer was scratched by a sterilized 10-μl pipette tip, followed by PBS washing to remove the detached cells. Subsequently, the obtained cells were cultured in RPMI-1640 medium containing 2% FBS for 24 h at 37˚C. Then, the images of cells were observed under a light microscope (Thermo Fisher Scientific, Inc.) and the migration distance was analyzed by ImageJ software (version 1.8.0; National Institutes of Health).

Transwell assay. Following transfection, AGS cells were collected and $5x10^4$ cells were added to the serum-free medium (200 μl) in the upper chambers coated with Matrigel at 37˚C for 1 h. RPMI-1640 medium containing 10% FBS was added to the lower chambers. Following culture for 24 h at 37˚C, the cells in the upper chamber were removed and the obtained cells were fixed with 95% ethanol, stained with 0.1% crystal violet for 10 min at room temperature and washed by PBS. The observation of the invasive cells was performed using an inverted microscope.

Dual luciferase reporter assay. Wild-type (WT) and mutant (mut) sequences of pleckstrin homology-like domain family A member 3 were cloned and inserted into the pGL4.13 reporter vector (Promega Corporation). Subsequently, AGS cells were transfected with the luciferase reporter, oe-KLF5 and oe-NC using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) for 48 h at 37˚C. At 48 h post-transfection, the Luc-Pair Duo-Luciferase HS Assay kit (cat. no. LF004, GeneCopoeia Expressway to Discovery) was used to calculate the luciferase activity referring to the standard curve of luciferin activity.

Chromatin immunoprecipitation (ChIP) assay. The binding ability of KLF5 to the CARD11 promoter was confirmed by a ChIP assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. Briefly, $1x10^6$ AGS cells were cultured in 1% formaldehyde at 37˚C for 10 min to cross-link the target protein with the corresponding genomic DNA. Cells were collected via centrifugation at 13,000 x g for 10 min at 4˚C and washed twice with pre-chilled PBS. A high intensity ultrasonic processor (Cole-Parmer; Antylia Scientific) was used to shear the genomic DNA on ice so that its majority was broken into 200-500 bp fragments. An equal amount of chromatin was immunoprecipitated at 4˚C overnight. ChIP was performed using 2 μg anti-KLF5 (1:5,000; cat. no. 21017-1-AP, Proteintech Group, Inc.). Total chromatin was used as the input. Immunoprecipitated products were collected after incubation with magnetic beads coupled with anti-rabbit IgG (1:100; cat. no. 30000-0-AP, Proteintech Group, Inc.). The precipitated chromatin DNA was recovered and analyzed by qPCR. The sequences of primers were as follows: CARD11 forward primer: 5'-CCCAGGAGGAGA GAGAATTTGAG-3', reverse primser: 5'-CGTTCATCA GGAAGTGCGTG-3'. The reaction conditions included an initial pre-denaturation step at 94˚C for 10 min followed by 50 cycles at 94˚C for 20 sec and 60˚C for 1 min. Data were analyzed using the $2^{-\Delta\Delta Cq}$ method and normalized to input samples (14).

Bioinformatics. HumanTFDB was used to predict the binding of the transcription factor KLF5 to the CARD11 promoter.

Statistical analysis. The experimental data were expressed as mean ± standard deviation of at least three independent experiments, which were analyzed using GraphPad Prism 8.0.1 (Dotmatics). An unpaired student's t-tests were used for comparisons between the two groups and one-way ANOVA with Tukey's post hoc test was used for multiple group comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

CARD11 is highly expressed in gastric cancer and is associated with poor prognosis. First, CARD11expression in patients with gastric cancer evaluated using ENCORI database. As shown in Fig. 1A, CARD11 was highly expressed in tissues of patients with gastric cancer. Additionally, it was also found from ENCORI database that high expression of CARD11 was significantly associated with low overall survival in patients with gastric cancer (Fig. 1B). Additionally, the expression of CARD11 was significantly upregulated in gastric cancer cells (MKN-45, KE-39 and AGS) compared with that in human normal gastric epithelial cell line GES-1 cells (Fig. 1C and D). As the highest CARD11 expression level was observed in AGS cells, this cell line was selected to perform the following experiments. These results indicated the abnormal expression of CARD11 in gastric cancer.

