Regular paper

MicroRNA-650 suppresses KLF12 expression to regulate growth and metastasis of human ovarian cancer cells

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MicroRNA-650 (miR-650) has been shown to regulate the development of human cancers. The present study investigated the role of miR-650 in ovarian cancer by targeting Krüppel-like factor 12 (KLF12). The results showed a down-regulation of miR-650 in tissues and cell lines. Overexpression of miR-650 caused a substaining decrease in the viability of CAOV3 cells by promoting apoptotic cell death. *In silico* analysis and dual luciferase assay revealed KLF12 as a potential target of miR-650. Unlike miR-650, KLF12 showed a substantial up-regulation in ovarian cancer tissues and cell lines. However, miR-650 overexpression suppressed KLF12 expression posttranscriptionally. Intrestingly, KLF12 knockdown inhibited the viability of CAOV3 cells by promoting apoptotic cell death. However, the expression of KLF12 eliminated the tumor suppressing effects of miR-650 in CAOV3 cells. Additionally, KLF12 knockdown or miR-650 overexpression suppressed CAOV3 cell migration and invasion. However, KLF12 overexpression eliminated the inhibitory effects of miR-650 on the migration and invasion of CAOV3 cells. Taken together, these results suggest that miR-650/KLF12 axis regulates the viability, migration, and invasion of CAOV3 cells an0d may prove to be an important therapeutic taregt.

Keywords: Krüppel-like factors, ovarian cancer, microRNA, KLF12, apoptosis, metastasis

INTRODUCTION

Ovarian cancer is the most aggressive type of gynecological cancer and the seventh most prevalent cancer among women worldwide, with 240 000 new cases diagnosed annually (Stewart *et al.*, 2019). Moreover, ovarian cancer is the second most common malignancy after breast cancer in women over 40 years of age (Jayson *et al.*, 2014). Around 22 000 new cases of ovarian cancer and 14 000 deaths related to ovarian cancer are reported annually in the United States (Torre *et al.*, 2018) (Momenimovahed *et al.*, 2019). The incidence of ovarian cancer has been shown to be higher in Central and Eastern European countries, but the incidence rates are comparatively lower in China (He *et al.*, 2021; Feng *et al.*, 2019). However, due to the large population of China, annual new ovarian cases and ovarian cancer-related deaths are higher than in the United States (Viale, 2020; Smith *et al.*, 2019). The 5-year survivability rate of ovarian cancer is around 47% (Smith *et al.*, 2019). Commonly referred to as a silent killer, ovarian cancer is often diagnosed at advanced stages (Cortez *et al.*, 2018). Around 70% of ovarian cancers are detected when the disease has already progressed to stage III and IV (Chandra *et al.*, 2019). The lack of reliable biomarkers and efficient therapeutic agents/traitors is one of the main bottlenecks in the treatment of ovarian cancer (Boussios *et al.*, 2020). MicroRNAs (miR) have been shown to be involved in crucial cellular processes (Bartel, 2004). The abnormal expression of miRs has been associated with the development of different human diseases such as cancer (Hayes *et al.*, 2014). MicroRNA-650 (miR-650) has been shown to participate in several cancer-related processes (Liu *et al.*, 2021; Xu *et al.*, 2017; Xu *et al.*, 2019). Lui *et al.* (2020) reported the involvement of miR-650 in the development of gastric carcinoma (Liu *et al.*, 2021). In epitheloid cells, miR-650 has been shown to promote inflammation-induced apoptosis (Xu *et al.*, 2017). Similarly, miR-650 has been reported to suppress proliferation, migration, and invasion of synovial fibroblasts (Xu *et al.*, 2019). However, the role of miR-650 has not been studied in ovarian cancer. Furthermore, *in silico* analysis revealed several taregts of miR-650. However, we selected zinc finger Krüppel-like factor 12 (KLF12) as a potential target of miR-650 for further investigation for two reasons. First, KLF12 has not been studied as a molecular target of miR-650, and secondly, KLF-12 has been shown to be involved in several cancer-related cellular processes such as proliferation, differentiation, cell death, migration, and invasion (Simmen *et al.*, 2010; Ding *et al.*, 2015; Zhang *et al.*, 2020). Dysregulation of KLFs has been shown to be associated with several human cancers in which they exhibit tumor suppressor or oncogenic regulatory functions (Yang *et al.*, 2005; Tetreault *et al.*, 2013; Ding *et al.*, 2019). Previously, KLF5 was shown to promote stem cell proliferation and drug resistance in ovarian cancer (Dong *et al.*, 2013). Thus, the present study was designed to study the role of the miR-650/KLF12 molecular axis in ovarian cancer and to explore its therapeutic applicability.

