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ORIGINAL ARTICLE

MicroRNA-613 inhibited ovarian cancer cell proliferation and invasion by regulating KRAS

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Abstract MicroRNAs (miRNAs) play several important roles in carcinogenesis, and the dysregulation of miRNAs is associated with cancer progression. Little is known about the role of miR-613 in ovarian cancer. In the present study, we demonstrate that miR-613 expression is downregulated in human ovarian cancer cell lines and tissues. Additionally, miR-613 overexpression suppressed ovarian cancer cell proliferation, colony formation, and invasion. Furthermore, KRAS was identified as a target of miR-613. Reintroducing KRAS rescued the inhibitory effects exerted by miR-613 on ovarian cancer cell proliferation and invasion. Taken together, our findings suggest that miR-613 functions as a candidate tumor suppressor miRNA in ovarian cancer by directly targeting KRAS. To the best of our knowledge, this is the first study to show that miR-613 affects the proliferation and invasion of ovarian cancer.

Keywords Ovarian cancer · MicroRNAs · miR-613 · KRAS

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Introduction

Ovarian cancer is the leading cause of death among women worldwide and is one of the most common gynecological malignancies, causing over 140,000 deaths annually [1–3]. Although advances have been made in its treatment by combining chemotherapy, surgery, and radiotherapy, the overall ovarian cancer survival rate has not changed in the past 50 years [4–7]. Therefore, the identification of new molecular biomarkers is important for improving the clinical outcome of patients suffering from ovarian cancer [8–10].

MicroRNAs (miRNAs) are a recently discovered class of small, non-coding RNAs that range in size from 19 to 25 nucleotides (nt) and are capable of regulating gene expression at both the transcriptional and translational levels [11–14]. miRNAs play important roles in various cellular processes, including cell proliferation, apoptosis, differentiation, migration, and invasion, that are important in cancer development [15–18]. Increasing evidence has shown that the deregulation of miRNAs is related to the initiation and development of various cancers such as bladder cancer, gastric cancer, lung cancer, breast cancer, and hepatocellular carcinoma [19–23]. miRNAs may also serve as potential biomarkers to aid in the prognostic assessment and diagnosis of various cancers [24–27].

miR-613 is a newly discovered miRNA, its function and mechanism of action in biological processes and diseases are not completely understood [28, 29]. A previous study showed that miR-613 acts as a tumor suppressor gene in papillary thyroid cancer by negatively regulating FN1 expression [30]. Moreover, miR-613 plays a role in cholesterol efflux by targeting the 3′-UTRs of LXRa and ABCA1 mRNAs [31]. However, the role of miR-613 is still uncovered. In the present study, we found that miR-613 is frequently downregulated in ovarian cancer cells and tissues and is a potential tumor-suppressing miRNA that regulates KRAS expression in ovarian cancer.
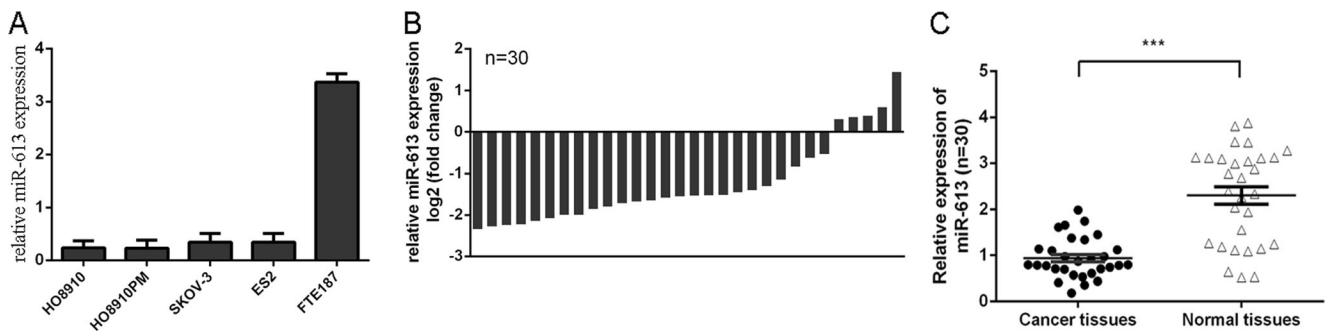
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Fig. 1 Reduced miR-613 expression in ovarian cancer cell lines and tissue specimens. **a** Relative miR-613 expression in ovarian cancer cell lines (HO8910, HO8910PM, SKOV-3 and ES2) and in non-cancerous FTE187 cells. **b** Relative miR-613 expression in 30 pairs of ovarian cancer tissues and adjacent normal counterpart tissues was detected using real-time RT-PCR. **c** A comparison of the average expression of miR-613 between ovarian cancer tissues and non-tumor tissues. ***$p < 0.001$

