YTHDF2, a protein repressed by miR-145, regulates proliferation, apoptosis and migration in ovarian cancer cells

Jie Li (lijiexj64@126.com)
First Affiliated Hospital of xi’an Jiaotong University
https://orcid.org/0000-0002-4433-5393

Lei Wu
First Affiliated Hospital of xi’an University

Meili Pei
First Affiliated Hospital of xi’an Jiaotong University

Yun Zhang
First Affiliated Hospital of xi’an Jiaotong University

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Abstract

RNA methylation can reverse the methylation modification at RNA level, which is a kind of extremely important epigenetic modification. YTHDF2, as a reader of m6A modification, the function and mechanisms of in epithelial ovarian cancer (EOC) have not been elucidated so far. In this study, we demonstrated that YTHDF2 was significantly upregulated in EOC tissues compared with normal ovarian tissues, further function studies confirmed that YTHDF2 significantly promoted the proliferation and migration of EOC cell lines, and reduced the global mRNA m6A levels. Next, we found that the expression levels of miR-145 and YTHDF2 were inversely correlated in ovarian cancer tissues and cells, and YTHDF2 is the direct target gene of miR-145. Interestingly, there was a crucial crosstalk between miR-145 and YTHDF2 via a double-negative feedback loop. Overexpression of YTHDF2 rescues miR-145-induced reduction of proliferation and migration in EOC cells. To conclude, YTHDF2 and miR-145, as two crucial m6A regulators, are involved in the progression of EOC by indirectly modulating m6A levels. In view of these promising results, YTHDF2 and miR-145 may provide new insights into the carcinogenesis and new potential therapeutic targets for EOC.

Introduction

Ovarian cancer is one of the most common malignant tumors in women, and its mortality rate ranks first in gynecological tumors[1]. There are many histopathological types of ovarian cancer, about 90% of which belong to epithelial ovarian cancer (EOC)[1]. Because EOC patients have no obvious symptoms and signs in the early stage and lack of effective means of early screening, most of them have reached the late stage when the diagnosis is clear. The main treatment of EOC is surgery, supplemented by intravenous chemotherapy or intraperitoneal perfusion chemotherapy or combination therapy. However, the positive treatment did not achieve satisfactory results, and the 5-year survival rate of EOC patients was not significantly improved[2]. In addition to the difficulty of early diagnosis, the poor prognosis of patients is also attributed to tumor recurrence and metastasis[3]. Therefore, it is an urgent task for ovarian cancer treatment to obtain early sensitive markers of EOC or to find the key molecules related to EOC recurrence and metastasis, and to develop a targeted and multi-functional gene therapy.

RNA methylation can reverse the methylation modification at RNA level, which is a kind of extremely important epigenetic modification[4]. Among them, 6-methyladenine (m6A) is the most abundant methylation modification of mRNA in eukaryotic cells, involving the participation of three types of molecules, including methyltransferase complex (METTL3, METTL14 and WTAP, etc.), namely “Writers”, demethylase (FTO, ALKBH5), namely “Erasers”, and m6A modified binding protein (YTHDF 1 / 2 / 3), namely “readers”, which can dynamically and reversibly regulate m6A level[5, 6]. In 2017, Professor He Chuan's research team first found that m6A is involved in the regulation of self-renewal and differentiation of glioblastoma stem cells (GSCs). In vitro and in vivo experiments show that knockout of FOXM1-AS and ALKBH5 can affect the tumorigenicity of GSCs related to FOXM1, revealing the important role of demethylase ALKBH5 and m6A in glioblastoma[7]. Shuhan sun et.al revealed the important role of METTL14 in inhibiting the metastasis of HCC[8]. Samie R Jaffrey et.al found that METLL3 regulates the
myeloid differentiation of normal hematopoietic stem cells and leukemic cells, which provides more basis for mettl3 as a potential target for myeloid leukemia treatment[9].