MATERIALS AND METHODS

Human tissues

A total of 60 ovarian cancer and normal adjacent tissues were collected at the Second Affiliated Hospital of Nantong University (First People's Hospital of Nantong City), Nantong, Jiangsu, China. The study protocols were approved by the Research Ethics Committee of the Second Affiliated Hospital of Nantong University, Nantong, Jiangsu, China. The tissue collection was made only after obtaining the written consent of the enrolled patients. The clinical specimens were immediately frozen in liquid nitrogen and stored at –80°C until use.

Immunohistochemical staining

For their immunohistochemical (IHC) staining, tissue samples were fixed for 5 days with 12% neutral buffer formalin. The tissues were then embedded in paraffin followed by sectioning. The sections obtained were baked on glass slides and subsequently deparaffinized. This was followed by blocking and incubation with anti-KLF12 (1:1000, Cell Signaling Technologies) at 4°C overnight. The tissues were then washed with cold PBS and incubated with the respective secondary antibody. Finally, a 3,3'-diaminobenzidine(DAB) Substrate Kit (Abcam) was used for color development.

Cell lines

Four different human ovarian cancer cell lines (A2780, CAOV3, HO8910, and OVCAR3) along with a normal ovarian epithelial cell line (IOSE-386)were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cell line culturing was carried out with RPMI-1640 (Sigma-Aldrich) carrying a supplementation of 100 U/ml of penicillin/streptomycin (both from Sigma-Aldrich) and fetal bovine serum (FBS, 10%; Thermo Fisher Scientific) at 37°C with 5% CO_2. The humidified atmosphere of 5% CO_2 was used to maintain cell lines in a humidified CO_2 incubator at 37°C.

Transfection

Transfection of 0.25 µg of si-NC (5'-CCAUCUCC-CGGUACAAAAUCUGCU-3'), si-KLF12 (5'-GCAATC-GAATGAATAATCA-3'), miR-NC (5'-GUAGGAGUA-GUGAAAGGCC-3') or miR-650 mimics (5'AGGAGG-CAGCUCUCAGGAC-3') (GenePharma Co. Shanghai, China) were transfected into CAOV3 cancer cells with the help of Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's protocol. KLF12 overexpression was achieved using the pcDNA3.1 overexpression vector.

Expression analysis

For the analysis of KLF12 and miR-650 expression, RNA isolation was performed from tissues and cell lines with the help of TRIzolreagent (Thermo Fisher Scientific). The iScriptTMcDNA synthesis kit (Bio-Rad Inc.) was used for the synthesis of cDNA from extracted RNA according to the manufacturer's instructions. Using cDNA as a template, quantitative real-time polymerase chain reaction (qRT-PCR) was performed on the QuantStudio Real-Time PCR system (Thermo Fisher Scientific). With human GADPH and snRNA U6 as endogenous control, the $2^{-\Delta\Delta Ct}$ method was used to examine relative expression levels of KLF12 and miR-650. The

Table 1. Sequences of primers used in the present study

Primer	Direction	Sequence
KLF12	Forward	5'-CTTTCCATAGCCAGAGCA-3'
	Reverse	5'-TGCATCCCTCAAAATCAC-3'
miR-650	Forward	5'-AGAGGAGGCAGCGCTCT-3'
	Reverse	5'-CAGTGCGTGTCGTGGATG-3'
GAPDH	Forward	5'-GTGTCCGTCGTGGATCTG-3'
	Reverse	5'-CACCTTCTTGATGTCATC-3'
U6	Forward	5'-CTCGCTTCGGCAGCACA-3'
	Reverse	5'-AACGCTTCACGAATTTGCGT-3'

primer sequences used in the present study are included in Table 1.

MTT cell viability assay

Cell viability was quantitatively assessed by performing a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In summary, transiently transfected CAOV3 cells were seeded in a 96-well plate at a density of 6×10^3 cells per well. After culture of cells for 0, 12, 24, 48, 72, and 96 hat 37°C each well was added with 25 µL MTT (0.5%, Sigma-Aldrich). The cells were again incubated for 2.5 h at 37°C Subsequently, 250 µL dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals. Finally, the optical densities (OD) were measured for each well at 570 nm with the help of a microplate spectrophotometer (Thermo Fisher Scientific) for analyzing the respective cell viabilities.