Materials and methods

Cell lines and tissues

Human ovarian cancer cell lines (HO8910, HG-SOC, HO8910PM, SKOV-3 and ES2) and an immortalized normal human fallopian tube epithelial cell line (FTE187) were obtained from the China Type Culture Collection (Shanghai, China). The cells were cultured in RPMI-1640 medium supplemented with 10 % fetal calf serum (FCS; Invitrogen, Carlsbad, CA, USA). Primary epithelial ovarian cancer tissues and adjacent normal ovarian tissues (located >3 cm from the tumor) were collected during surgery in our hospital. None of the patients had received chemotherapy before surgical resection. The characteristics of the patients are described in Table S1.

RNA isolation and quantitative RT-PCR

Total RNA was isolated from cells or tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcription was performed with 1 mg of total RNA using M-MLV Reverse Transcriptase (Promega). Real-time PCR (qRT-PCR) was performed with TaqMan Fast Advanced Master Mix (Applied Biosystems) using an ABI Prism 7700 system (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The following primers were used: KRAS forward, 5′-GACTCTGAAGATGTACCTATGGTC CTA-3′; KRAS reverse, 5′-CATCATCAACACCCTGTC TTGTC-3′; GAPDH forward, 5′-TTGGTATCGTG GAAGGACTCA-3′; GAPDH reverse, 5′-TGTCATCATATTTGGCAGGTT-3′. The reaction conditions were as follows: 95 °C for 30 s, followed by 48 cycles of 95 °C for 5 s, 60 °C for 10 s, and 72 °C for 30 s. Relative miR-613 expressions were calculated with normalization to U6 and GAPDH was used to as internal controls for KRAS mRNAs.

Western blot analysis

Total proteins were isolated from cells or tissues, and protein concentrations were detected using the BCA Assay Kit (Thermo Scientific). Protein samples were separated by 10 % SDS-PAGE and transferred onto PVDF membranes (Millipore). The membranes were blocked with 5 % non-fat milk for 2 hours and then incubated overnight at 4 °C with the primary antibody (KRAS or GAPDH, Sigma-Aldrich, USA). Signals were detected using the enhanced chemiluminescence (ECL) luminol reagent (PerkinElmer Inc.). GAPDH was used as a loading control.

Oligonucleotide transfection

miR-613 mimics (5′-AGGAAUGUUCCUUCUUUGCC-3′) and negative control oligonucleotides (scramble control, 5′-UUCUCCGAACGUGUCACGUTT-3′) were synthesized by Dharmacon (Austin, TX) and transfected into cells with Lipofectamine 2000 (Invitrogen) at a final concentration of 20 nm, according to the manufacturer's instructions.

Cell proliferation and colony formation assays

For the cell proliferation assay, cells were plated into 96-well plates and cultured for 0, 1, 2, or 3 days, after which CCK-8 (10 μL) was added to each well. The cells were then incubated for an additional 2 h, and the absorbance was then detected at a wavelength of 450 nm. For the colony formation assay, cells were seeded onto six-well plates at a density of 500 cells per well. After 10 days, the colonies were fixed with methanol and stained with 1 % crystal violet, and the number of colonies was then counted.