Readers are responsible for "reading" the information of RNA methylation modification, and participating in the process of downstream RNA translation and degradation. There are two modes of "reading", one is direct reading, which refers to the selective binding with the m6A site of RNA. The YTH domain family with RNA binding domains was the first "reader" to be found to read directly[10]. YTHDF2 can accelerate the decay of m6A methylated mRNA. [11, 12]. YTHDF2 was found to regulate m6A levels in HCC[13], however, the expression and mechanism of YTHDF2 in most tumors, especially in ovarian cancer, have not been elucidated

MicroRNAs (miRNAs) are a kind of endogenous non coding microRNAs with a length of about 22 nt, which widely exist in eukaryotes. It was found that there was an interaction between the methylation modification of RNA m6A and miRNA. On the one hand, the miRNA targeting site found that m6A was enriched, and miRNA could positively regulate the activity of METTL3; on the other hand, the miRNA synthesis process depended on m6A methylation modification[14, 15]. Our previous studies have confirmed that miR-145 can regulate different biological functions of ovarian cancer by targeting different target genes[16–19]. However, the expression pattern and its m6A-regulated role of miR-145 in ovarian cancer was still unclear.

In our study, We first found that YTHDF2 and miR-145 form a negative feedback pathway to regulate ovarian cancer progression in a m6A modification way. Our results provide a theoretical basis for the application of YTHDF2 and miR-145 in the diagnosis and treatment of ovarian cancer.

**Materials And Methods**

2.1 Human tissue specimens and cell culture

Methods described in previous studies[16]. This study was approved by the Ethics Committee of The First Affiliated Hospital of Xi’an Jiaotong University, China.

2.2 Plasmid transfection

The human YTHDF2 expression vector pcDNA3-flag-YTHDF2 were obtained from Addgene. Cells were seeded into 6-well plates until 70%-90% confluency and transiently transfected with pcDNA3-flag-YTHDF2 or empty vector using the X-treme GENE HP DNA Transfection Reagent(Roche, Indianapolis, IN, USA) following the manufacturer's protocol.

2.3 siRNA and transient transfection

Human YTHDF2 siRNA were purchased from GenePharma(Shanghai, China). YTHDF2 siRNA was transiently transfected 100nM per well using the X-treme GENE siRNA Transfection Reagent(Roche,
Indianapolis, IN, USA) following the manufacturer's protocol. RNA was extracted 48 hours later and protein was extracted 72 hours later for subsequent experiments.

2.4 miR transient transfection

Methods described in previous studies[16].

2.5 Quantitative real-time PCR (qRT-PCR)

Methods described in previous studies[16].

2.6 Western blot

Total proteins were extracted by RIPA lysis buffer (Roche, Indianapolis, IN, USA) and 1 mM PMSF on ice, proteins were separated by SDS-PAGE and then transmembrane. 5% skimmed milk was sealed at room temperature for 2 hours. First antibody was added to the membrane. TBST membrane was washed for 8 minutes and 5 times, and the corresponding second antibody (1:2000) was added, incubated for 2 hours, and TBST membrane was washed for 8 minutes and 5 times.

2.7 Luciferase reporter assay

Methods described in previous studies[16]

2.8 RNA m6A quantitative experiment

In this experiment, the total RNA content of m6A was determined by using the m6A RNA metrology Quantification Kit (ab185912, Abcam) of Abcam company. We measure m6A level following the manufacturer's protocol. The absorbance of the measuring plate at 450 nm was measured by the enzyme scale instrument, and the RNA m6A content of each sample was calculated according to the standard curve. The formula is m6A% = [(sample OD-NC OD)/S] / [(PC OD-NC OD)/P]×100%, where S is the ng amount of sample RNA and P is the ng amount of positive control RNA.

2.9 Cell viability assay

Methods described in previous studies[18].