AO/EB and DAPI staining methods

Transiently transfected CAOV3 cancer cells were cultured for 24 h at 37°C with an initial density of 5×10^4 cells/well of a 12-well plate. Subsequently, 20 µL of cell suspension was placed on a clean glass slide and then stained with 1 µl of acridine orange/ethidium bromde (AO/EB) staining mixture or 4',6-diamidino-2-phenylindole (DAPI) staining solution. The coverslips were placed on the glass slides and finally the cells were examined under a fluorescent microscope (Olympus).

Transwell Assays

For the analysis of the migration of ovarian cancer cells, 2×10^5 transfected cells suspended in 250 µL culture medium were placed in the upper chamber of a 24-well Transwell plate with a pore size of 8.0-µm. The lower chamber was added with 500 µL of serum-free RPMI-1640 medium supplemented with 10% FBS. After incubation of 24 h at 37°C, the cells migrating to the lower chamber were washed with PBS, fixed with 70% ethanol for 15 min at room temperature, and then stained with 0.2% crystal violet. Finally, the migrating cells were photographed under an inverted light microscope and the relative percentage of the migrating cells was determined using 6 random microscopic fields. The invasion of transfected CAOV3 cancer cells was also assayed using the same procedure. However, the upper chambers were precoated with 100 µL Matrigel (BD Biosciences, USA).

Taregt identification and dual luciferase assay

In silico analysis was performed using the online TargetScan database (http://www.targetscan.org/) to predict

miR-650 targets and KLF12 was selected as the potential taregt of miR-650. The miR identification was validated using dual luciferase activity assay. Here, the 3'UTR segment of KLF2 with the wild-type (WT) or mutated (MUT) binding site for miR-650 was amplified by PCR and then cloned into the PGL3 vector. The reporter plasmid PGL3-KLF12-WTor PGL3-KLF12-MUT was co-transfected with miR-650 mimics or miR-NC into COAV3 cells with the help of lipofectamine 2000 and cells were cultured for 48 h at 37°C The Dual luciferase Reporter Assay System (Promega) was used for the analysis of luciferase activities of transfected cells to analyze miR-650 binding to KLF12 3'-UTR.

Western blotting

Proteins were extracted from tissues and cells on ice by treating them with RIPA lysis and extraction buffer (Thermo Fisher Scientific). Bradford's assay was performed in estimating protein concentrations. Next, equal protein concentrations were resolved using SDS-PAGE and then electrophoretically transferred to nitrocellulose membranes (Sigma-Aldrich). Nonfat milk, 5%, was used to block the membranes for 60 min at room temperature. The membranes were then subjected to overnight incubation with (KLF12 (sc-134373, Santa Cruz, CA, USA) Bax (sc-7480, Santa Cruz, CA, USA), Bcl-2 (sc-23960, Santa Cruz, CA, USA) and β-actin (sc-58673, Santa-Cruz, CA, USA) at 4°C overnight (Dilution for all antibodies 1:1000). This was followed by incubation with horseradish peroxidase-conjugated anti-rabbit secondary antibody (sc-2357-CM; Santa Cruz, CA, USA) secondary antibody for an hour. An enhanced chemiluminescence (ECL) detection system (Thermo Fisher Scientific) was used for visualization of protein signals following the manufacturer's recommendations. Human β-actin was used as a reference protein in western blotting.

Statistical analysis

The offline Graphpad Prism 7.0 software was used to perform all statistical analyzes. Three experimental replicates were used to perform the study procedures and the results were given as mean ± standard deviation (S.D.). Student's t-test (unpaired and two-tailed) and one-way ANOVA were performed to analyze statistical differences. $P<0.05$ was considered to represent a statistically significant difference.