Invasion assays

Cells were plated in the upper chambers of Matrigel-coated wells (1:5 dilution in serum-free medium), and medium supplemented with 10 % serum was added to the lower chamber.
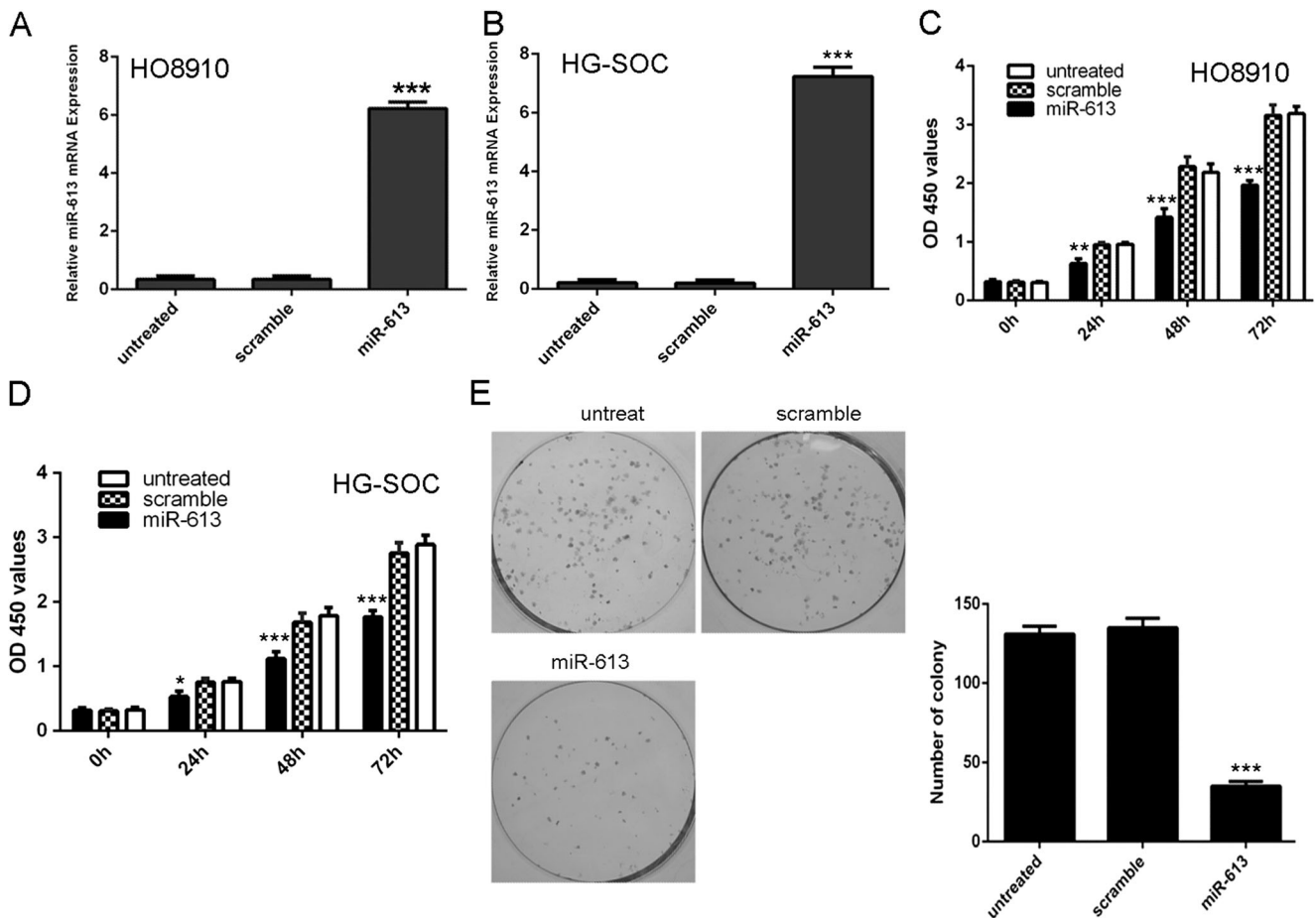
Fig. 2 miR-613 inhibits ovarian cancer cell proliferation. **a** Relative miR-613 expression in HO8910 cells was measured after the cells were transfected with miR-613 mimics or scramble control miRNA using real-time RT-PCR. **b** Relative miR-613 expression in HG-SOC cells was measured after the cells were transfected with miR-613 mimics or scramble control miRNA using real-time RT-PCR. **c** Cell proliferation was measured using a CCK8 assay. HO8910 cells were transfected with miR-613 mimics or scramble control miRNA. **d** Overexpression of miR-613 inhibited HG-SOC cells proliferation. **e** A colony formation assay was performed with HO8910 cells transfected with miR-613 mimics or scramble control miRNA. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$

After culturing for 24 h, the cells remaining on the upper surface of the membranes were removed, and the cells on the lower surface of the chamber were then stained with 0.1 % crystal violet (Sigma) and counted.