2.10 Transwell assay

Methods described in previous studies[16]

2.11 Cell apoptosis assay

Cell apoptosis analysis was performed using an Annexin V-FITC/propidium iodide (PI) Apoptosis Detection kit (KeyGEN Biotech, Nanjing, China). Normal culture cells were selected in the logarithmic growth period, and the growth state was good for the experiment. After culture for 24 hours, the
supernatant was introduced into EP tube, and cells were digested with trypsin without EDTA, then cell suspension was made and transferred to new EP tube. After centrifuging for 10 minutes at 1000rpm and 4 °C, discard the supernatant; add 1ml of precooled PBS, gently blow to suspend the cells for 1000rpm, centrifuging for 10 minutes at 4°C, discard the supernatant; repeat step 3 and step 4 twice; re suspend the cells in 400ul x binding buffer; add 5ul annexin V-FITC to each sample to be tested, and add PI after mixing 5ul, mix well, react at room temperature for 15min, pay attention to avoid light, and try to get on the machine within 1h. The results were analyzed using the Cell-Quest™ Pro software (BD Biosciences, Bedford, MA, USA)

2.12 Statistical analysis

Data were presented as the means±SE and were analyzed using SPSS 22.0 software(Chicago, IL, USA). Statistical differences were tested by Chi-square test, two-tailed t-test, one-way ANOVA test or Fisher’s Exact test. Differences were considered significant at P<0.05(*) or highly significant at P<0.001 (**).

Results

3.1 The expression of YTHDF2 in ovarian cancer tissues

More and more studies have shown that m6A modification plays an important role in the occurrence and development of human complex diseases, especially in cancer. It is reported that YTHDF2 is closely related to the malignant degree of HCC, and it can regulate mRNA degradation by recognizing the m6A site, leading to the enhancement of HCC cell proliferation[20-22]. However, its specific expression pattern in EOC are still unclear. We detected mRNA expression of YTHDF2 in ovarian cancer tissues and normal ovarian cancer tissues. The result demonstrated that the expression of YTHDF2 was higher than in normal ovarian tissues(Fig.1A). Clinicopathological correlation analysis of DNMT3A level to ovarian cancer showed that the later the clinical stage, the higher the pathological grade, the higher the expression of YTHDF2, and the higher the expression of YTHDF2 in patients with metastasis(Fig.1B-D). The above results indicated YTHDF2 promoted ovarian cancer progression.

3.2 YTHDF2 significantly downregulates global mRNA m6A levels to promotes EOC cell proliferation and migration

RNA m6A quantitative experiment was performed to investigate the global mRNA m6A levels. After downexpression of YTHDF2(Fig.2A), we found the global mRNA m6A level was upregulated(Fig.2B). CCK8 test results showed that the proliferation of ovarian cancer cells decreased after knocking down YTHDF2(Fig.2C). Accordingly, the ability of cell apoptosis increased (Fig.2D) and migration decreased(Fig.2E). Next, after overexpression of YTHDF2(Fig.2F), global mRNA m6A level was decreased(Fig.2G), the proliferation of ovarian cancer cells increased(Fig.2H), the ability of cell apoptosis decreased (Fig.2I) and migration increased(Fig.2J). In conclusion, YTHDF2, as an important m6A reader, significantly promoted proliferation and migration by decreasing the global mRNA m6A levels in ovarian
cancer cells, which suggested the m6A modification and the reader protein YTHDF2 were involved in carcinogenesis of EOC.

### 3.3 YTHDF2 is the direct target gene of miR-145

Our previous study demonstrated that miR-145 was low expression in ovarian cancer. We further analyzed the mRNA expression levels of miR-145 and YTHDF2 in ovarian cancer tissues. The results showed that the expression levels of miR-145 and YTHDF2 were inversely correlated in ovarian cancer (Fig.3A). Then we found that the YTHDF2 level in the miR-145 low-expression cell line (3AO) was higher than in the miR-145 high-expression cell line (SKOV3) (Fig.3B). After overexpression of miR-145 (Fig.3C), the mRNA and protein expression level of YTHDF2 decreased (Fig.3D). Interestingly, the expression level of miR-145 also decreased (Fig.3E) after overexpression of YTHDF2 (Fig.2F), suggesting a crucial crosstalk between miR-145 and YTHDF2 via a double-negative feedback loop. Biological software (http://www.targetscan.org/, http://www.mirdb.org/) predicts that YTHDF2 is a target gene of miR-145, A luciferase reporter assay revealed miR-145 targeted YTHDF2 directly (Fig.3F). In a word, YTHDF2 is the direct target gene of miR-145.