RESULTS

miR-60 is down-regulated in ovarian cancer

The isolation of RNA was carried out from 60 ovarian cancer and normal adjacent tissues. Following cDNA synthesis, qRT-PCR was performed to examine miR-650 expression levels from tissue samples. The miR-650 transcript levels were significantly ($P<0.05$) downregulated in tissues with ovarian cancer compared to adjacent normal adjacent tissue (Fig. 1A). Furthermore, the expression of miR-650 was evaluated in four different human ovarian cancer cell lines (A2780, CAOV3, HO8910 and OVCAR3). The expression of miR-650 was found to be significantly ($P<0.05$) downregulated in human ovarian cancer cells compared to normal ovarian epithelial cells (IOSE-386) (Fig. 1B). To gain insight into the role of miR-650, it was over-
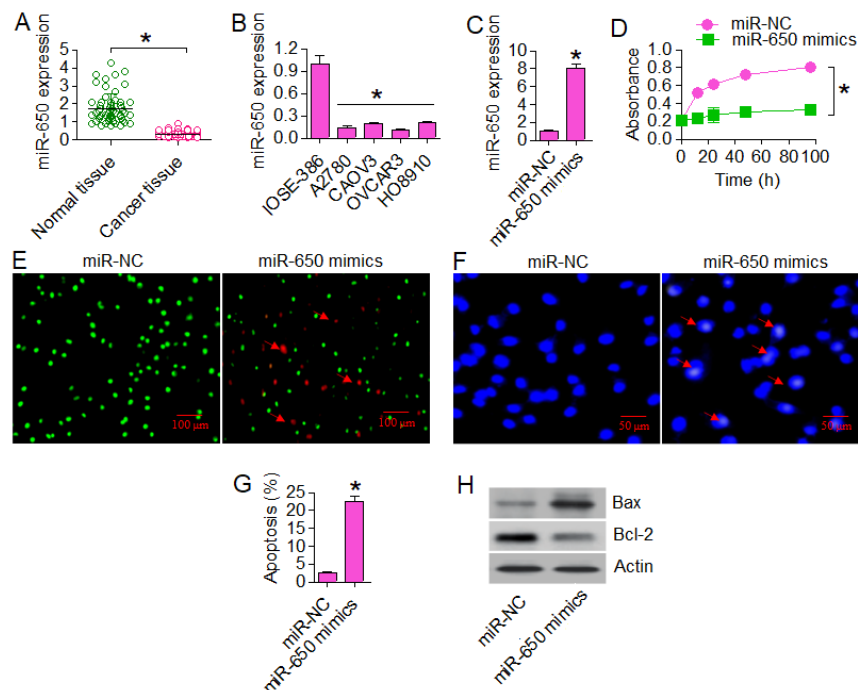

