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TRIP13 promotes proliferation and invasion of epithelial ovarian cancer cells through Notch signaling pathway

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Abstract. – OBJECTIVE: Growing evidence supports the involvement of Thyroid hormone Receptor Interactor 13 (TRIP13) in the progression and metastasis of multiple cancers. However, the roles of TRIP13 in epithelial ovarian cancer (EOC) remains unknown. The present study aimed to investigate the expression pattern and biological function as well as the underlying molecular mechanism.

PATIENTS AND METHODS: The expression patterns of genes in EOC tissues and normal ovarian tissues via microarray from GEO and TCGA datasets. The expression levels of TRIP13 in EOC cell lines were detected by Real Time-Polymerase Chain Reaction (RT-PCR). Next, we investigated the effect of TRIP13 on the proliferation, apoptosis, migration and invasion in the EOC cells. Western blot assay was used to explore the role of TRIP13 on the Notch signaling pathway proteins (Notch1, P21, Hes1).

RESULTS: Bioinformatics analysis showed that TRIP13 was one of the most significantly upregulated in EOC. The results of RT-PCR also indicated that TRIP13 expression was markedly upregulated in EOC cell lines (SKOV-3, HEY and OVCAR-3) compared to normal ovarian cell lines. Functionally, our data revealed that silencing TRIP13 in EOC cells inhibits cell proliferation, decreases cell invasion and migration, and stimulates EOC cell apoptosis *in vitro*. Mechanistically, the knockdown of TRIP13 suppressed the Notch signaling pathway activation and subsequently inhibited EMT progression.

CONCLUSIONS: The present study provided the first evidence that TRIP13 acted as an onco-promotive regulator in EOC development by modulating the Notch signaling pathway. Our findings enlarged our knowledge in the molecular pathology of TRIP13 tumorigenesis.

Key Words

TRIP13, Epithelial ovarian cancer, EMT, Notch signaling pathway, Tumorigenesis.

Introduction

Ovarian cancer is cancer that forms in or on an ovary and is one of the most leading causes of death among women with malignant gynecological cancer[1,2]. Epithelial ovarian cancer (EOC) is the most common subtype of ovarian cancer[3]. In China, patients with ovarian cancer are expected with about 43.2% mortality, which is still increasing[4]. Despite the significant achievements in surgery, chemotherapy and radiotherapy, the 5 years survival rate of advanced-stage EOC remains below 30%, mainly due to most patients diagnosed in advanced stages and metastasis[5,6]. There are many risk factors for EOC, such as family history, environmental factors and smoking tobacco[7]. Importantly, dysregulation of several genes is significantly associated with development and progression of EOC[8]. Thus, the identification of abnormal genes which participate in the biological progression is very critical to develop novel diagnostic and prognostic biomarker as well as potential treatment targeting for EOC.

Thyroid receptor-interacting protein 13, encoded by the TRIP13 gene, is located at 5p15.33 and a member of the AAA + ATPase super-family which play a functional role in various cellular processes, such as protein degradation and DNA replication[9,10]. As a highly conserved gene in a wide range of species, TRIP13 was reported to be involved in meiotic recombination and chromosome synapsis[11]. Interestingly, recent evidence showed that TRIP13 was abnormally expressed in several tumors, revealing the important roles of TRIP13 in cancer biology[12,13]. The functional role of TRIP13 was also reported. Sheng et al[14] referred that TRIP13 was highly expressed in colorectal cancer and its overexpression in colorectal

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cancer patients was markedly associated with advanced clinical progression and poor prognosis. The functional investigation indicated that TRIP13 served as a tumor promoter in this disease because its overexpression promoted colorectal cancer cell proliferation, invasion and EMT both *in vitro* and *in vivo*. On the other hand, the similar oncogenic role of TRIP13 was also reported in several other tumors[15,16]. However, to our best knowledge, the expression pattern and potential function of TRIP13 in EOC has not been investigated. In this work, by analyzing the microarray data from GEO and TCGA datasets, we found that TRIP13 was an up-regulated gene in EOC, which was also confirmed by Real Time-Polymerase Chain Reaction (RT-PCR) in cells experiments. *In vitro* assay displayed that TRIP13 acted as an oncogene in the progression of EOC. To explore the potential mechanism, our attention focused on the Notch-1 signaling pathway whose activation is implicated in tumorigenesis. Our present results first provided evidence that TRIP13 might be a therapeutic target in EOC.