Interference of CARD11 expression inhibits the proliferation of gastric cancer cells and induces cell cycle arrest. To investigate the effects of CARD11 in the progression of gastric cancer, AGS cells were transfected with si-CARD11#1 and si-CARD11#2 to silence CARD11 expression. As shown in Fig. 2A and B, when compared with the si-NC group, the expression levels of CARD11 in AGS cells transfected with si-CARD11#1 and si-CARD11#2 were significantly downregulated. si-CARD11#2 demonstrated a stronger inhibitory effect on CARD11 expression and was therefore selected for subsequent experiments. When AGS cells were transfected with si-CARD11, their viability and proliferation were suppressed (Fig. 2C-E). Furthermore, interference of CARD11 expression increased the proportion of cells at the G_0/G1 phase and decreased the proportion of the cells at the S and G_2/M phases, indicating that the cell cycle was arrested (Fig. 2F). These data suggested that CARD11 silencing inhibits the proliferation and induces cell cycle arrest of gastric cancer cells.

Interference of CARD11 expression inhibits gastric cancer cell metastasis. To study the effects of CARD11 knockdown on the metastasis of gastric cancer cells, the capacities of cell migration, invasion and epithelial-to-mesenchymal transition (EMT) were determined. The migratory and invasive abilities of AGS cells were apparently suppressed by the downregulation of CARD11 expression compared with those noted in the control and si-NC groups (Fig. 3A and B). Meanwhile, the expression levels of E-cadherin were increased and those of N-cadherin and vimentin were decreased in AGS cells following transfection with si-CARD11 (Fig. 3C). These data

