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*Original Article*

# NF-1 suppresses proliferation and migration of epithelial ovarian cancer cell via regulation of autophagy

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**Abstract:** Epithelial ovarian cancer (EOC) is the most lethal gynecological malignancy worldwide. Neurofibromatosis type 1 (NF-1) is a tumor-suppressor gene that encodes neurofibromin. However, whether NF-1 plays a role in the development of EOC is not known. The present study was designed to investigate the possible function of NF-1 in EOC and to examine the probable mechanisms. The mRNA and protein level of NF-1 was significantly reduced in EOC tissues, compared with that in normal ovarian tissues. In addition, in all the EOC cell lines, including 8910, SKOV3, A2780 cell lines, mRNA and protein level of NF-1 was markedly lower than that in the normal human ovarian cell line (IOSE80). In A2780 cells, EdU staining was markedly reduced by overexpression of NF-1. In wound healing assay, migration of cells transfected with pCMV-NF-1 was remarkably decreased compared with control cells. Overexpression of NF-1 significantly increased the protein level of Atg 5, Beclin 1 and LC3, indicating the increase of autophagy in EOC cells. The phosphorylation of mTOR and P70S6K was markedly reduced by overexpression of NF-1. The data suggested that downregulation of mTOR/P70S6K signaling may play a role in NF-1-induced autophagy and the subsequent inhibition of cell proliferation and invasion. Overall, we found that NF-1 inhibited EOC cell proliferation and migration through increase of autophagy. Our findings identify NF-1 as a novel therapeutic target for the inhibition of EOC growth.

**Keywords:** Epithelial ovarian cancer, neurofibromatosis type 1, autophagy, mTOR

## Introduction

Epithelial ovarian cancer (EOC) is the most lethal gynecological malignancy worldwide [1]. EOC accounts for 25% of all malignancies affecting the female genital tract and 4.2% of all cancer-related deaths in women. It was estimated that EOC was responsible for 14,000 deaths in the United States in 2015 [1]. EOC is characterized by difficulty for early diagnosis, high rate of recurrence and migration, intractable treatment and poor prognosis [2, 3]. Current standard treatment for EOC includes a combination of surgical resection and platinum and taxane-based chemotherapy. However, about 75% of the patients ultimately recur within 5 years after initial treatment [4, 5]. Therefore, exploration of novel mechanisms of the development and metastasis and finding new target for effective therapies are emergent for EOC treatment and management.

Neurofibromatosis type 1 (NF-1) is a tumor-suppressor gene that encodes neurofibromin [6]. Neurofibromin is a Ras-GTPase-activating protein, which converts active Ras-GTP to its inactive form, thereby negatively regulating Ras signaling [7, 8]. Germline mutations in the NF-1 gene causes neurofibromatosis type 1, a common genetic disorder that affects over 2 million people worldwide [8]. Moreover, NF-1 acts as a tumor suppressor in several other types of cancers. Abnormal expression of NF-1 could result in constitutive activation of Ras, which can facilitate multiple signal transduction pathways, leading to various cancer phenotypes [9-11]. Wang et al. reported that NF-1 functioned as a tumor suppressor in gastric cancer

which is the target of miR-107 [12]. In contrast, positive neurofibromin expression in colorectal carcinoma was suggested to be a sign of aggressive disease and poor outcome [13]. However, whether NF-1 plays a role in the development of EOC is not known.

The present study was designed to investigate the possible function of NF-1 in EOC and to examine the probable mechanisms. We found that NF-1 expression was decreased in EOC tissues and cell lines and functioned to suppress EOC cell proliferation and migration through enhancement of autophagy.

## Materials and methods

### *Chemicals and materials*

β-actin was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz). Atg5, Beclin 1, LC3, mTOR, p-mTOR, P70S6K and p-P70S6K antibodies were obtained from Cell Signaling Technology Technology. Most of the other chemicals used were obtained from Sigma.

### *Ethics statement and clinical specimens*

The study was approved by the Ethical Review Committee of Xijing Hospital, Fourth Military Medical University and complied with the Declaration of Helsinki. 5 EOC tissue specimens and 4 normal ovarian tissue specimens were collected from surgery during Feb 2015 to Nov 2016, snap-frozen in liquid nitrogen and then stored at -80°C, at the Department of Obstetrics and Gynecology, Xijing Hospital, Fourth Military Medical University (Xi'an, China). All patients provided their informed consent. The EOC was diagnosed by two independent pathologists according to the WHO classification.