Materials and Methods

Cell Lines and Cell Transfection

This study was approved by the Ethics Committee of Huai'an First People's Hospital. A normal ovarian epithelial cell line, IOSE80, and three human EOC cell lines, SKOV-3, HEY and OVCAR-3, were obtained from the cell bank of the Chinese Academy of Sciences (Xuhui, Shanghai, China). All the cells were cultured using Roswell Park Memorial Institute-1640 (RPMI-1640; Gibco, Grand Island, NY, USA) complete medium containing 10% fetal bovine serum (FBS) as well as the penicillin-streptomycin solution. The FBS and penicillin-streptomycin solution were purchased from Jin Ma Experimental Equipment Co., Ltd. (Minhang, Shanghai, China). Small interfering RNAs (siRNAs) specific targeting TRIP13 (si-TRIP13-1 and si-TRIP13-2) as well as negative control siRNAs (si-NC) were obtained from iGene Biotechnology Co., Ltd. (Guangzhou, Guangdong, China). Subsequently, a transfecting reagent, EndoFectin Max (iGene Biotechnology, Guangzhou, Guangdong, China), was utilized to transfect the siRNAs into EOC cells.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

The qRT-PCR assays were conducted by the use of a SYBR FAST one-Step qRT-PCR kit (Sigma-Aldrich, St. Louis, MO, USA) after total RNA was isolated from EOC cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Primer sequences were as follows: for TRIP13, 5'-ACTGTTGCACTTCACATTTTCCA-3' (sense) and 5'-TCGAGGAGATGGGATTTGACT-3'(antisense); for GAPDH, 5'-CTGGGCTACACTGAGCACC-3' (sense) and 5'-AAGTGGTCGTTGAGGGCAATG-3' (antisense). The qRT-PCR was performed using CFX96 Real Time-PCR Detection System (Bio-Rad, Hercules, CA, USA) and the expression levels of TRIP13 were normalized to GAPDH. The relative quantification values for each miRNA were calculated by the $2^{-\Delta\Delta Ct}$ method.

Western Blot Analysis

Total protein was extracted from TRIP13 siRNAs transfected SKOV3 and OVCAR-3 cells using radioimmunoprecipitation assay (RIPA) buffer which was purchased from G-CLONE Co., Ltd. (Yizhuang, Beijing, China). Afterward, equal amounts of proteins were resolved by 8%-12% SDS-PAGE and subsequently transferred to polyvinylidene difluoride (PVDF) membranes (Renoldbio, Suzhou, Jiangsu, China). Then, the membranes were sequentially incubated with the primary antibodies, the secondary antibodies and Super ECL kit (Comiike, Nantong, Jiangsu, China) for visualizing the corresponding protein bands. The primary antibodies against Vimentin, N-Cadherin, P21 and GAPDH were purchased from Boster Biological Technology Co., Ltd. (Wuhan, Hubei, China). The primary antibodies against Notch1 and Hes1 were obtained from Cell Signaling Technology Co., Ltd. (Danvers, MA, USA).

Cell Viability and Colony Formation Assays

The growth curves of SKOV3 and OVCAR-3 cells were assessed by Cell Counting Kit-8 (CCK-8) which was obtained from Biomedical Technology Co., Ltd. (Wuxi, Jiangsu, China). In brief, after plating SKOV3 or OVCAR-3 cells (2000 cells/well) in 96-well plates for 24 h, 10 μl of CCK-8 solution was added into each well and subsequently the plates were maintained at 37°C with 5% CO_2 for 1 h. Then, the absorbance of 450 nm was read by a SpectraMax iD5 microreader system (Molecular Devices, Sunnyvale, CA, USA). For cell colony formation assays, the SKOV3 or OVCAR-3 cells transfected with TRIP13 siRNAs or negative control siRNAs were plated in 6-well plates (500 cells/well). After two weeks, the colonies of SKOV3 or OVCAR-3 cells were fixed with methanol and subsequently stained with 0.1% crystal violet solution.

