miR-454 suppresses the proliferation and invasion of ovarian cancer by targeting E2F6

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Abstract

**Background:** It has been reported that hypoxia is closely related to the tumor malignancy and recurrence and regulates multiple hub genes in ovarian cancer. MicroRNA-454 (miR-454) has been confirmed to be involved in tumorigenesis and tumor development. However, the functional role of miR-454 in ovarian cancer remains unclear.

**Methods:** The expression of miR-454 in ovarian cancer cells and serum of ovarian cancer patients was detected by RT-PCR. CCK8, colony formation, transwell, and flow cytometry assays were conducted to assess the effects of miR-454 on ovarian cancer cell proliferation, migration, invasion, and apoptosis. Dual-luciferase reporter assay was used to confirm the targeting relationship between miR-454 and E2F6. The expression pattern of E2F6 in ovarian cancer tissues was detected using immunohistochemistry assay. The relative expression of related proteins was examined using western blot analysis.

**Results:** miR-454 was markedly down-regulated by hypoxia in ovarian cancer cells. Compared with normal serum, the expression of miR-454 was up-regulated in the serum of ovarian cancer patients, and was correlated with the clinicopathological stage of ovarian cancer patients. Next, we found that miR-454 overexpression inhibited the proliferation, migration and invasion of OVCAR3 and SKOV3 cells, as well as promoted apoptosis. In addition, the Akt/mTOR and Wnt/β-catenin signaling pathway were inhibited by miR-454. Bioinformatic analysis and dual-luciferase reporter assay confirmed that E2F6 was a target of miR-454 and negatively regulated by miR-454 in ovarian cancer cells. Moreover, immunohistochemical analysis showed that E2F6 was highly expressed in ovarian cancer tissues. Finally, we found that the increasing cell proliferation and migration triggered by E2F6 overexpression were abolished by miR-454 overexpression.

**Conclusion:** Taken together, these results highlight the role of miR-454 as a tumor suppressor in ovarian cancer by targeting E2F6, indicating that the hypoxia/miR-454/E2F6 pathway may be a novel therapeutic approach for ovarian cancer.

**Background**

Ovarian cancer has the highest mortality rate in gynecological malignancies, with approximately
140,000 deaths worldwide each year [1, 2]. Ovarian cancer is an occult tumor with early symptoms that are not obvious and easy to metastasize early. In addition, most patients with ovarian cancer are in advanced stages at the time of diagnosis [3]. Despite significant improvements in surgical techniques and chemotherapy in recent decades, the 5-year survival rate for patients with ovarian cancer is still below 50% [4, 5]. Therefore, it is urgent to develop novel therapeutic targets for ovarian cancer treatment.

Increasing number of studies reveal that microRNAs (miRNAs) are closely involved in tumorigenesis and tumor progression [6-8]. miRNAs negatively regulate expression of target gene by binding to the 5'-UTR of target gene to inhibit mRNA translation or promote mRNA degradation [9, 10]. Many miRNAs have been proved to be dysregulated in ovarian cancer, and act as tumor suppressor or promoter in the growth and metastasis of ovarian cancer [11-13]. The study has also revealed that miRNAs are closely related to the prognosis of patients with ovarian cancer and might function as potential prognostic biomarkers [14, 15]. Thus, miRNAs have the potential as therapeutic targets and prognostic biomarkers, and are expected to become important diagnostic and therapeutic tools for ovarian cancer. Of them, miR-454 has been reported to be implicated in the progression of many types of cancer. However, previous studies have demonstrated that miR-454 plays different roles in different tumors, functioning as an oncogene in colorectal cancer [16], hepatocellular carcinoma [17] and non-small cell lung cancer [18], but servers as a tumor suppressor in osteosarcoma [19] and glioblastoma [20]. However, the function and mechanism of miR-454 in ovarian cancer remains largely unclear.