Figure 1. miR-650 inhibits viability of ovarian cancer cells
(**A**) qRT-PCR analysis showing miR-650 transcript levels in ovarian cancer tissues relative to normal adjacent tissues (**B**) qRT-PCR expression analysis showing miR-650 transcript levels in ovarian cancer cell lines relative to normal cells (**C**) qRT-PCR showing miR-650 expression in miR-NC or miR-650 mimics transfected CAOV3 cells (**D**) MTT assay showing viability of mi-RNC or miR-650 transfected CAOV3 cells (**E**) AO/EB staining of miRNC or miR-650 transfected CAOV3 cells (**F**) DAPI staining of miRNC or miR-650 transfected CAOV3 cells. Arrows represent apoptotic cells (**G**) Bar diagram showing percentage of apoptotic miR-NC or miR-650 mimics transfected CAOV3 cells (**H**) Western blots showing expression of Bax and Bcl-2 in miR-NC or miR-650 transfected CAOV3 cells. Three replicates were used for each experiment and the results were considered statistically significant at *$P<0.05$.
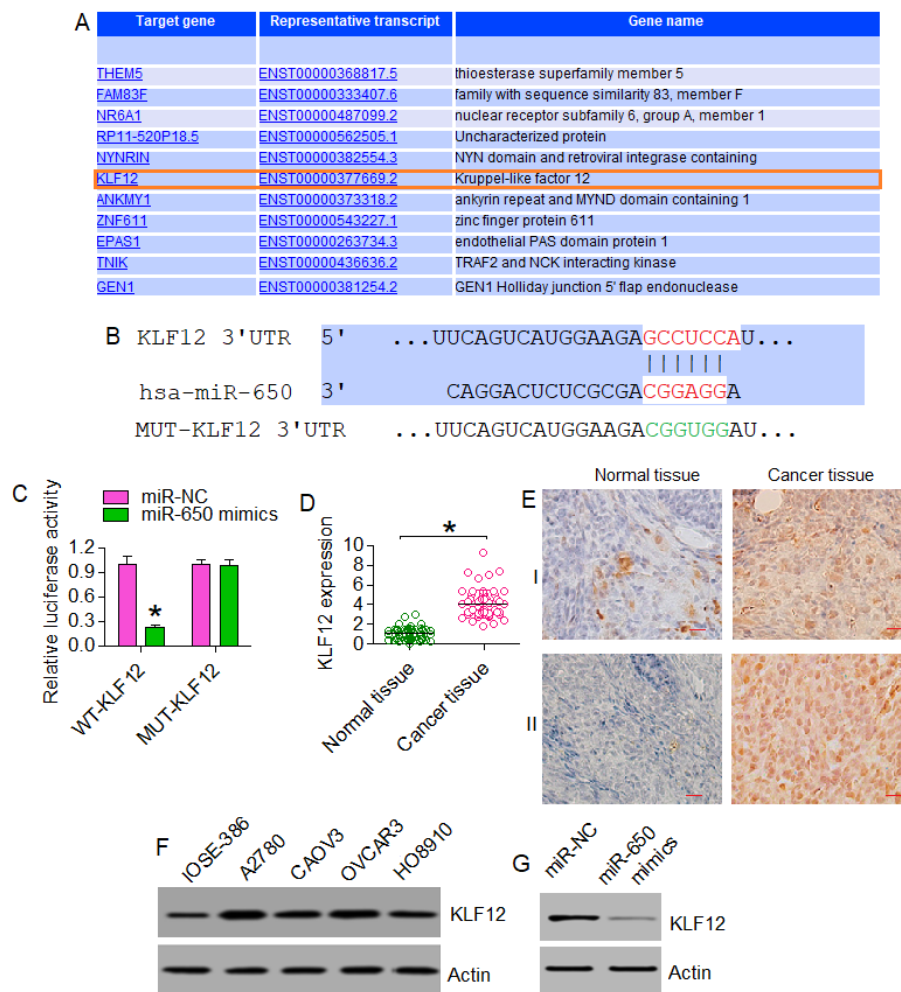
Figure 2. miR-650 tatgets KLF12 in ovarian cancer cells
(**A**) TargetScan analysis showing miR-650 potential targets (**B**) TargetScan predicting miR-650 binding with a specific seeding sequence in 3'-UTR of KLF12 (**C**) Dual luciferase assay showing the interaction of miR-650 with KLF12 (**D**) qRT-PCR showing KLF12 expression in ovarian cancer tissues relative to normal tissues CAOV3 cells (**E**) IHC analysis showing KLF12 expression in two (I and II) ovarian cancer and adjcant normal tissues (**F**) Western blots showing KLF12 expression in normal and ovarian cancer cell lines (**G**) Western blots showing KLF12 expression in ovarian cancer tranfected with miR-NC or miR-650 mimics. Three replicates were used for each experiment and the results were considered statistically significant at *$P<0.05$.

expressed in CAOV3 cells and overexpression was confirmed by qRT-PCR (Fig. 1C). Overexpression of miR-650 was found to suppress the viability of CAOV3 ovarian cancer cells (Fig. 1D). Next, the AO/EB and DAPI staining assays revealed that apoptosis is responsible for the tumor suppressing effects of miR-650 (Fig. 1E and 1F). The percentage of apoptotic cells was found to be around 23% in miR-650 mimcs transfected relative to 3% in CAOV3 cells transfected with miR-NC (Fig. 1G). This was accompanied by up-regulation of Bax and down-regulation of Bcl-2 (Fig. 1H). Thus, miR-650 suppresses the viability of CAOV3 cells by inducing apoptosis.

miR-650 taregts KLF12 in ovarian cancer

In silico analysis revealed several taregts of miR-650 (Fig. 2A). However, KLF12 was selected as miR-650 taregt for further study because it has not been previously studied as miR-650 taregt and, second, it has previously been shown to be involved in cancer related problems (17-19). KLF12 was found to have a specific binding sequence for microR-650 in 3'-UTR of KLF12 (Fig. 2B).

The dual luciferase reporter assay showed that that the luciferase activity of CAOV3 cancer cells was significantly decreased ($P<0.05$) once transfected with the KLF12 3'-UTR the KLF12 3'-UTR reporter plasmid with the wild-type binding site of miR-650 (pGL3-KLF12-WT) and mimics of miR-650,mimics of miR-650, indicative of sequence-specific binding of miR-650 to the 3'-UTR of KLF12 (Fig. 2C). The expression of KLF12 was found to be significantly ($P < 0.05$) upregulated in ovarian cancer tissues relative to normal tissues (Fig. 2D and 2E) and in ovarian cancer cell lines relative to normal IOSE-386 epithelial cells (Fig. 2F). However, miR-650 overexpression caused suppression of KLF12 in CAOV3 cells (Fig. 2G). Thus, the results indicate miR-650 taregts KLF12 in ovarian cancer.