Cell Apoptosis Assay

A DxFLEX flow cytometer (Beckman-Coulter Inc., Brea, CA, USA) was employed to analyze the apoptosis of SKOV3 and OVCAR-3 cells transfected with siRNAs using Aposcreen Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (AmyJet Scientific Inc, Wuhan, Hubei, China). Briefly, after SKOV3 and OVCAR-3 cells were transfected with TRIP13 siRNAs for 24 h, the cells were re-suspended in the banding buffer containing Annexin V and propidium iodide (PI). Thereafter, the cells were washed with Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA) three times and then analyzed by the flow cytometer.

Wound Healing Assays

The SKOV3 or OVCAR-3 cells transfected with TRIP13 siRNAs or corresponding negative control siRNAs were seeded into the wells of an Ibidi 3.5cm μ-dish with culture insert (ChemBio, Hongkou, Shanghai, China). After culturing for 24 h, the culture inserts were gently removed and the cells were washed with PBS three times. Afterward, the wounded areas at 0 h and 24 h were imaged by a microscope (CSW-DY01, Coosway, Shenzhen, Guangdong, China).

Transwell Assays

Transwell invasive assays were applied to evaluate the effects of TRIP13 knockdown expression on cell invasion of SKOV3 and OVCAR-3 cells. In short, SKOV3 or OVCAR-3 cells transfected with TRIP13 siRNAs or si-NC were re-suspended in the serum-free medium at a density of 5×10^5 cells/ml. Then, 100 μl of the cells were seeded into the upper side of a polycarbonate membrane Boyden chamber insert (8.0 μm pore size; BioGenius, Minhang, Shanghai, China) which was pre-treated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). In addition, the lower chamber contained 500 μl of medium plus 10% FBS. After culturing for 24 h, the invaded cells at the bottom of the chamber were fixed with methanol and subsequently stained with 0.1% crystal violet solution for 20 min. Finally, after washing with PBS three times, the cells were imaged by a microscope (CSW-DY01, Coosway, Shenzhen, Guangdong, China).

Statistical Analysis

We used the SPSS 19.0 (IBM, Armonk, NY, USA) in the present study to conduct the statistical analysis. GraphPad Prism 5.0 version X (GraphPad, La Jolla, CA, USA) was used for image editing. The differences between the two groups were analyzed by the Student's *t*-test. One-way ANOVA and Tukey's post-hoc test was performed to analyze the difference among three or above groups. p-value < 0.05 was defined as statistically significant.

Results

TRIP13 Was Highly Expressed in EOC Tissues and Cell Lines

To screen aberrantly expressed genes in EOC, we investigated the raw microarray data downloaded from the GEO dataset GSE26712 and GSE66957. The dysregulated genes were shown in heat map and Volcano plot (Figure 1A and 1B). The results of the Venn diagram indicated that 345 up-regulated genes and 40 down-regulated genes were found in both GSE26712 and GSE66957 (Figure 1C). Interestingly, TRIP13 was one of the most significantly upregulated genes in both two datasets (Figure 1D). On the other hand, we also analyzed TCGA datasets to further confirm the above results, finding that TRIP13 expression was also markedly up-regulated in TCGA datasets (Figure 1E). In addition, we performed RT-PCR to detect the expression of TRIP13 in EOC cell lines (SKOV-3, HEY and OVCAR-3) and a normal ovarian epithelial cell line, IOSE80, finding that TRIP13 expression was remarkably upregulated in three EOC cell lines (Figure 1F). Thus, our online analysis revealed that TRIP13 was one of the up-regulated genes in EOC and may play a functional role in the progression of this disease.

Silence of TRIP13 Suppressed EOC Cell Proliferation and Accelerated Cell Apoptosis

To investigate the effects of TRIP13 on cellular growth, EOC cells, including SKOV3 and OVCAR-3, were evaluated by CCK-8 and cell colony formation assays after being transfected with TRIP13 siRNAs (si-TRIP13-1 or si-TRIP13-2) or negative control siRNAs (si-NC). The qRT-PCR assays suggested that knockdown efficiency of TRIP13 siRNAs was high in SKOV3 and OVCAR-3 cells (Figure 2A). The enforced expression of TRIP13 resulted in a remarkable decrease in the proliferation of the SKOV3 and OVCAR-3 cells (Figure 2B). Meanwhile, the cell colony assays revealed that TRIP13 siRNAs dramati-