### 3.4 overexpression of YTHDF2 rescues miR-145-induced reduction of proliferation and migration in EOC

Overexpression of miR-145 inhibited the proliferation, migration and apoptosis of ovarian cancer cells, which was attenuated by overexpression of YTHDF2 (Fig.4A-C). Similarly, the effect of overexpression of miR-145 on the global mRNA m6A levels was offset by overexpression of YTHDF2 (Fig.4D). qRT-PCR and western blot assays results showed after overexpression of miR-145, the expression of YTHDF2 decreased, which was reversed by overexpression of YTHDF2 (Fig.4E,F). To conclude, overexpression of YTHDF2 rescues miR-145-induced reduction of proliferation and migration in EOC.

### Discussion

Ovarian cancer is one of the most common malignant tumors in women, and its mortality rate ranks first in gynecological tumors[1]. In addition to the difficulty of early diagnosis, the poor prognosis of patients is also attributed to tumor recurrence and metastasis[2]. However, the specific mechanisms of EOC are not fully elucidated. At present, the research on the relationship between the methylation of m6A mRNA and tumor has been widely concerned. More and more evidences show that the methylation of m6A mRNA is closely related to the occurrence and development of tumor. The expression level of m6A related protein is an important regulatory factor in the development of tumor, which directly determines the pathological process of tumor[6]. However, The m6A modification in regulation of EOC is still poorly understood. In this study, we investigated how YTHDF2 and miR-145 modulated EOC progression in a m6A modification way.

Methylation of m6A is a dynamic and reversible process, which is composed of writers, erasers and readers[23]. YTHDF2, as a reader of m6A modification, belongs to a member of YTH domain family. YTHDF2 is closely related to the malignancy of HCC, and can be adjusted by recognizing m6A site. It has
been found that the down regulated miR-145 in HCC patients can directly target the 3'UTR of YTHDF2 mRNA to inhibit the expression of YTHDF2, which may be a new target for the treatment of HCC[13]. However, the role of YTHDF2 in ovarian cancer has not been elucidated. In this study, we detected that the expression of YTHDF2 was significantly upregulated in EOC tissues compared with normal ovarian tissues, which clarified YTHDF2 might play a role in promoting ovarian cancer. In addition, we confirmed that YTHDF2 promoted the proliferation and migration, inhibited the apoptosis of EOC cell lines, and reduced the global mRNA m6A levels. Taken together, these results confirmed that YTHDF2 could promote the proliferation and migration by decreasing global m6A levels.

miR-145 is a newly discovered miR, which is significantly underexpressed in breast cancer, cervical cancer, glioma, colon cancer, esophageal cancer and non-small cell lung cancer[24, 25]. In recent years, many studies have found that microRNA is closely related to ovarian cancer[26, 27]. Our previous studies have confirmed that miR-145 can regulate different biological functions of ovarian cancer by targeting different target genes[16–19]. However, the expression pattern and its m6A-regulated role of miR-145 in ovarian cancer was still unclear. In this study, we found that the expression levels of miR-145 and YTHDF2 were inversely correlated in ovarian cancer tissues and cells. There was a crucial crosstalk between miR-145 and YTHDF2 via a double-negative feedback loop, and YTHDF2 is the direct target gene of miR-145. However, we have not studied how YTHDF2 regulates miR-145, which needs further exploration.