Knockdown of KLF12 inhibits the viability of ovarian cancer cells

Next, KLF12 was silenced in CAOV3 cells and knockdown was confirmed by qRT-PCR (Fig. 3A). The elimination of KLF12 was found to cause inhibition of CAOV3 cell proliferation (Fig. 3B). Apoptosis was found
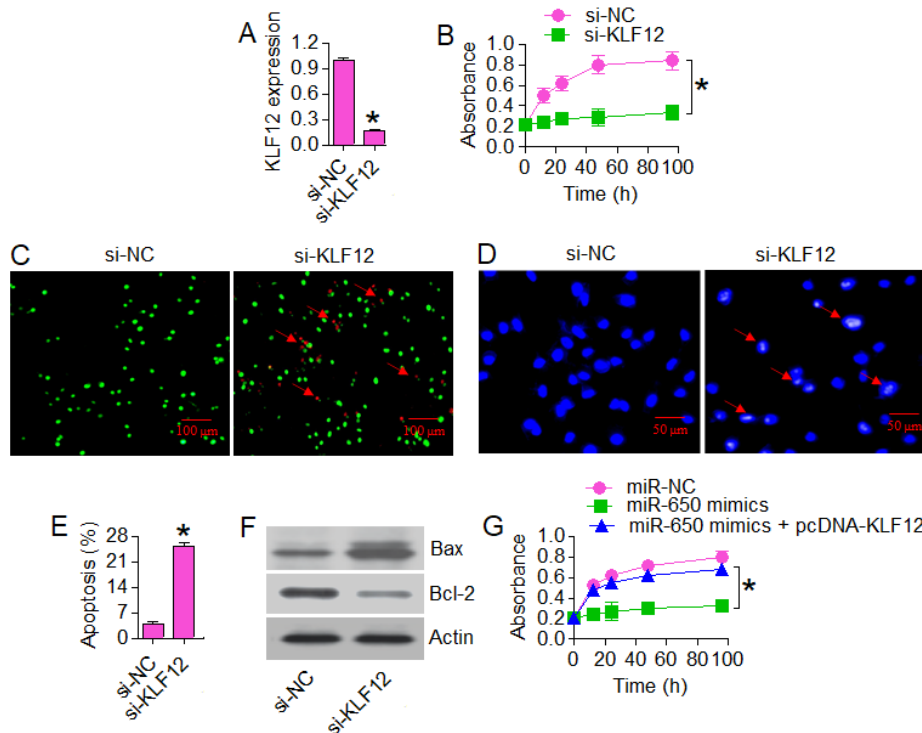
Figure 3. Knockdown of KLF12 inhibits the proliferation of ovarian cancer cells.
(A) qRT-PCR showing the expression of KLF12 in si-NC or si-KLF12 transfected CAOV3 cells (**B**) MTT assay showing the viability of si-NC or si-KLF12 transfected CAOV3 cells (**C**) AO/EB staining of si-NC or si-KLF12 transfected CAOV3 cells (**D**) DAPI staining of si-NC or si-KLF12 transfected CAOV3 cells (**E**) Percentage of apoptosis in si-NC or si-KLF12 transfected CAOV3 cells. Arrows show apoptotic cells (**F**) Western bltos showing the expression of Bax and Bcl-2 in si-NC or si-KLF12 transfected CAOV3 cells (**G**) MTT assay showing viability of miR-NC, miR-650 mimcs or miR-650 mimcs + pcDNA-KLF12 transfected cells. Three replicates were used for each experiment and the results were considered statistically significant at *$P<0.05$

to be responsible for the inhibitory effects observed under KLF12 silencing (Fig. 3C and 3D). The percentage of apoptotic cells was found to be around 25% in si-KLF12 transfected compared to 4% in si-NC transfected CAOV3 cells (Fig. 1E). Furthermore, KLF12 knockdown-induced apoptosis was accompanied by up-regulation of Bax and down-regulation of Bcl-2 (Fig. 3F). Intrestingly, overexpression of KLF12 could eliminate the tumor-suppressive effects of miR-650 in ovarian cancer cells (Fig. 3G).