The results of the current study showed that miR-454 was down-regulated by hypoxia in ovarian cancer cells. We also demonstrated that miR-454 overexpression suppressed the proliferation, migration and invasion of ovarian cancer cells, as well as promoted cell apoptosis. Further, E2F6 was identified as a target of miR-454 and up-regulated in ovarian cancer tissues, which is involved in the tumor suppressive role of miR-454. This study advances the understanding of the mechanism of hypoxia affecting the occurrence and development of ovarian cancer, and suggest that the hypoxia/miR-454/E2F6 pathway may be a novel therapeutic approach for ovarian cancer.
Materials And Methods

Cell lines and cell culture

OVCAR3 and SKOV3 cells were obtained from Cell Bank of Chinese Academy of Sciences (Shanghai, China) and maintained in RPMI-1640 medium (HyClone, USA) supplemented with 10% FBS. Cells were transfected with pCMV-MIR-miR-454 (Ribobio, Guangzhou, China) using Lipofectamine 2000 (Invitrogen, CA, USA). pCMV-MIR vector was used as negative control (NC). The E2F6 cDNA sequences were cloned into pcDNA3.1 vector and the pcDNA3.1-E2F6 was transfected into cells using Lipofectamine 2000.

Clinical samples

Seventy-five cases of ovarian cancer tissues and fifteen cases of tumor-adjacent tissues were obtained from Beijing Anzhen Hospital, Capital Medical University. All patients gave informed consent in written, and this study was approved by the ethics committee of Beijing Anzhen Hospital, Capital Medical University. Venous blood was collected from 13 patients with ovarian cancer and 6 normal subjects from Beijing Anzhen Hospital, Capital Medical University. All blood samples were taken before patient treatment.

Quantitative RT-PCR analysis

Total RNA extracted from OVCAR3 and SKOV3 cells or serum samples using TRizol Reagent. The SYBR PrimeScript miRNA RT PCR kit (Takara, Shiga, Japan) was performed for RT-PCR reaction to examine the expression of miR-454. For detection of E2F6 mRNA expression, a HiFiScript cDNA Synthesis Kit (CWBIO, Beijing, China) was used to reverse transcribe RNA to cDNA, and a SYBR Premix Ex Taq II kit (Takara) was performed for RT-PCR reaction. Primers were obtained from Ribobio. The relative expression of miR-454 or E2F6 mRNA was calculated using the $^{2-\Delta\Delta CT}$ method and normalized to NC group.

CCK8 assay

Cells were seeded in a 96-well plate at a density of 1000 cells/well and cultured for 0, 24, 48, and 72 h, respectively. Then, 10μl of CCK8 reagent (Beijing Solarbio Science & Technology, Beijing, China) was added into each well. Following incubation for 1.5 h, the absorbance was detected at 450 nm.
Colony formation assay

Cells were cultured in 60-mm dishes (500 cells/dish) and incubated for 1-2 weeks. After that, cell colonies were fixed with 4% paraformaldehyde for 30 min and dyed with 0.1% crystal violet for another 30 min. Finally, the number of cell colonies was counted.

Transwell migration and invasion assay

Transwell chambers (Millipore, MA, USA) were used to measure cell migration and invasion. About $1 \times 10^5$ cells were seeded in the upper chamber and the lower chamber was filled with RPMI-1640 medium containing 20% FBS. Following incubation of 12 h, the migrated or invaded cells were fixed with 4% paraformaldehyde, and stained with 0.1% crystal violet for 20 min. In invasion experiment, the upper chamber was coated with Matrigel (BD Bioscience, CA, USA) before seeding cells.

Flow cytometry assay

Cells transfected with plasmids of 24 h were cultured in serum-free medium for 24 h, and re-suspended in loading buffer after PBS washing. An Annexin V/FITC-propidium iodide kit (BioVision, USA) was used to stain cells. The apoptotic rate was examined by a flow cytometer (BD FACSCanto II, BD Biosciences, USA).

Western blot analysis

Total proteins were extracted from transfected cells by RIPA lysis buffer, and quantitated by a BCA kit. Proteins were subjected to 10% SDS-PAGE and transferred to the membrane of PVDF. After blocked with 5% dried skimmed milk for 1 h, the member was probed with primary antibodies (1:1000; Proteintech Group, USA). Then, the blots were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000), and developed using the Enhanced Chemiluminescence kit (CWBIO, Beijing, China).