Luciferase assay

pGL3-KRAS-3′UTR-MUT or pGL3-KRAS-3′UTR-WT constructs were transfected into cells together with the miR-613 mimics or scramble control and the pRL-TK vector (Promega). After 48 h of transfection, luciferase activity was measured using the Dual Luciferase Reporter Assay kit (Promega) according to the manufacturer's instructions. Renilla luciferase activity was detected for normalization.

Statistical analysis

The statistical analysis was performed using SPSS 17.0 software. Data are expressed as the means with standard deviations (SD). Differences between groups were evaluated using Student's t test when comparing only two groups or using one-way analysis of variance (ANOVA) when comparing more than two groups.

Results

miR-613 expression is reduced in ovarian cancer tissues and cell lines

miR-613 expression was detected by qRT-PCR in ovarian cancer cell lines (HO8910, HO8910PM, SKOV-3 and ES2) and an immortalized normal human fallopian tube epithelial cell line (FTE187). All four ovarian cancer cell lines tested had lower miR-613 levels than did the FTE187 cells (Fig. 1a). Of 30 ovarian cancer samples, miR-613 was downregulated in 25 cases (25/30; 83 %) compared with the adjacent tissues (Fig. 1b). Moreover, miR-613 expression in ovarian cancer

tissues was significantly lower than that in adjacent tissues (Fig. 1c).

miR-613 inhibits ovarian cancer cell proliferation

We generated miR-613-overexpressing HO8910 and HG-SOC cells by transiently transfecting cells with miR-613 mimics. miR-613 expression was confirmed by RT-PCR (Fig. 2a, b). miR-613 overexpression in the ovarian cancer cells resulted in significantly reduced cell proliferation in both HO8910 and HG-SOC cells (Fig. 2c, d). The colony-forming assay results also showed that miR-613 overexpression inhibited colony formation by the HO8910 cells (Fig. 2e).

miR-613 inhibits ovarian cancer cell invasion

A Matrigel invasion assay was performed to assess the effects of miR-613 on the invasive capacity of ovarian cancer cells. miR-613 overexpression repressed the invasive capacity of HO8910 and HG-SOC cells compared with that of cells transfected with the scramble control (Fig. 3a–d).

KRAS is a candidate target of miR-613

A putative miR-613-binding site was detected at the end of the KRAS 3′UTR using TargetScan v5.1 (Whitehead Institute for Biomedical Research, Cambridge, MA, USA) target prediction software (Fig. 4a). The luciferase reporter activity decreased by approximately 60 % in HO8910 cells or 65 % in HG-SOC cells containing the KRAS wild-type 3′UTR fragment (Fig. 4b, d). Additionally, miR-613 overexpression

suppressed the KRAS protein expression in both HO8910 and HG-SOC cells, according to western blot analysis (Fig. 4c, e).

KRAS overexpression rescues the inhibitory effects of miR-613 on cell proliferation and invasion

KRAS mRNA and protein expression in ovarian cancer cell lines (HO8910, HO8910PM, SKOV-3 and ES2) and FTE187 cells was detected by qRT-PCR and western blotting. All four ovarian cancer cell lines had higher KRAS levels compared with the FTE187 cells (Fig. 5a, b). KRAS overexpression via KRAS plasmid transfection enhanced KRAS protein expression (Fig. 5c). KRAS expression in miR-613-overexpressing HO8910 cells was rescued by transfecting the cells with KRAS plasmids. The CCK8 proliferation assay showed that KRAS overexpression rescued the proliferation ability of the HO8910 cells (Fig. 5d). Furthermore, an invasion assay showed that KRAS overexpression rescued the invasive capacity of HO8910 cells (Fig. 5e).