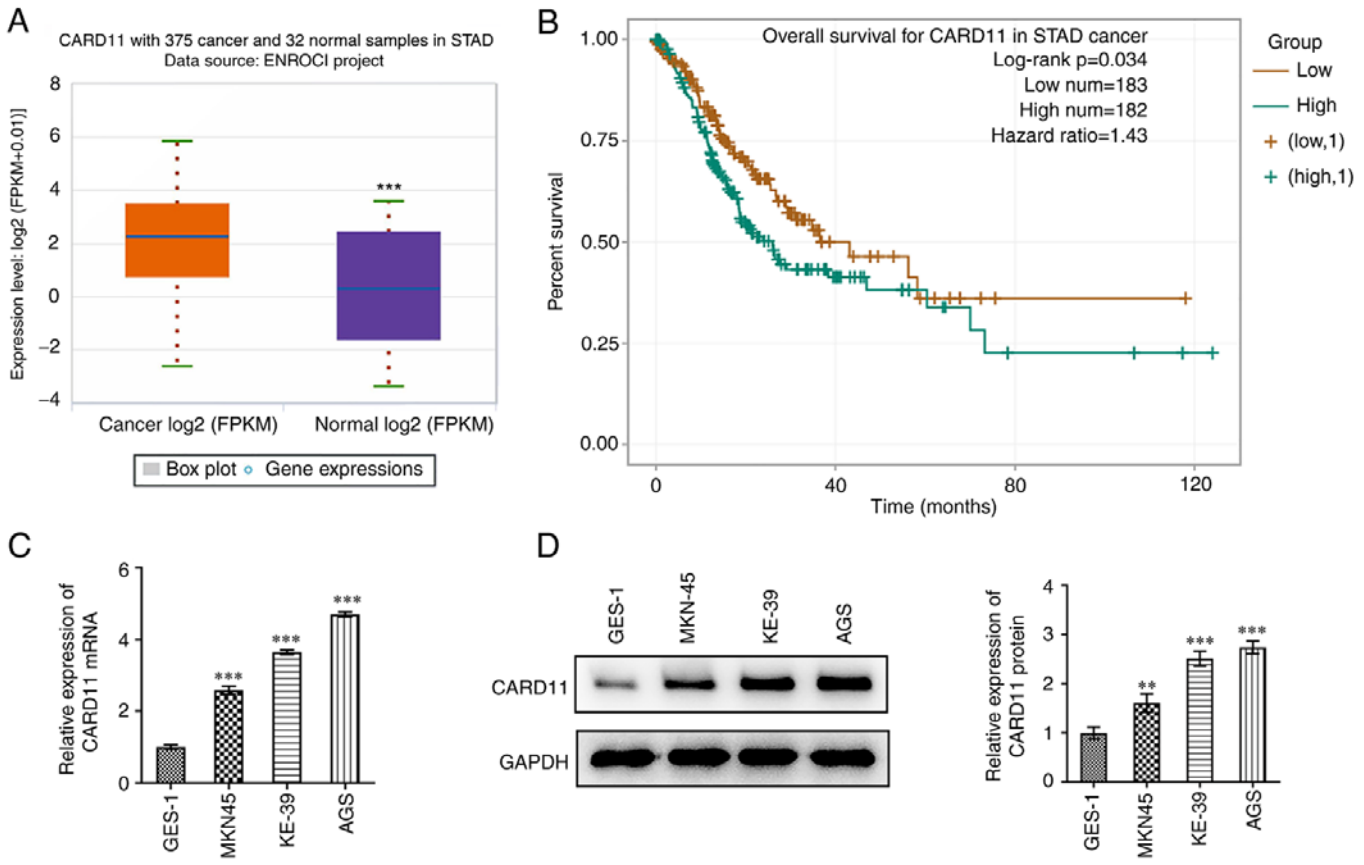

Figure 1. CARD11 is highly expressed in gastric cancer and is associated with poor prognosis. (A) The ENCORI database predicted the expression of CARD11 in tissues of patients with gastric cancer. ***P<0.001 vs. the cancer group. (B) The ENCORI database predicted the association of CARD11 overexpression with overall survival. Cutoff-High, median 50% and Cutoff-Low, median 50%. The expression levels of CARD11 in normal gastric epithelial cells and in gastric cancer cells were detected by (C) reverse transcription-quantitative PCR and (D) western blotting. **P<0.01 and ***P<0.001 vs. the GES-1 group. CARD, caspase recruitment domain-containing protein; ENCORI, the encyclopedia of RNA interactomes.

revealed that CARD11 silencing suppressed the metastasis of gastric cancer cells.

Transcription factor KLF5 positively regulates the transcription of CARD11 in gastric cancer. To explore the potential mechanism of CARD11 on the progression of gastric cancer, HumanTFDB predicted the binding of the transcription factor KLF5 to the CARD11 promoter (Fig. 4A). The binding sites between the transcription factor KLF5 and CARD11 are shown in Fig. 4B. The expression levels of KLF5 and CARD11 in AGS cells were also upregulated (Fig. 4C and D). When AGS cells were transfected with oe-KLF5, the expression levels of KLF5 and CARD11 were upregulated (Fig. 4E and F). Additionally, the luciferase activity of AGS cells co-transfected with CARD11-WT and oe-KLF5 were increased compared with those in AGS cells co-transfected with CARD11-mut and oe-KLF5 (Fig. 4G). Moreover, ChIP assay indicated that the CARD11 promoter was specifically pulled down by a KLF5-specific antibody; however, this was not noted for the control antibody (Fig. 4H). Furthermore, the expression levels of CARD11 in AGS cells were upregulated following transfection of the cells with oe-KLF5 (Fig. 4I and J). These observations suggested that transcription factor KLF5 positively regulated the transcription of CARD11 in gastric cancer.

KLF5 regulates CARD11 to promote malignant progression of gastric cancer perhaps by activating the mTOR pathway. Subsequently, KLF5 was overexpressed to study the mechanism of KLF5 regulation on CARD11 in the progression of gastric cancer. It was found that KLF5 overexpression improved the viability and proliferation of AGS cells transfected with si-CARD11 (Fig. 5A-C). The proportion of cells at the G_0/G1 phase was decreased and that of the cells corresponding to the S and G_2/M phases was increased in AGS cells transfected with si-CARD11 and oe-KLF5 (Fig. 5D). Meanwhile, KLF5 overexpression improved the migration and invasion of AGS cells transfected with si-CARD11 (Fig. 5E and F). Additionally, KLF5 overexpression suppressed the expression of E-cadherin, while it promoted the expression levels of N-cadherin and vimentin in AGS cells transfected with si-CARD11 (Fig. 5G). Interference of CARD11 expression downregulated the expression levels of p-mTOR and p-P70S6K, which were activated by KLF5 overexpression (Fig. 5H). Above findings demonstrated that KLF5 regulated CARD11 to promote malignant progression of gastric cancer might by activating the mTOR pathway.

Discussion

At present, the CARD11 protein in mammals has been studied extensively. The CARD11 protein is not only a member of the