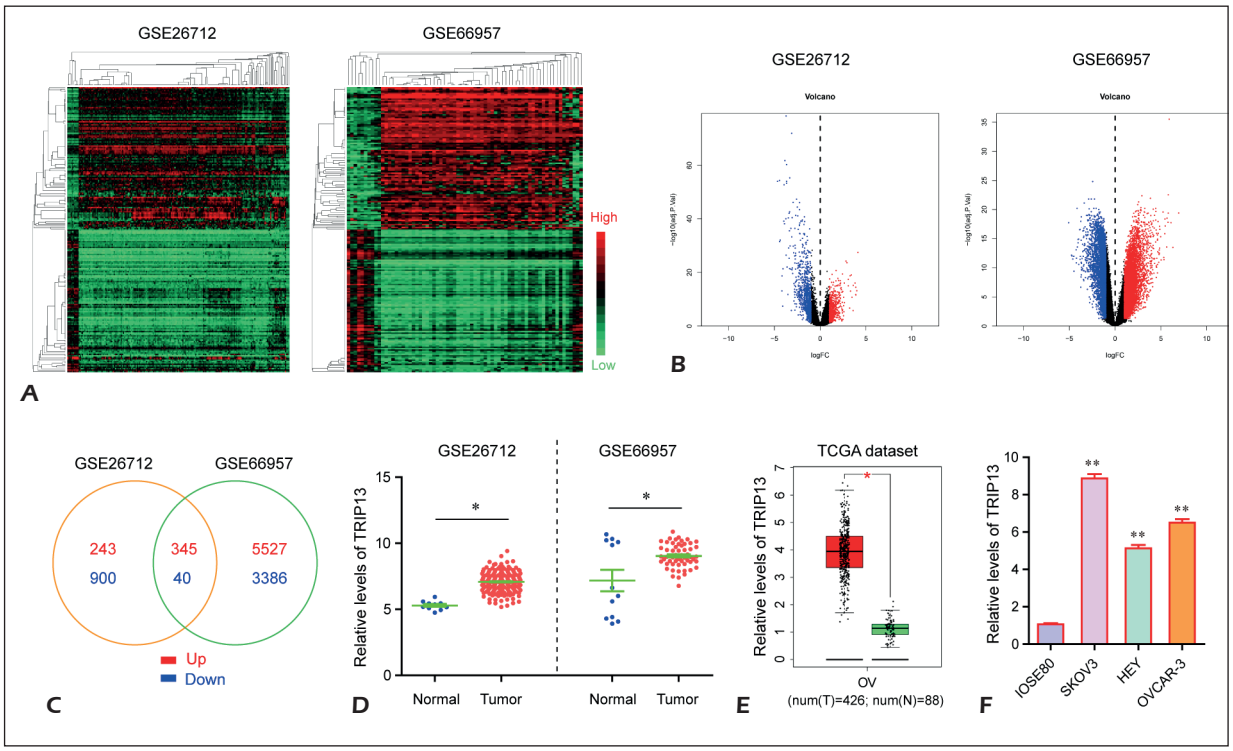

Figure 1. Bioinformatics analysis of differentially expressed genes in ovarian carcinoma. **A**, Heat map from microarray demonstrated differently expressed genes by analyzing GES26712 and GSE66957 datasets. Red represents high relative expression levels while green represents low relative expression levels. **B**, Volcano plot of differentially expressed genes by analyzing GES26712 and GSE66957. **C**, Venn diagram of differentially expressed genes in GES26712 and GSE66957 datasets. **D**, The relative expression of TRIP13 mRNA in EOC tissues compared with normal tissue was analyzed using GEO datasets GES26712 and GSE66957. **E**, The relative expression of TRIP13 mRNA in EOC tissues compared with normal tissue was analyzed using TCGA datasets.

cally suppressed the number of SKOV3 and OVCAR-3 cell colonies (Figure 2C). Furthermore, the analysis of cell apoptosis by flow cytometry revealed that transfection of TRIP13 siRNAs notably promoted the apoptotic rates of SKOV3 and OVCAR-3 cells (Figure 2D and E). Collectively, these results suggested that TRIP13 was an essential regulator in the development of EOC cell proliferation and apoptosis.