Discussion

Increasing evidence has shown that miRNAs are involved in various aspects of carcinogenesis and function as either tumor suppressors or oncogenes [18, 32–36]. In this study, we first demonstrated that miR-613 levels are lower in ovarian cancer cells compared with normal human fallopian tube epithelial cells. We also showed that miR-613 expression was downregulated in ovarian cancer

Fig. 3 miR-613 inhibits ovarian cancer cell invasion. **a** The invasive capacity of HO8910 cells was measured by an invasion assay after transfecting the cells with miR-613 mimics or scramble control miRNA for 48 h. **b** The relative ratio of invasive cells per field is shown. **c** Overexpression of miR-613 inhibited the HG-SOC cells invasion. **d** The relative ratio of invasive cells per field is shown. ***p < 0.001
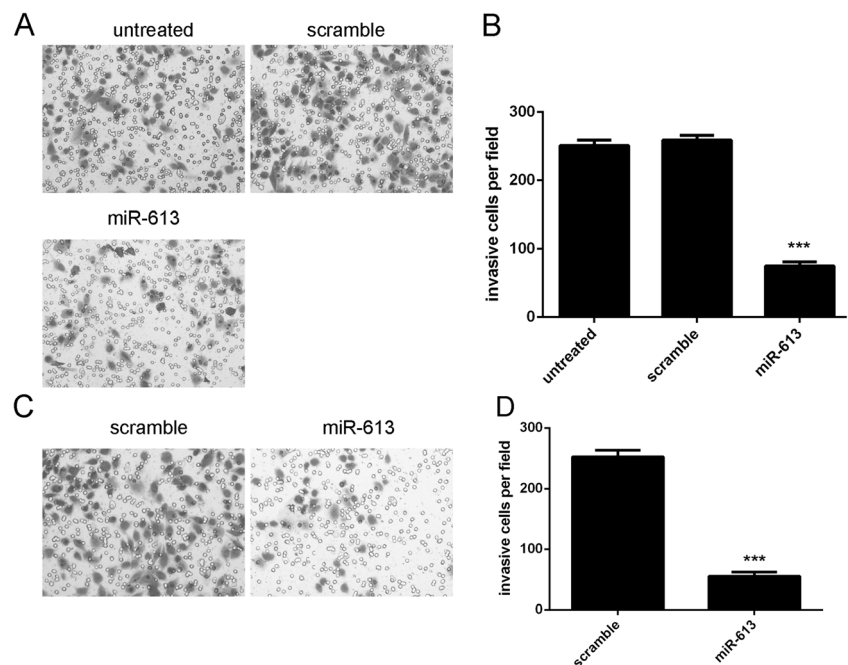
Fig. 4 KRAS is a candidate target of miR-613. **a** The predicted miR-613 target sequence in the 3′UTR of KRAS (KRAS-3′-UTR-WT) and a mutant containing seven altered nucleotides in the 3′UTR of KRAS (KRAS-3′-UTR-MUT). **b** The miR-613 mimic inhibited the luciferase activity controlled by wild-type KRAS-3′-UTR but did not affect the luciferase activity controlled by mutant KRAS-3′-UTR in HO8910 cells. **c** Western blot analysis was performed to detect KRAS protein expression in HO8910 cells transfected with miR-613 mimics or scramble control miRNA. **d** The miR-613 mimic inhibited the luciferase activity controlled by wild-type KRAS-3′-UTR but did not affect the luciferase activity controlled by mutant KRAS-3′-UTR in the HG-SOC cells. **e** Overexpression of miR-613 inhibited KRAS protein expression in the HG-SOC cells. GAPDH was used as a loading control. ***$p < 0.001$
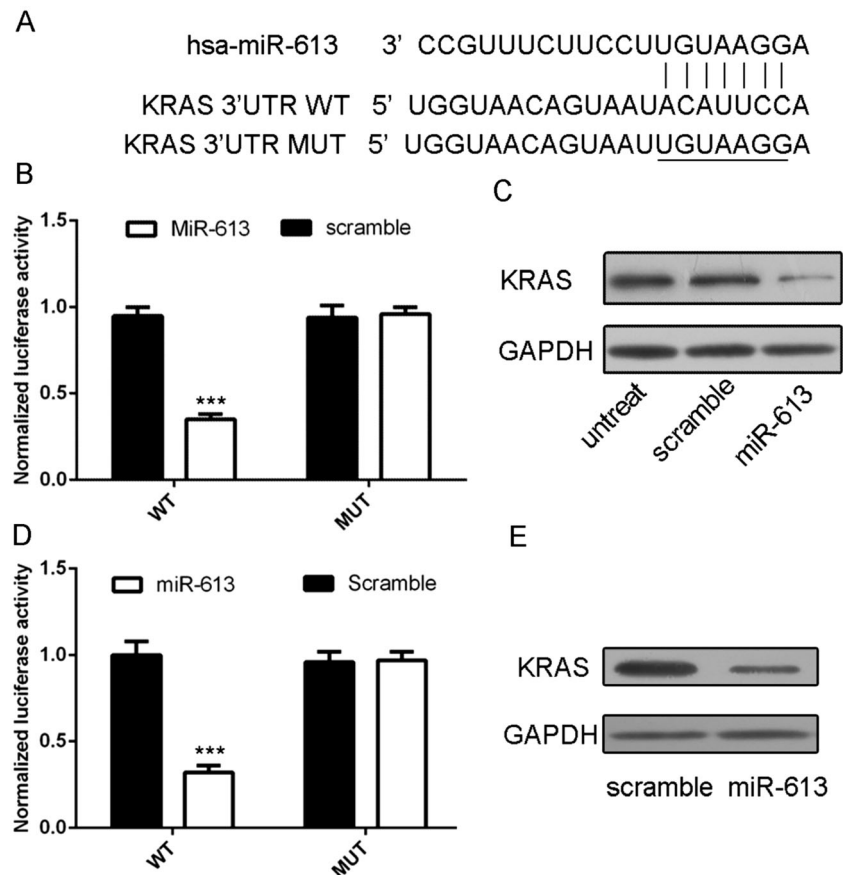