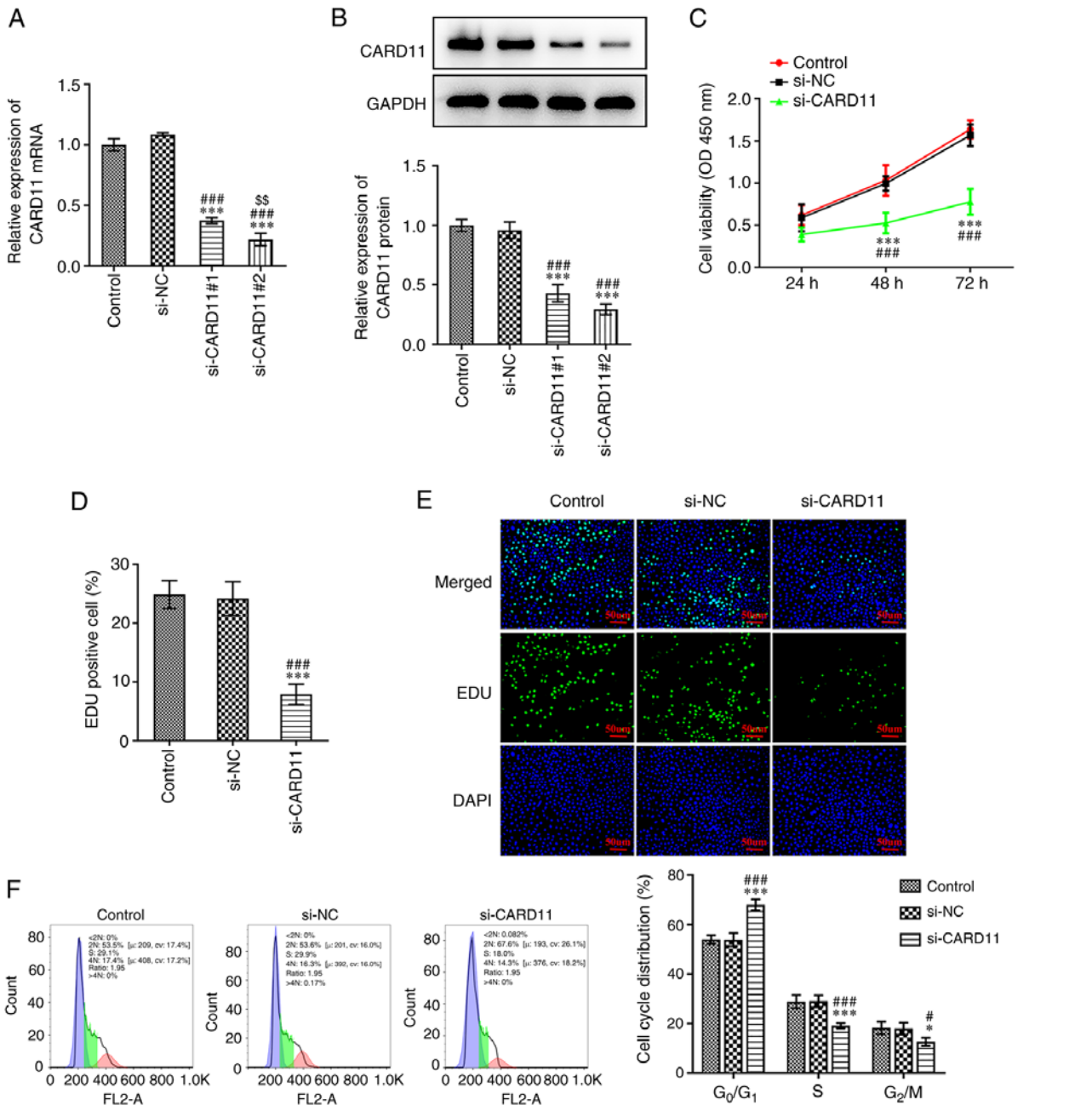

Figure 2. Interference of CARD11 expression inhibits the proliferation of gastric cancer cells and induces cell cycle arrest. The expression levels of CARD11 in gastric cancer cells transfected with si-CARD11#1 or si-CARD11#2 were detected (A) reverse transcription-quantitative PCR and (B) western blotting. ***P<0.001 vs. the control group. $^{###}$P<0.001 vs. the si-NC group. $^{\$\$}$P<0.01 vs. the si-CARD11#1 group. The viability, proliferation and cell cycle of gastric cancer cells transfected with si-CARD11 was analyzed by (C) CCK-8 assay, (D and E) EdU staining and (F) flow cytometry analysis. *P<0.05 and ***P<0.001 vs. the control group. $^{\#}$P<0.05 and $^{###}$P<0.001 vs. the si-NC group. CARD, caspase recruitment domain-containing protein; si, small interfering; NC, negative control; CCK-8, Cell Counting Kit-8; EdU, 5-ethynyl-2'-deoxyuridine.

membrane-associated guanylate kinase (MAGUK) family of enzymes, but also a member of the CARD-MAGUK (CARMA) protein family, which exists in the cytoplasm of the host cell. It is especially abundant in hematopoietic cells and immune organs and is the only member of the MAGUK family specifically expressed in lymphocytes (15). Members of the CARMA family were originally discovered using bioinformatics methods based on the CARD functional domain and named CARD11 (CARMA1), CARD14 (CARMA2) and CARD10 (CARMA3) (16-18). CARMA1 is highly expressed in specific tumor cell lines, such as the chronic myelogenous leukemia cell K562, acute promyelocytic leukocytosis HL-60 and Burkitt's lymphoma cell Raji (16). In addition, CARMA1 can activate NF-κB through the CBM complex to cause excessive cell proliferation, invasion and metastasis and tumor angiogenesis, which is an important cause of autoimmune diseases or of lymphatic hematopoietic system tumors (19-21). Furthermore, high CARD11 expression in uveal melanoma is associated with poor overall survival and CARD11 is associated with autophagy, cell senescence and apoptosis (22). EMT is a cellular process by which epithelial cells gain a mesenchymal phenotype through specific changes in gene expression (23).