Knockdown of TRIP13 Inhibited the Metastatic Potentials of EOC Cells

To further explore the effects of TRIP13 on cellular metastasis of EOC cells, wound healing and transwell assays were then conducted using SKOV3 and OVCAR-3 cells. From the data of wound healing assays in Figure 3A and B, it is apparent that suppressive expression of TRIP13 remarkably impeded the migratory capacities of SKOV3 and OVCAR-3 cells. Furthermore, transwell invasion assays demonstrated that the invasive cell number of TRIP13 siRNAs transfected SKOV3 and OVCAR-3 cells was significantly decreased compared with that of cells transfected with negative control siRNAs (Figure 3C). In addition, we examined the alteration of N-cadherin and Vimentin which were involved in epithelial-mesenchymal transition (EMT). The results of Western blot assays indicated that depression of TRIP13 markedly reduced the protein levels of N-cadherin as well as Vimentin in SKOV3 and OVCAR-3 cells (Figure 3D and E). Overall, these data validated that TRIP13 played critical roles in the cellular metastasis of EOC cells by modulating the expression of EMT related molecules.

Depletion of TRIP13 Suppressed the Activity of Notch Signaling in EOC Cells

We next aimed to study the molecular mechanism of TRIP13 in regulating the development and progression of EOC cells. As the Notch signaling