The miR-650/KLF12 axis regulates the migration and invasion of ovarian cancer cells

Next, the effects of KLF12 silencing on CAOV3 cell migration and invasion were also examined by transwell assays. The elimination of KLF12 was found to significantly ($P<0.05$) suppressed migration (Fig. 4A) and invasion (Fig. 4B) of CAVO3 cells ($P<0.05$). Both migration and invasion of CAOV3 cells was affected by more than 70% after KLF12 knockdown. Intrestingly, overexpression of miR-650 also caused inhibtion of migration and invasion of CAOV3 cells. However, overexpression of KLF12 could nullify the inhibitory effects of miR-650 on migration and invasion of CAOV3 ovarian cancer cells (Fig. 4A and 4B). Thus, the miR-650/KLF12 axis regulates the migration and invasion of ovarian cancer cells.

DISCUSSION

Ovarian cancer is a highly lethal gynecological disorder with poorly understood molecular pathogenesis

(Stewart *et al.*, 2019). The lack of efficient molecular markers and therapeutic targets prevents early macular cancer of ovarian cancer (Boussios *et al.*, 2020). Here, we investigate the role and explore the therapeutic implications of the miR-650/KLF12 axis in ovarian cancer. The results showed a downregulation of miR-650 in tissues and ovarian cancer cell lines and its overexpression inhibited the viability of ovarian cancer cells. Previously, miR-650 was shown to be down-regulated in glioma (Xu *et al.*, 2018), and leukemia (Yuan *et al.*, 2018) and functions as a tumor suppressor in these cancers. Defective apoptosis is one of the hallmarks of cancer cells, whereby induction of apoptosis may prevent cancer development (Wang *et al.*, 2019). Several miRs have been shown to prevent tumorigenesis by promoting apoptosis (Lee *et al.*, 2020). Consistently, we found that miR-650 exerts tumor suppressive effects on ovarian cancer cells by promoting apoptosis. Bax is a pro-apoptotic protein that is a transcriptional target for p53. Bax can inhibit Bcl-2 activity and induce cell apoptosis (Azimian *et al.*, 2018). Here, we found that miR-650 increased Bax and decreased Bcl-2 protein levels, thus favoring apoptosis. Generally, miRs regulate various cellular processes by modulating the expression of target genes. A single miR may transcribe several genes post-transcriptionally (Hashimoto *et al.*, 2013). For example, miR-650 has been shown to target Gfi1 in leukemia (Yuan *et al.*, 2018) and oral cancer (Ningning *et al.*, 2019), FAM83F in glioma (Xu *et al.*, 2018) and CSR1 in prostate cancer (Zuo *et al.*, 2015). Herein, we found that miR-650 regulates the viability of ovarian cancer cells by post-transcriptionally regulating the expression of KLF12. KLF proteins are the transcription factors that regulate crucial cellular pro-
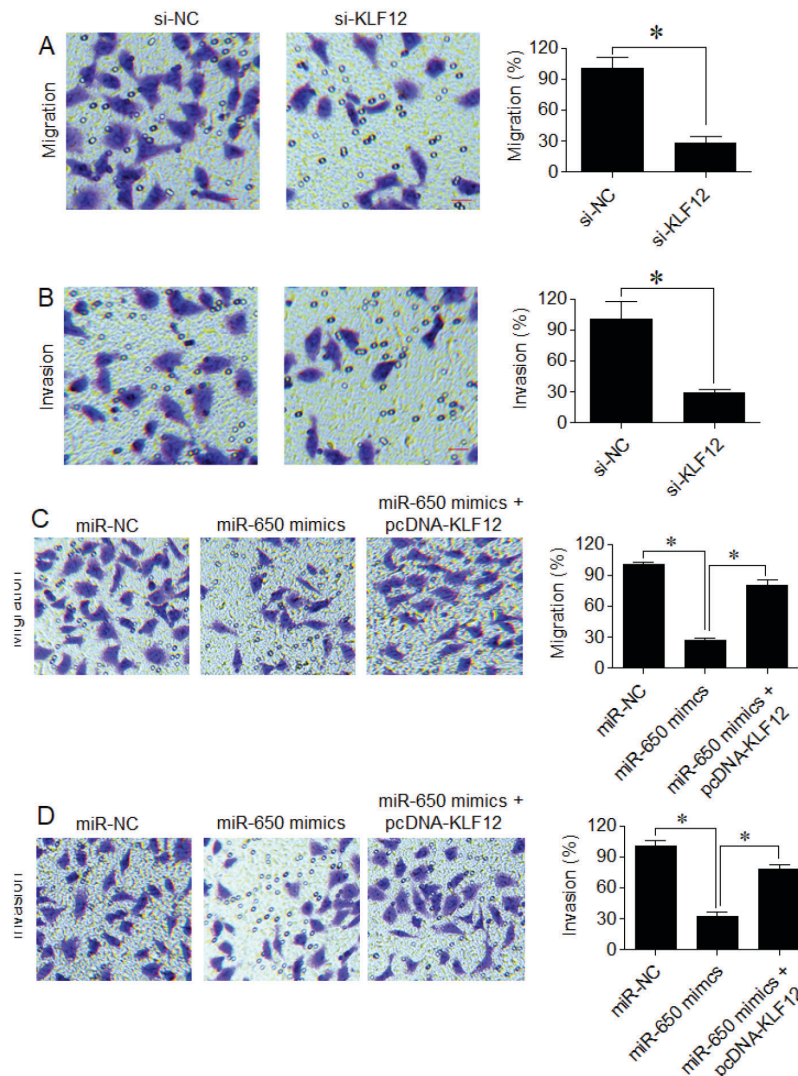
Figure 4. The miR-650/KLF12 axis regulates migration and invasion of ovarian cancer cells
(**A**) Transwell assay showing migration of si-NC or si-KLF12 transfected CAOV3 cells (**B**) Transwell assay showing invasion of si-NC or si-KLF12 transfected CAOV3 cells (**C**) Transwell assay showing migration of miR-NC, miR-650 mimics or miR-650 mimics + pcDNA-KLF12 transfected CAOV3 cells (**D**) Transwell assay showing invasion of miR-NC, miR-650 mimics or miR-650 mimics + pcDNA-KLF12 transfected CAOV3 cells. Three replicates were used for each experiment and the results were considered statistically significant at *$P<0.05$.