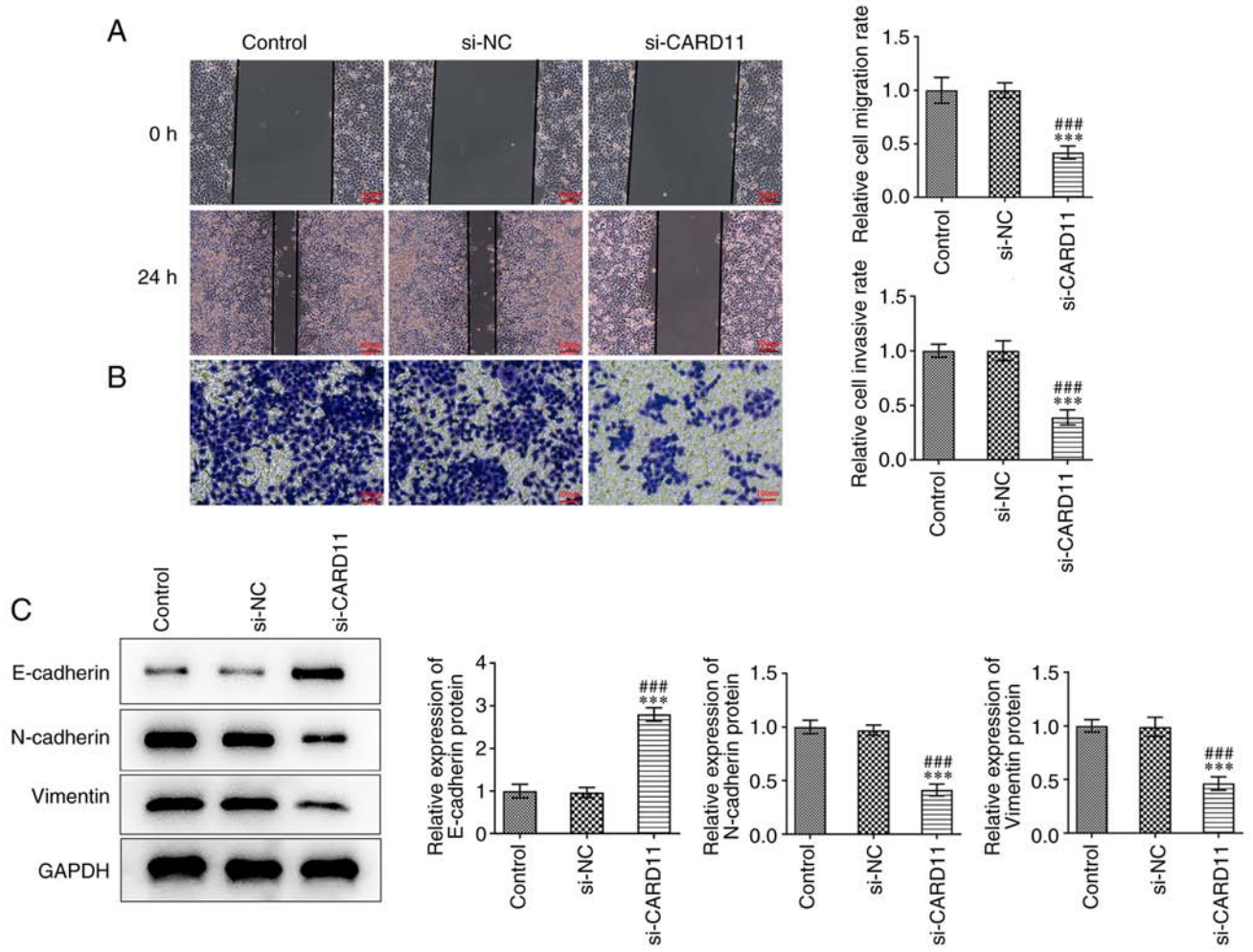

Figure 3. Interference of CARD11 expression inhibits gastric cancer cell metastasis. The (A) migratory and (B) invasive abilities of gastric cancer cells transfected with si-CARD11 were detected by wound healing and Transwell assays. (C) The expression levels of EMT-related proteins in gastric cancer cells transfected with si-CARD11 were detected by western blot analysis. ***P<0.001 vs. the control group. $^{###}$P<0.001 vs. the si-NC group. CARD, caspase recruitment domain-containing protein; si, small interfering; EMT, epithelial-to-mesenchymal transition.