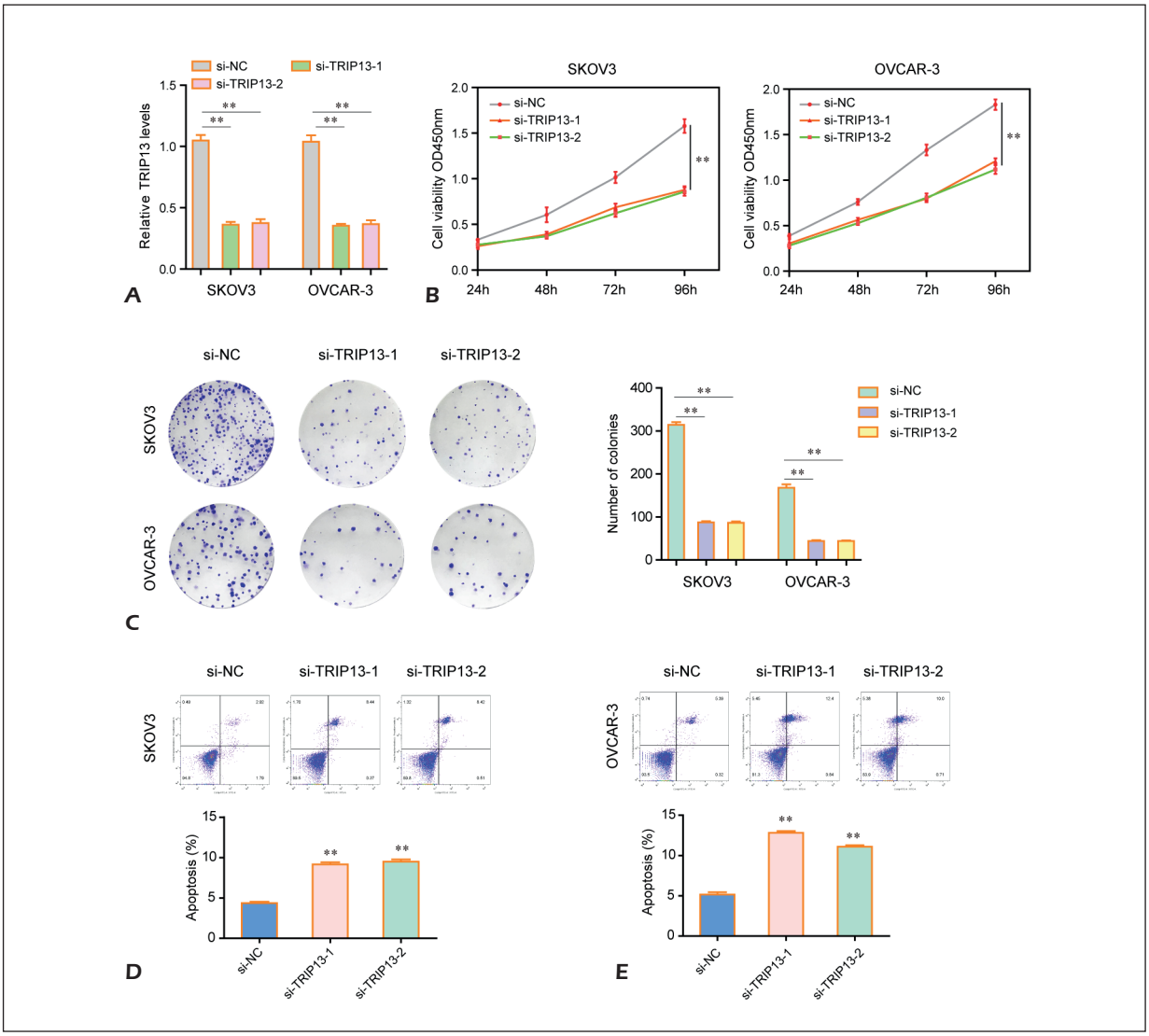

Figure 2. The effects of TRIP13 on the proliferation and apoptosis of SKOV3 and OVCAR-3 cells. **A**, qRT-PCR determined the relative mRNA levels of TRIP13 in SKOV3 and OVCAR-3 cells. **B**, CCK-8 assays detected the cellular growth of SKOV3 and OVCAR-3 cells transfected with TRIP13 siRNAs (si-TRIP13-1 and si-TRIP13-2) or negative control siRNAs (si-NC). **C**, Transfection of TRIP13 siRNAs reduced colony formation capabilities of SKOV3 and OVCAR-3 cells. **D**-**E**, Flow cytometry analysis evaluated the cells apoptosis analysis of SKOV3 and OVCAR-3 cells. *p<0.05, **p<0.01.

pathway was involved in modulating a plethora of biological functions of multiple cancers, we next carried out Western blot assays to evaluate the Notch signaling-related molecules. The results indicated that transfection of TRIP13 siRNAs significantly reduced the protein levels of Notch1 as well as its downstream target molecules, P21 and Hes1, in SKOV3 and OVCAR-3 cells (Figure 4). Therefore, these data provided evidence that the suppressive effects of TRIP13 knockdown on the proliferation and metastatic potentials of EOC cells might be *via* impairing the activity of Notch signaling.

Discussion

EOC has become a serious burden worldwide, resulting in 161,100 deaths worldwide in 2015[17]. The prognosis of EOC patients depends on the extent of the disease, the subtype of tumor present, and other medical conditions[18,19]. In China, the incidence and mortality rates of EOC are increasing and death from EOC is more common in developed countries than in developing countries[20,21]. Surgery and chemotherapy are effective for the patient at an early stage; however, treatments for advanced or recurring cases have been