Dual-luciferase reporter assay

The wild-type of E2F6 3′-UTR (E2F6-wt) or mutant E2F6 3′-UTR (E2F6-mut) was cloned in pmir-GLO fluorescein enzyme vector. 293T cells were co-transfected with luciferase reporter plasmids (wt or mut) and pCMV-MIR-miR-454 or pCMV-MIR. Luciferase activity of each group was measured by the dual-luciferase assay system kit (Promega, WI, USA).
Immunohistochemical staining analysis

Immunohistochemical staining was performed to evaluate E2F6 expression as described previously [21]. E2F6 expression levels were scored by staining intensity and percentage of positive stained cells [22]. The staining intensity was graded as follows: 0, no staining; 1, pale yellow staining; 2, buffy staining; 3, intense brown staining. The percentage of positive stained cells was scored as follows: 0=0–10%, 1=11–25%, 2=26–50%, 3=51–75%, 4=> 75%. Samples were scored by multiplying synchronically the two sections, and score > 6 as E2F6 high expression level.

Statistical analysis

Data was presented as Mean± SD and statistical analyses were conducted using GraphPad Prism 7.0 software (GraphPad, CA, USA). The chi-square test was performed for continuous or discrete data analysis, and the Student’s t test or one-way ANOVA analysis was used for comparisons between groups. Differences were considered significant when P value was less than 0.05.

Results

MiR-454 is down-regulated by hypoxia and suppresses the proliferation, migration and invasion of ovarian cancer cells

We found that miR-454 expression was obviously down-regulated by hypoxia (after 6 or 24 h in 1% O₂) in OVCAR3 and SKOV3 cells compared with corresponding normoxic condition (Figure 1A). These data indicated the specificity of miR-454 in hypoxic induction in ovarian cancer. Next, we examined the expression of miR-454 in the serum of ovarian cancer patients (n=13) and normal human serum (n=6). The expression of miR-454 was found to be significantly up-regulated in the serum of ovarian cancer patients compared with normal human serum (Figure 1B). Additionally, our data demonstrated that the expression of miR-454 was correlated with the clinicopathological stage of ovarian cancer patients (P=0.01619), the expression of miR-454 in the serum of patients in stage I and II was significantly higher than that in stage III and IV (Table 1). But miR-454 expression in serum of ovarian cancer patients was not found to be correlated with age and lymph node metastasis (Table 1). Therefore, miR-454 might be associated with the progression of ovarian cancer.

To elucidate the functional role of miR-454 in ovarian cancer, pCMV-MIR-miR-454 or pCMV-MIR
plasmid was transfected into OVCAR3 and SKOV3 cells. RT-PCR analysis showed that miR-454 expression was markedly up-regulated in OVCAR3 and SKOV3 cells transfected with pCMV-MIR-miR-454 (Figure 1C). As shown in Figure 1D and E, miR-454 overexpression significantly reduced the proliferation of OVCAR3 and SKOV3 cells compared with corresponding control group. Consistently, OVCAR3 cells transfected with pCMV-MIR-miR-454 formed fewer colonies than control group (Figure 1F). Similar results were also observed in SKOV3 cells, up-regulation of miR-454 inhibited colony formation ability of SKOV3 cells (figure 1F). Moreover, transwell assay showed that up-regulation of miR-454 dramatically inhibited the migration and invasion abilities of both OVCAR3 and SKOV3 cells (Figure 1G, H). These results suggest that miR-454 suppresses the proliferation, migration and invasion of ovarian cancer cells.

**miR-454 promotes cell apoptosis and inhibits the Akt/mTOR and Wnt/β-catenin signaling pathways in ovarian cancer cells**

As indicated by flow cytometry assay, miR-454 overexpression significantly induced apoptosis in both OVCAR3 and SKOV3 cells (Figure 2A). Then, expression of apoptosis-related proteins was detected by western blot analysis to investigate the underlying mechanism of induced apoptosis by miR-454. As shown in Figure 2B, the expression of anti-apoptotic protein Bcl-2 was significantly down-regulated by miR-454 in both OVCAR3 and SKOV3 cells, while expression of pro-oncogenic proteins Bax, cleaved Caspase 9 and cleaved Caspase 3 was up-regulated by miR-454, indicating that miR-454 induces apoptosis in ovarian cancer cells by regulating the Bcl-2/Bax axis and Caspase cascade.