A number of studies have found that the occurrence of EMT biological behavior plays an important role in the metastasis of malignant tumors (24-26). The epithelial cells undergoing EMT lose epithelial characteristics, such as loss of E-cadherin expression and gain mesenchymal features, such as overexpression of vimentin and N-cadherin (27). The present study found that high CARD11 expression in tissues of patients with gastric cancer was predicted in the ENCORI database and CARD11 expression was increased in gastric cancer cells. Downregulation of CARD11 expression suppressed the proliferation, invasion, migration and EMT of gastric cancer cells and induced their cell cycle arrest.

However, the regulatory models that mediate CARD11 expression and the mechanisms by which it modulates global gene expression have not been well characterized in gastric cancer cells. In the present study, a positive correlation between the KLF5 transcription factor and CARD11 was noted by analyzing HumanTFDB. The results derived from ChIP and luciferase reporter assays confirmed high enrichment of KLF5 in the promoter of CARD11. It was shown that KLF5 could activate CARD11 expression via an interaction with its promoter. KLF5 is a basic transcription element binding protein 2 in eukaryotes and a zinc finger protein transcription factor (28). It can regulate the tissue and time specificity of gene expression by activating or inhibiting the transcription of target genes and serves an important role in cell proliferation, differentiation and apoptosis (28-30). KLF5 expression is markedly upregulated in gastric cancer tissues and knockdown of its expression suppresses proliferation and arrested gastric cancer cell cycle at the G_0/G1 phase (31). Upregulation of KLF5 expression attenuated the function of crocin to promote the migration, invasion and EMT of gastric cancer cells (32). Depletion of KLF5 expression reduces gastric cancer proliferation *in vitro* and *in vivo* (33). The present study further indicated that KLF5 expression was increased in gastric cancer cells and that its upregulation weakened the function of CARD11 interference to promote the migration, invasion and EMT; it also decreased cell cycle arrest of gastric cancer cells.

CARD11 is activated by phosphorylation and forms the CBM signalosome with the two downstream signaling molecules Bcl-10 and MALT1 (34-36). Following the formation of CBM, the MALT1 protein recruits the downstream signaling molecules to activate NF-κB, JNK and the mTOR signaling pathways (35,37-39). It was hypothesized that