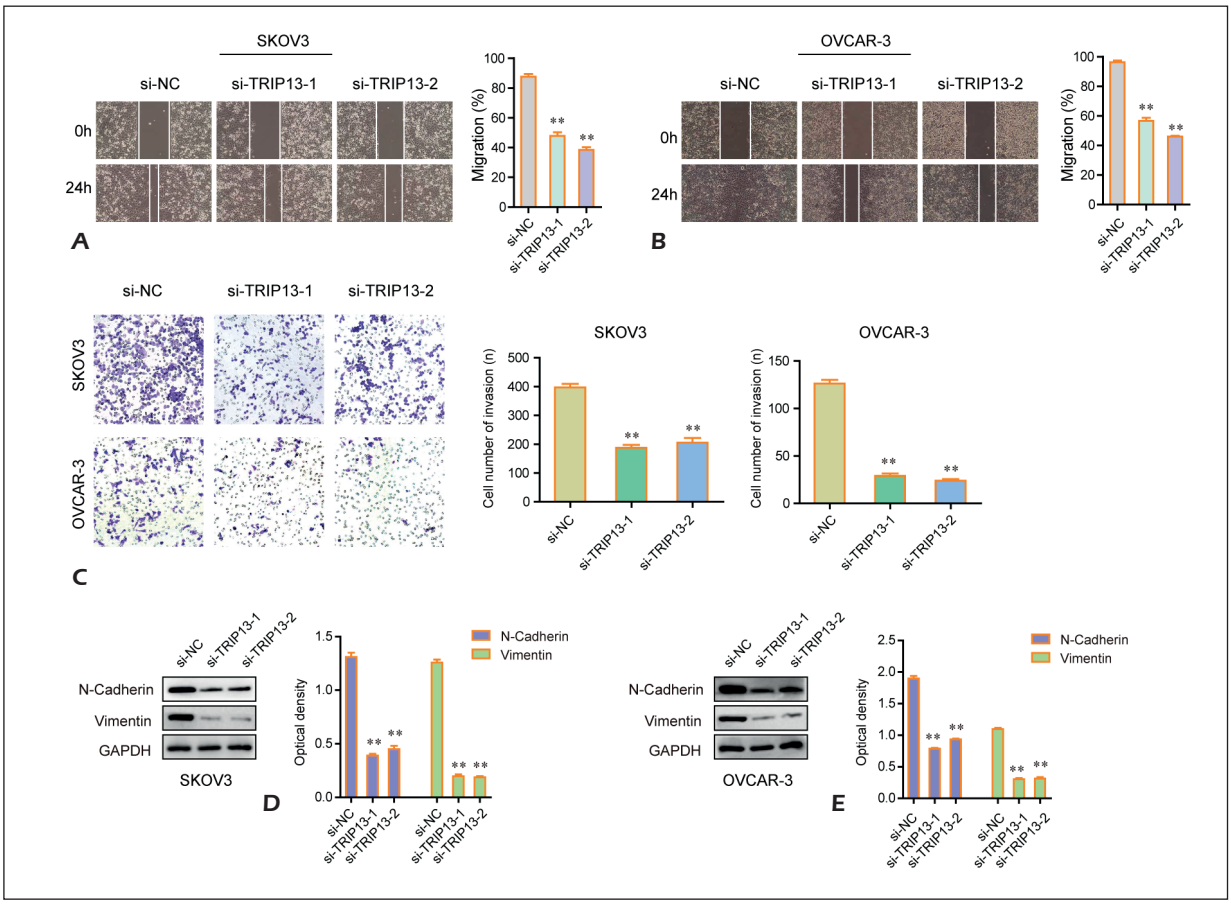

Figure 3. Silence of TRIP13 affected the migration and invasion of SKOV3 and OVCAR-3 cells. **A**-**B**, Transfection of TRIP13 siRNAs (si-TRIP13-1 and si-TRIP13-2) significantly reduced the migratory capacities of SKOV3 and OVCAR-3 cells. **C**, The invasive abilities of SKOV3 and OVCAR-3 cells transfected with TRIP13 siRNAs (si-TRIP13-1 and si-TRIP13-2) were markedly decreased compared with the control cells. **D**-**E**, The protein levels of N-cadherin and Vimentin detected by Western blot. *p<0.05, **p<0.01.

most often unsuccessful[22]. On the other hand, outcome following surgery differs substantially and such large variation is mostly unexplained[23]. Thus, understanding the molecular mechanisms for carcinogenesis is valuable to develop effective therapies and novel sensitive biomarkers for this disease. Previously, TRIP13 has been confirmed to be a kinetochore-localized protein which en-