cesses including proliferation, apoptosis, migration, and pluripotency (Miller & Bieker, 1993; McConnell & Yang, 2010). As reflected in a number of research studies, several members of the KLF family have been implicated in a key regulatory role in human cancers (Tetreault *et al.*, 2013). KLF12 has been shown to promote the growth and progression of human colorectal cancer (Kim *et al.*, 2016). The transcription factor has also been shown to be involved in gastric cancer progression (Nakamura *et al.*, 2009). Furthermore, KLF12 overexpression was previously indicated to be associated with the progression of human endometrial cancer (Ding *et al.*, 2019). There are also reports that KLF12 regulates ovarian cancer growth and metastasis (Mak *et al.*, 2017; Zhang *et al.*, 2021). Herein, KLF12 was shown to be significantly up-regulated in ovarian cancer tissues and cell lines, suggesting its possible involvement in the regulation of ovarian cancer tumorigenesis. While KLF12 knockdown suppresses ovarian cancer cell viability through induction of apoptosis, its overexpression nullifies the tumor suppressive effects of miR-650 on ovarian cancer cells. Xun and others (Xun *et al.*, 2019) have recently shown that

KLF12 negatively regulates gastric cancer cell apoptosis, which is in agreement with the findings of the present study. It is noteworthy that KLF12 knockdown or miR-650 overexpression was initiated, while KLF12 overexpression promoted the migration and invasion of ovarian cancer cells even under miR-650 overexpression. These results indicate the regulatory role of miR-650/KLF12 axis in the metastatic behavior of ovarian cancer cells. Taken together, these findings suggest that the miR-650/KLF12 axis regulates the proliferation and metastasis of ovarian cancer cells. However, more studies are required involving *in vivo* systems for further confirmation.

CONCLUSION

Taken together, the results revealed a downregulation of miR-650 in ovarian cancer. Overexpression of miR-650 suppressed viability and induced apoptosis in ovarian cancer cells by targeting KLF2. In addition, the miR-650/KLF12 axis regulates migration and invasion of ovarian cancer cells. Therefore, the study revealed the

possible therapeutic importance of the miR-650/KLF12 molecular axis in human ovarian cancer.

Declarations

Conflict of interest. The authors declare that there are no conflicts of interest.

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