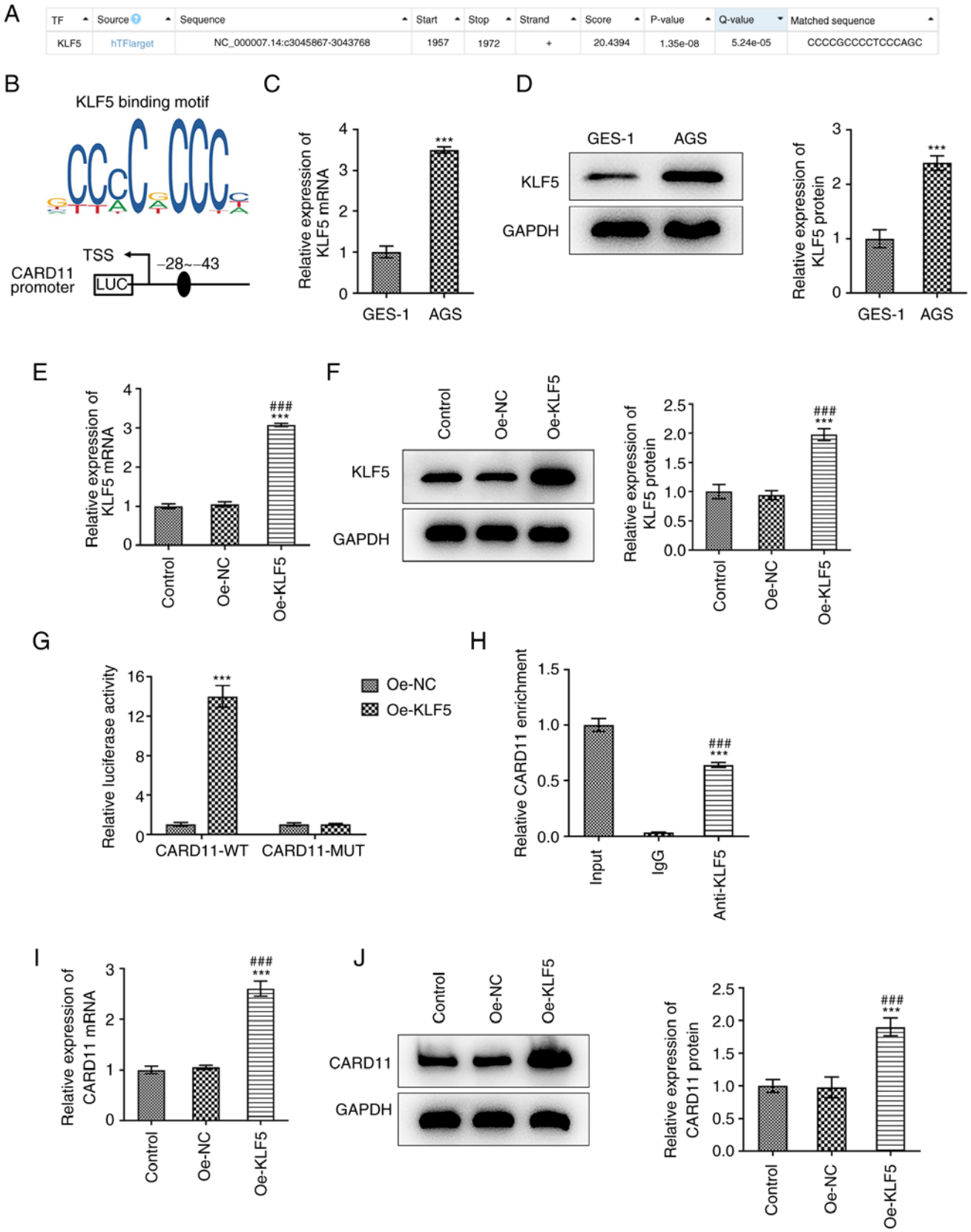

Figure 4. The transcription factor KLF5 positively regulates the transcription of CARD11 in gastric cancer. (A) HumanTFDB predicted the binding of the transcription factor KLF5 to the CARD11 promoter. (B) The binding sites between KLF5 and CARD11. The expression of KLF5 in gastric cancer cells was detected by (C) RT-qPCR and (D) western blotting. ***P<0.001 vs. GES-1 group. The expression of KLF5 in gastric cancer cells transfected with oe-KLF5 was detected by RT-qPCR (E) and western blot (F). ***P<0.001 vs. Control group. ###P<0.001 vs. oe-NC group. (G) The luciferase activity of gastric cancer cells co-transfected with oe-KLF5 and CARD11-WT or CARD11-MUT was detected by dual luciferase reporter assay. ***P<0.001 vs. oe-NC group. (H) The expression of CARD11 in gastric cancer cells adding anti-KLF5 was detected by ChIP assay. ***P<0.001 vs. Control group. ###P<0.001 vs. IgG group. The expression of CARD11 in gastric cancer cells transfected with oe-KLF5 was detected by (I) RT-qPCR and (J) western blotting. ***P<0.001 vs. Control group. ###P<0.001 vs. oe-NC group. KLF5, Krüppel-like factor 5; CARD, caspase recruitment domain-containing protein; RT-qPCR, reverse transcription-quantitative PCR; oe, overexpression; NC, negative control; WT, wild type; MUT, mutant; ChIP, chromatin immunoprecipitation.