tissues compared with adjacent tissues. Additionally, miR-613 overexpression inhibited ovarian cancer cell proliferation, colony formation, and invasion, and we identified KRAS as a new target of miR-613. Our data showed that KRAS overexpression rescued the inhibitory effects exerted by miR-613 on cell proliferation and invasion. In summary, our results revealed that miR-613 may act as a tumor suppressor in ovarian cancer, at least partly by regulating KRAS expression.

Because miR-613 is a relatively newly discovered miRNA, its function and mechanism of action in biological processes and diseases are not completely understood [28, 29]. A previous study showed that miR-613 acts as a tumor suppressor gene in papillary thyroid cancer by negatively regulating FN1 expression [30]. Moreover, miR-613 plays a role in cholesterol efflux by targeting the 3′-UTRs of LXRa and ABCA1 mRNAs [31]. In the present study, we first detected miR-613 expression in several ovarian cancer cell lines. We found that the miR-613 levels were lower in ovarian cancer cells compared with normal human fallopian tube epithelial cells. We also found that miR-613 expression was downregulated in ovarian cancer tissues compared with adjacent tissues. We subsequently exploited this in vitro model to validate the tumor suppressor role of miR-613 in ovarian cancer.

Our data showed that miR-613 overexpression repressed ovarian cancer cell proliferation, colony formation, and invasion.

In this study, we identified KRAS as a new target of miR-613. We demonstrated that miR-613 inhibited KRAS expression by targeting its 3′UTR, indicating that KRAS is a direct target of miR-613. Furthermore, we performed western blot experiments to demonstrate that the overexpression of miR-613 could inhibit KRAS protein expression. KRAS is a crucial molecule involved in ovarian cancer progression and development [37]. Mutations of the KRAS oncogene are significantly associated with a lower grade ($p = 0.001$), the mucinous histological subtype ($p < 0.001$) and progesterone receptor expression ($p = 0.035$) in ovarian cancer [38]. The KRAS oncogene is a critical regulator of cell proliferation and is frequently mutated in ovarian cancer [39]. In line with this knowledge, we found here that KRAS expression was higher in ovarian cancer cells compared with normal human fallopian tube epithelial cells. Furthermore, KRAS overexpression was shown to reverse the effects of miR-613 on ovarian cancer cell proliferation and invasion. Taken together, the present data demonstrate that miR-613 regulates KRAS expression and thus functions as a tumor suppressor gene in ovarian cancer.