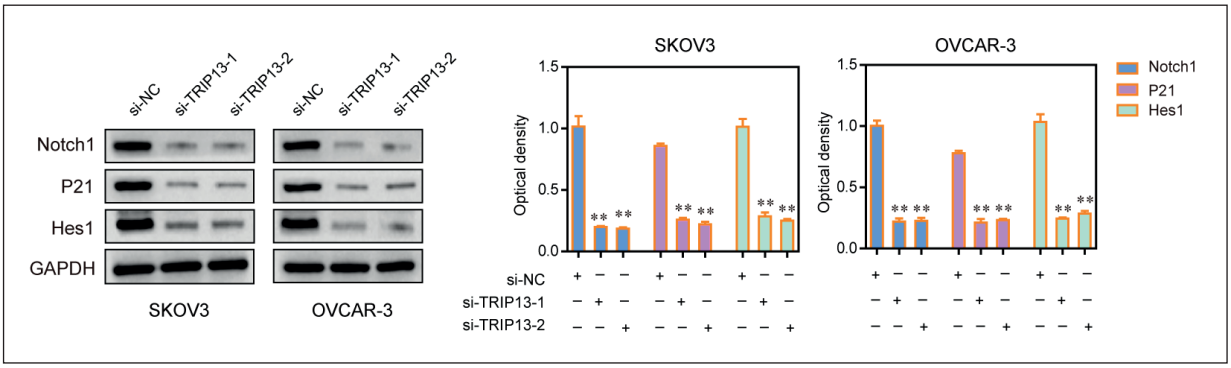

Figure 4. TRIP13 regulated the activity of Notch signaling in SKOV3 and OVCAR-3 cells. Western blot assays were performed to evaluate the protein levels of Notch1, P21 and Hes1 in SKOV3 and OVCAR-3 cells. The relative optical density of the protein bands was assessed by Image J software (version 1.46; NIH, Bethesda, MD, USA). *p<0.05, **p<0.01.

sures the accurate progression of cell division. Evidence has shown that kinetochore-localized proteins play an important role in the progression of tumors[24]. The expression pattern and biological function of TRIP13 were also reported in several tumors. For instance, Li et al[16] first showed that the overexpression of TRIP13 was associated with poor prognosis in lung adenocarcinoma patients, and the knockdown of TRIP13 suppressed lung adenocarcinoma cell proliferation and migration by modulating PI3K/Akt signaling. Dong et al[25] showed that TRIP13 was significantly up-regulated in human prostate cancer tissues, which was correlated with distant metastasis and unfavorable prognosis. Function assay showed that TRIP13 plays a positive role in regulating prostate cancer cell proliferation and metastasis. However, the expression and roles of TRIP13 in EOC have not been investigated. In this work, we analyzed the microarray data from GEO (GSE26712 and GSE66957) and TCGA dataset and found overexpressed TRIP13 expression in EOC tissues. Moreover, we performed RT-PCR to further confirm online data, finding that the levels of TRIP13 were up-regulated in EOC cell lines. Thus, our results, together with the results of GEO and TCGA dataset, showed that TRIP13 may act as a tumor promoter in EOC. Then, we performed a lost-function assay to explore the biological function of TRIP13 in EOC and the results indicated that the knockdown of TRIP13 suppressed EOC cells proliferation, migration and invasion. On the other hand, flow cytometry indicated that the down-regulation of TRIP13 markedly promoted apoptosis. The epithelial-mesenchymal transition (EMT) is an essential step in invasion and metastasis of human cancers[26]. To explore whether TRIP13 promoted the ability of migration and invasion, we further performed Western blot to explore the effect of TRIP13 on EMT, finding that the knockdown of TRIP13 lead to decreased expression of E-cadherin and Vimentin, indicating that TRIP13 promoted the EMT in EOC cells. Our findings indicated that TRIP13 acted as a tumor promoter in EOC by promoted EOC cells proliferation, migration, invasion and EMT. The Notch signaling pathway, a highly conserved cell signaling system present in human organisms, controls cell fate in metazoans through local cell-cell interactions[27,28]. Notch signaling is critical for maintaining the balance between cell proliferation, differentiation, and apoptosis as well as migration and invasion, which suggests that Notch signaling was involved in the regulation of various tumors[29-31]. It was also reported[32] that Notch signaling promotes migration and invasion in carcinoma by inducing EMT. Indeed, the activation of the Notch signaling pathway may contribute to the progression of EOC[33,34]. However, Notch can display opposite effects in the same tumor under differing microenvironmental conditions which suggested the diversity of roles of Notch signaling pathway[35]. In this work, we wondered whether TRIP13 may display its role by modulating the Notch signaling pathway. As expected, we found that EOC cells transfected with si-TRIP13 displayed an increase in expression levels of Notch1, P21 and Hes1, suggesting that TRIP13 may promote the activity of the Notch signaling pathway. Thus, our findings first showed that TRIP13 may be an important contributor to EOC cancer development by modulating the Notch signaling pathway.

Conclusions

We showed that TRIP13 acts as a tumor promoter gene in EOC, and can promote cell migration and invasion by promoting the Notch signaling pathway to inhibit EMT. Therefore, TRIP13 may serve as a potential anti-metastatic therapeutic target for EOC treatment.

Conflict of Interests

The authors declare that they have no conflict of interest.

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