It is well known that the Akt/mTOR and Wnt/β-catenin signaling pathways play critical roles in tumor genesis and progression. To further investigate the role of miR-454 in ovarian cancer, we examined the effect of miR-454 on activation of the Akt/mTOR and Wnt/β-catenin signaling pathways in ovarian cancer cells. As indicated by western blot analysis, the phosphorylation levels of Akt and mTOR were reduced in miR-454 transfected cells, and expression of downstream proteins Cyclin D1 and p70s6k was inhibited correspondingly (Figure 2C, D). Similarly, the expression of Wnt3 and β-catenin was also down-regulated by miR-454 overexpression in OVCAR3 and SKOV3 cells, the expression of downstream protein E-cadherin was up-regulated correspondingly (Figure 2E).
miR-454 directly binds to E2F6

In order to elucidate the molecular mechanism of miR-454, we identified the potential target of miR-454. Bioinformatics analysis (TargetScan) suggested E2F6 mRNA had possible binding sites for miR-454 in its 3′-UTR (Figure 3A). To confirm that, the wild-type (wt) or mutant (mut) E2F6 3′-UTR was cloned in a luciferase reporter vector and co-transfected with pCMV-MIR-miR-454 into 293T cells. As shown in Figure 3B, the relative luciferase activity of E2F6-wt was markedly decreased in the presence of miR-454, while luciferase activity of E2F6-mut was not impaired by miR-454. Moreover, the expression of E2F6 protein was significantly inhibited in OVCAR3 and SKOV3 cells transfected with pCMV-MIR-miR-454 (Figure 3C). Therefore, miR-454 could directly target E2F6 and negatively regulate its expression.

E2F6 is up-regulated in ovarian cancer tissues and mediates the effects of miR-454 on ovarian cancer cells

The IHC analysis was performed to determine the expression of E2F6 in 75 cases of ovarian cancer tissues and 15 cases of tumor-adjacent tissues. The rate of E2F6-high expression in ovarian cancer tissues was 57.3% (43/75), which was 20% (3/15) in tumor-adjacent tissues (P<0.05). As shown in Figure 4, E2F6 expression was up-regulated in ovarian cancer tissues compared with tumor-adjacent tissues.

To investigate the role of E2F6 in ovarian cancer progression and anti-tumor function of miR-454, pcDNA3.1-E2F6 plasmid was constructed and transfected separately into OVCAR3 and SKOV3 cells, or co-transfected with pCMV-MIR-miR-454 (Figure 5A). As shown in Figure 5B and C, E2F6 overexpression significantly enhanced the proliferation ability of OVCAR3 and SKOV3 cells compared with NC group, while miR-454 overexpression attenuated the promoting effect of E2F6 on cell proliferation. Moreover, the migration of OVCAR3 and SKOV3 cells was also promoted by E2F6 overexpression, and the promoting effect of E2F6 on cell migration was also abolished by miR-454 overexpression (Figure 5D). Collectively, miR-454 suppresses the progression of ovarian cancer by targeting E2F6.

Discussion
Hypoxia is one of the important hallmarks of tumor microenvironment. Increasing studies have revealed that tumor hypoxia is closely related to the progression of cancer, such as tumor metastasis, recurrence and drug resistance [23-26]. It has been confirmed that hypoxia in vivo is associated with poor prognosis and high mortality in ovarian cancer patients, and involved in ovarian cancer cell proliferation [27, 28]. By bioinformatics analysis, Zhang et al. showed that hypoxia could regulate some hub genes involved in cell proliferation, invasion and adhesion, and key pathways in ovarian cancer, including ErbB signaling pathway [29]. However, most of the human transcriptome is non-coding genes, which have been confirmed to play critical roles in tumorigenesis and development. Among them, miRNA is the most widely studied, but hypoxic miRNAs are an emerging research topic. Only a few miRNAs have been found to participate in the development of tumors under hypoxia. For example, Zhang Q et al. report that hypoxia induces miR-10b-3p expression in esophageal squamous cell carcinoma, which promotes tumor growth and metastasis [30]. Zheng H et al. show that miR-196-5p is down-regulated by hypoxia in hepatocellular carcinoma and serves as a tumor suppressor [31]. However, whether miRNAs are implicated in hypoxic ovarian cancer is poorly studied.