Conclusions

In summary, we concluded that YTHDF2, a protein repressed by miR-145, promotes proliferation and migration in ovarian cancer cells. Our results reveal for the first time the regulatory mechanism of YTHDF2 in ovarian cancer, and determine that miR-145 and YTHDF2 are involved in the m6A modification and progression of EOC. We hope this study of YTHDF2 and miR-145 may provide potential therapeutic targets of EOC.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets during and/or analysed during the current study available from the corresponding author on reasonable request.
Competing interests

The authors declare that they have no competing interests

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Authors’ contributions

Jie Li conceived and designed the experiments. Lei Wu and Yun Zhang performed the experiments. Jie Li and Meili Pei analyzed the data. Jie Li, Lei Wu and Meili Pei wrote the paper. All authors read and approved the final manuscript.

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Figures
Figure 1

The expression of YTHDF2 in ovarian cancer tissues. (A) Relative expression of YTHDF2 in ovarian cancer tissues and normal ovarian tissues. (B) The relationship between the expression level of YTHDF2 and clinical stage. (C) The relationship between the expression level of YTHDF2 and pathological grade. (D) The relationship between the expression level of YTHDF2 and metastasis. All experiments were carried out in triplicate and the results were presented as means ± SE. *P < 0.05, **P < 0.001, t-test. N normal ovarian tissue, C ovarian cancer tissues.
Figure 2

YTHDF2 significantly downregulates global mRNA m6A levels to promotes EOC cell proliferation and migration. (A) The mRNA and protein level of YTHDF2 was downregulated after transfection of YTHDF2 siRNA. (B) Knocking down YTHDF2 increased global mRNA m6A level. (C) CCK8 assay result showed knock-down of YTHDF2 decreased proliferation of ovarian cancer cells. (D) Knock-down of YTHDF2 promoted apoptosis of ovarian cancer cells. (E) Knock-down of YTHDF2 inhibited migration of ovarian cancer cells. (F) The mRNA and protein level of YTHDF2 was upregulated after overexpression of YTHDF2. (G) Overexpression of YTHDF2 decreased global mRNA m6A level. (H) CCK8 assay result showed upregulated of YTHDF2 increased proliferation of ovarian cancer cells. (I) Overexpression of YTHDF2 inhibited apoptosis of ovarian cancer cells. (J) Overexpression of YTHDF2 promoted migration of ovarian cancer cells. All experiments were carried out in triplicate and the results were presented as means ± SE. *P<0.05, **P<0.001, t-test.
YTHDF2 is the direct target gene of miR-145. (A) Scatter diagram showing YTHDF2 expression and miR-145 expression by qRT-PCR and their correlations (R2=0.914, P=0.000) in 31 EOC tissue samples. (B) qRT-PCR results showed a negative correlation between YTHDF2 and miR-145 in both SKOV3 and 3AO cells. (C) qRT-PCR showed that transfection of miR-145 mimic rescued miR-145 level in SKOV3 and 3AO cells. (D) The mRNA and protein levels of YTHDF2 decreased after overexpression of miR-145. (E) The expression level of miR-145 increased after overexpression of YTHDF2. (F) Luciferase reporter assays showed that miR-145 targeted YTHDF2 directly. All experiments were carried out in triplicate and the results were presented as means ± SE. *P<0.05, **P<0.001, t-test.
Figure 4

overexpression of YTHDF2 rescues miR-145-induced reduction of proliferation and migration in EOC. (A) overexpression of YTHDF2 reversed the proliferation repressed by overexpression of miR-145. (B) miR-145 promoted apoptosis of ovarian cancer cells, which was counteracted by overexpression of YTHDF2. (C) overexpression of YTHDF2 reversed the migration repressed by overexpression of miR-145. (D) The effect of overexpression of miR-145 on the global mRNA m6A levels was offset by overexpression of YTHDF2. (E) The expression of YTDHF2 at mRNA levels after overexpression of miR-145 and YTHDF2. (F) The expression of YTDHF2 at protein levels after overexpression of miR-145 and YTHDF2. All experiments were carried out in triplicate and the results were presented as means ± SE. *P<0.05, **P<0.001, t-test.