Fig. 5 KRAS overexpression rescues the inhibitory effect of miR-613 on ovarian cancer cell proliferation and invasion. **a** Relative KRAS mRNA expression in ovarian cancer cell lines (HO8910, HO8910PM, SKOV-3 and ES2) and non-cancerous FTE187 cells. **b** Relative KRAS protein expression in ovarian cancer cell lines (HO8910, HO8910PM, SKOV-3 and ES2) and non-cancerous FTE187 cells. **c** Western blot analysis showed that transfecting the cells with a KRAS vector could restore KRAS expression. KRAS expression was normalized to that of GAPDH. **d** The proliferation of miR-613-overexpressing HO8910 cells was partially rescued after the cells were transfected with a KRAS vector. **e** The invasive capacity of miR-613-overexpressing HO8910 cells was partially rescued after the cells were transfected with a KRAS vector. **p < 0.01 and ***p < 0.001
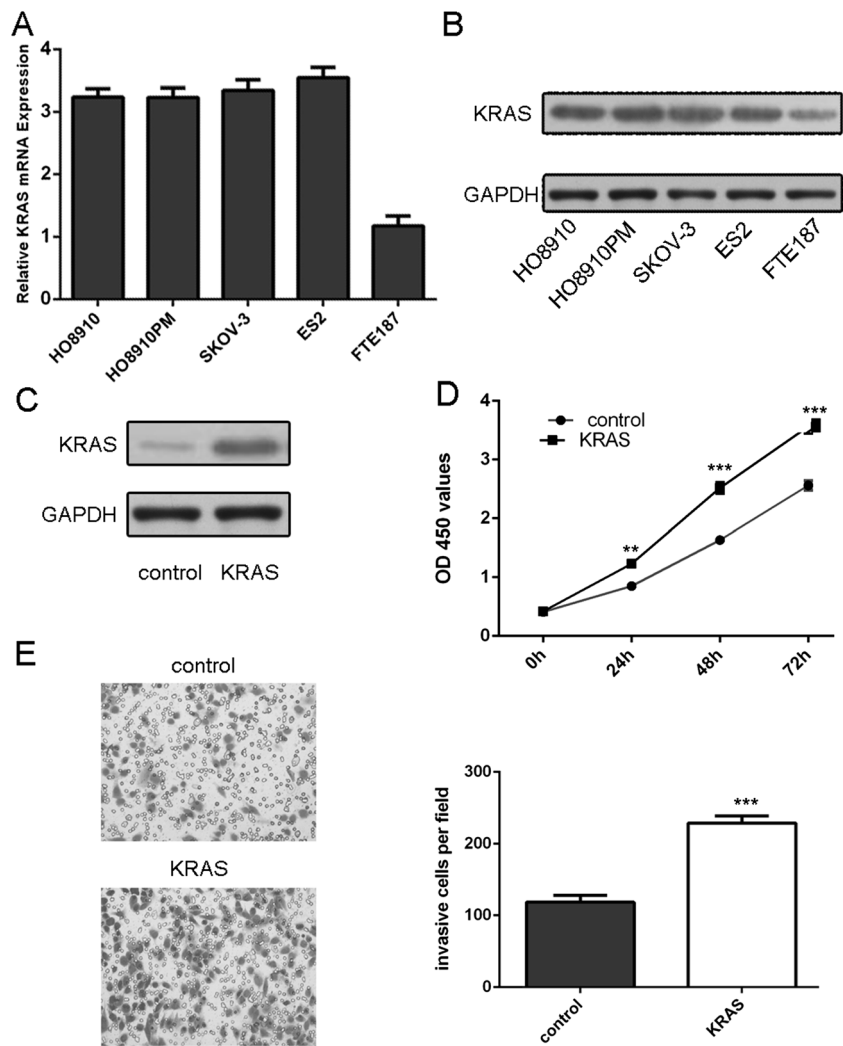
In summary, our work demonstrates that miR-613 is frequently downregulated in ovarian cancer and is a potential tumor-suppressing miRNA that may decrease ovarian cancer development and/or progression. KRAS regulation by miR-613 might play a role in its regulation of the malignant behavior of ovarian cancer. This study therefore provides a novel and promising therapeutic target for ovarian cancer.

Compliance with ethical standards

Ethics statement All of the patients provided written informed consent. This study was approved by the Ethics Committee of Tianjin Medical University Cancer Institute and Hospital and complied with the Declaration of Helsinki.

Conflicts of interest None

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