In the present study, we identified that miR-454 was markedly down-regulated by hypoxia in ovarian cancer cells, and up-regulated in the serum of ovarian cancer patients compared with normal human serum. The expression of miR-454 was correlated with the clinicopathological stage of ovarian cancer patients. These results indicate that miR-454 might be involved in the progression of ovarian cancer. However, the functional role and underlying mechanism of miR-454 in ovarian cancer remains unclear. To date, there are plenty of studies demonstrating that miR-454 is involved in the growth and metastasis of several types of cancers. Fu Q et al. report that miR-454 is highly expressed in prostate cancer tissues and promotes prostate cancer cell proliferation and invasion by regulating NDRG2 [32]. It has been revealed that miR-454 acts as an oncogene in triple negative breast cancer, and its high expression is associated with poor prognosis in patients with triple negative breast cancer [33, 34]. However, in glioblastoma, Fang B et al. demonstrate that miR-454 is down-regulated in tumors and cell lines, and miR-454 overexpression induces cell cycle arrest and inhibits cell proliferation [20]. miR-454 suppresses the growth, angiogenesis and metastasis of pancreatic ductal adenocarcinoma.
by targeting SDF-1 and LRP6 [35, 36]. To further reveal the functional role of miR-454 in the progression of ovarian cancer, OVCAR3 and SKOV3 cells were transfected with pCMV-MIR-miR-454. Applying gain-of-function experiments, our data revealed that miR-454 overexpression suppressed the proliferation, migration and invasion of ovarian cancer cells, and promoted cell apoptosis by regulating the Bcl-2/Bax axis and Caspase cascade. In addition, the Akt/mTOR and Wnt/β-catenin signaling pathways were inhibited by miR-454 overexpression. Collectively, our data support the view that miR-454 functions as a tumor suppressor in ovarian cancer. The controversial role of miR-454 might be due to different cancer cell types, sources and target genes [32]. Further study is required to confirm the precise role of miR-454 in the development of different types of tumors.

To determine the mechanism underlying the tumor-suppressive role of miR-454 in ovarian cancer, we identified the potential target gene of miR-454. By bioinformatics analysis and dual-luciferase reporter assay, we verified that E2F6 was a direct target of miR-454 in ovarian cancer. As a member of E2F family of transcription factors, E2F6 plays critical roles in regulating cellular biological activities. Previous studies have revealed that E2F6 exerts an oncogenic role in the progression of cancers and functions as a target gene of some miRNAs [37-39]. Li Y et al. report that up-regulated expression of E2F6 is observed in gastric cancer tissues and E2F6 knockdown inhibits cell proliferation and invasion [38]. In renal cell carcinoma, miR-425 suppresses cell proliferation and induced apoptosis by targeting E2F6 [37]. In the present study, we found that miR-454 negatively regulates the expression of E2F6. In addition, we demonstrated that E2F6 was significantly up-regulated in ovarian cancer tissues. Moreover, E2F6 overexpression significantly enhanced the proliferation and invasion of ovarian cancer cells. Further, these changes triggered by E2F6 overexpression were abolished by miR-454 overexpression. Therefore, our findings suggest that miR-454 impedes the growth and metastasis of ovarian cancer by targeting E2F6.

Conclusion
In summary, our data clearly for the first time demonstrate that hypoxia down-regulates miR-454 expression in ovarian cancer, and miR-454 acts as a tumor suppressor in ovarian cancer by directly targeting E2F6. These findings provide a new insight into the mechanism of ovarian cancer.
development under hypoxia, and suggest that the hypoxia/miR-454/E2F6 pathway may be a therapeutic strategy for ovarian cancer.

Abbreviations
Akt, protein kinase B; CCK8, cell Counting Kit-8 reagent; miRNA, microRNA; mTOR, mammalian target of rapamycin

Declarations
Authors’ contributions
YA, JZ and XZ designed the concept and experiments. BL, XC, YT, and FZ performed the experiments, collected the data, and analyzed the results. YA wrote the manuscript.