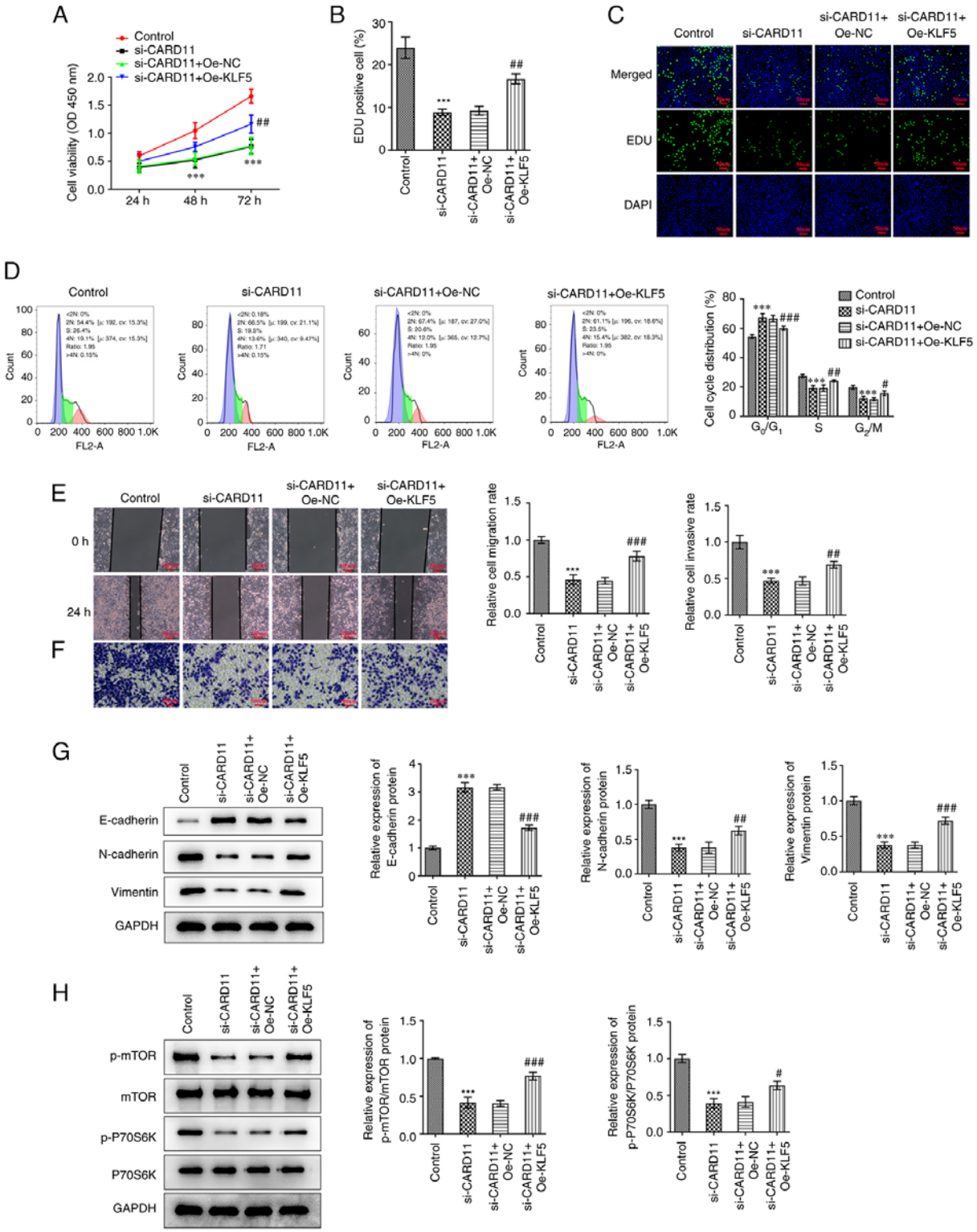

Figure 5. KLF5 regulates CARD11 to promote malignant progression of gastric cancer might by activating mTOR pathway. The viability, proliferation and cell cycle of gastric cancer cells transfected with si-CARD11 and oe-KLF5 was analyzed by (A) CCK-8, (B and C) EdU staining and (D) flow cytometry analysis. The (E) migration and (F) invasion of gastric cancer cells transfected with si-CARD11 and oe-KLF5 was detected by wound healing and Transwell assays. (G) The expression of EMT-related proteins in gastric cancer cells transfected with si-CARD11 and oe-KLF5 was detected by western blotting. (H) The expression of mTOR pathway related proteins n gastric cancer cells transfected with si-CARD11 and oe-KLF5 was detected by western blotting. ***P<0.001 vs. Control group. $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. si-CARD11 group. KLF5, Krüppel-like factor 5; CARD, caspase recruitment domain-containing protein; si, small interfering; oe, overexpression; EMT, epithelial-to-mesenchymal transition; p-. phosphorylated.

CARD11 may regulate the mTOR signaling pathway. mTOR and its upstream PI3K-protein kinase B (Akt) constitutes the PI3K/Akt/mTOR signaling pathway. This pathway is highly activated in gastric cancer and regulates cell proliferation, apoptosis, transcription, translation, metabolism and other important cell biological processes in the occurrence and

development of gastric cancer (40). Lang *et al* (41) also found that the expression of p-mTOR in gastric cancer was closely related to lymphatic infiltration. Another study showed significant differences in the expression of p-mTOR in gastric cancer tissues of different TNM stages, whereas the positive expression of the p-mTOR protein in gastric cancer tissues of stage III-IV is significantly higher than that noted in tissues of stage I-II (42). The present study observed that interference of CARD11 expression suppressed the mTOR expression in gastric cancer cells, which was reversed by upregulation of KLF5 expression.

In conclusion, the present study demonstrated that KLF5-mediated CARD11 promoted the proliferation, migration and invasion of gastric cancer cells possibly by activating the mTOR pathway. To the best of the authors' knowledge, the present study is the first to demonstrate the suppressive role of CARD11 silencing in gastric cancer and the mechanism related to KLF5. Overall, these findings may provide efficient therapeutic target for the treatment of gastric cancer. Nevertheless, the present study has a limitation. The present study only discussed the regulatory effect of CARD11 and KLF5 on the progression of gastric cancer cells. *In vivo* experiments involving transgenic animals and the in-depth investigation of CARD11 and mTOR pathway will be used in future investigations to support the conclusion obtained in the present study.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

QL conceived and designed the current study. QL, SL and ZL performed the experiments and QL, HX and WZ performed the data analysis. QL wrote the manuscript. ZL and HX confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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