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Competing interests
The authors declare that they have no competing interests.

Ethics approval and consent to participate
All patients gave informed consent in written, and this study was approved by the ethics committee of Beijing Anzhen Hospital, Capital Medical University.

Availability of data and materials
The data supporting the conclusions of this paper are included within the manuscript.

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Tables
Table 1 The correlation between the expression of serum miR-454 and the clinicopathological characteristics of ovarian cancer patients

| Characteristics               | n  | miR-454 expression | P-value     |
|------------------------------|----|-------------------|-------------|
| Age                          |    |                   |             |
| 59                           | 7  | 6.41±5.33         | 0.691166    |
| ≥59                          | 6  | 8.37±11.39        |             |
| Clinical stage               |    |                   |             |
| I-II                         | 7  | 12.13±8.92        | 0.01619*    |
| III-IV                       | 6  | 1.70±0.77         |             |
| Lymph node metastasis        |    |                   |             |
| Yes                          | 7  | 5.69±10.59        | 0.472306    |
| No                           | 6  | 9.21±4.88         |             |

*P<0.05.

Figures
miR-454 overexpression inhibits the proliferation, migration and invasion of ovarian cancer cells. A. Expression of miR-454 was examined in OVCAR3 and SKOV3 cells under hypoxic culture conditions (1% O2, 5% CO2, 94% N2) for 6 or 24 h. B. Expression of miR-454 in the serum of ovarian cancer patients (n=13) and normal samples (n=6). C. Expression of miR-454 in OVCAR3 and SKOV3 cells transfected with pCMV-MIR-miR-454 or pCMV-MIR plasmid. D, E. CCK8 assay was used to examine the proliferation of OVCAR3 (D) and SKOV3 (E) cells after indicated transfection. F. Colony formation ability of OVCAR3 and SKOV3 cells after indicated transfection. G, H. Transwell assay was performed to assess the migration (G) and invasion (H) abilities of OVCAR3 and SKOV3 cells after indicated transfection. NC, cells transfected with pCMV-MIR plasmid; miR-454, cells transfected with pCMV-MIR-miR-454.
miR-454 overexpression promotes apoptosis of ovarian cancer cells and inhibits the Akt/mTOR and Wnt/β-catenin signaling pathways. A. Apoptosis rate of OVCAR3 and SKOV3 cells transfected with pCMV-MIR-miR-454 or pCMV-MIR plasmid. B. Expression of apoptosis-related proteins in OVCAR3 and SKOV3 cells after transfection. C. Expression of the Akt/mTOR signaling pathway related proteins in OVCAR3 and SKOV3 cells. D. Quantitative analysis of Western blot results in Figure 2C. E. Expression of the Wnt/β-catenin signaling pathway related proteins in OVCAR3 and SKOV3 cells. *P<0.05.
miR-454 directly target E2F6. A. Bioinformatics analysis revealed predicted binding sites between miR-454 and E2F6. B. Relative luciferase activity in cells co-transfected with pCMV-MIR-miR-454 or pCMV-MIR and pmir-GLO-E2F6 wild type (wt) or pmir-GLO-E2F6 mutant (mut). C. Expression of E2F6 protein in OVCAR3 and SKOV3 cells transfected with pCMV-MIR-miR-454 or pCMV-MIR plasmid. **P<0.01.

Figure 3

E2F6 is highly expressed in ovarian cancer tissues. IHC analysis was performed to detect E2F6 expression in ovarian cancer tissues (Tumor) and tumor-adjacent tissues (Normal).

Figure 4
miR-454 overexpression abolishes the biological effects of E2F6 on ovarian cancer cells A.

Relative expression of E2F6 mRNA in OVCAR3 and SKOV3 cells co-transfected with pcDNA3.1-E2F6 and pCMV-MIR-miR-454 or transfected with pcDNA3.1-E2F6 alone. B, C. CCK8 assay was performed to assess the proliferation of OVCAR3 (B) and SKOV3 (C) cells after indicated treatment. D. Migration ability of OVCAR3 and SKOV3 cells after indicated treatment. *P<0.05, **P<0.01 vs NC group, ΔP<0.05 vs E2F6 group.