### *Cell culture*

The normal ovarian epithelial cells (IOSE80) and ovarian cancer cell lines (8910, SKOV3, A2780) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientic, Inc., Waltham, MA, USA) supplemented with 10% fetal calf serum (Gibco; Thermo Fisher Scientific, Inc.), 100 μg/ml streptomycin, and 100 U/ml penicillin in a humidified atmosphere of 5% $CO_2$ at 37°C.

### *Plasmids and cell transfection*

The full-length NF-1 cDNA was obtained by PCR using an expressed sequence tag clone as template, and constructed into the pCMV vector to express NF-1. The cells were transfected using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols.

### *EdU staining*

Cell proliferation was evaluated by EdU staining using an EdU Assay Kit (RIBBIO, Guangzhou, China). Briefly, cell dishes were added with 50 μmol reagent A and incubated for 2 h at 37°C. Then, cells were fixed in 4% paraformaldehyde for 30 min and neutralize with 2 mg/mL glycine for 5 min. After that, cells were permeabilized by 0.5% Trixon-100. Subsequently, cells were incubated with Reagent B, C, D, E mixture and then were rinsed with 0.5% Trixon-100 for 2, 3 times, washed with methanol for 1, 2 times and stained with DAPI. After washing with PBS, images were captured using a fluorescence microscope.

### *RNA isolation and real-time PCR*

Total RNA was isolated using Trizol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). cDNA was synthesized using 500 ng total RNA by a commercial kit according to the manufacturer's instructions (Takara, Tokyo, Japan). 1 μl cDNA was used for the performance of real-time PCR with SYBR Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) on an ABI Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The PCR reaction conditions were as follows: initial denaturation at 95°C for 10 min followed by 30 cycles at 95°C for 1 min, annealing at 53°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 5 min. Gene expression was normalized to the expression of house-keeping gene, and fold-changes were calculated using the $2^{\Delta\Delta Cq}$ method.

The primer sequences used in the qRT-PCR were: NF-1: Forward primer 5'-CAGAATTCCCCCCTCAACTTCGAAGT-3'; Reverse primer 5'-TGCGTGCTGCATCAAAGTTGCTTTTCAC-3'; GAPDH: Forward primer 5'-AGGTCGGTGTGAACGGATTTG-3'; Reverse primer 5'-TGTAGACCATGTAGTTGAGGTCA-3'.

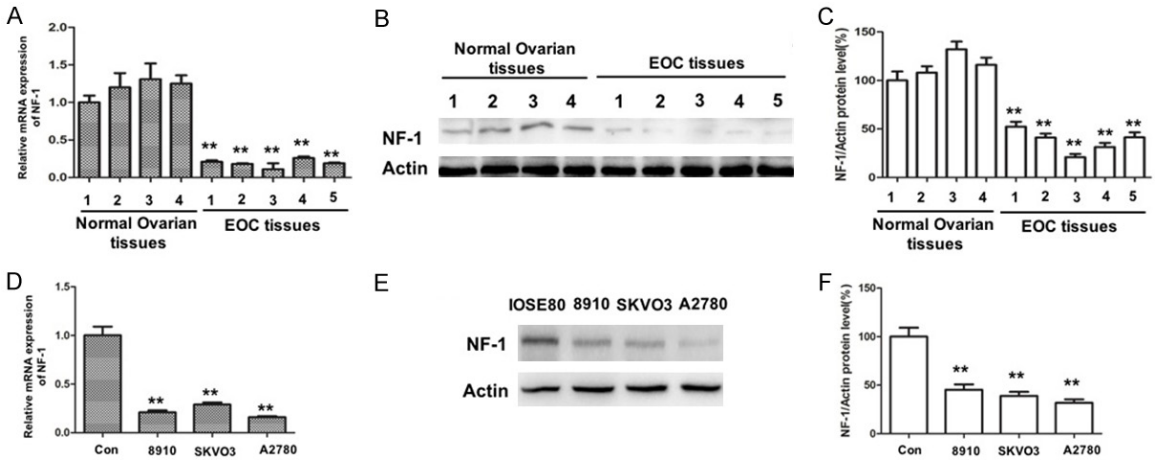


**Figure 1.** Relative expression of NF-1 in EOC tissues and cell lines. A: MRNA expression of NF-1 in EOC tissues and human normal ovarian tissues. B and C: Protein expression of NF-1 in EOC tissues and human normal ovarian tissues. D: MRNA expression of NF-1 in EOC cell lines, including 8910, SKOV3, A2780 cells, and human normal ovarian cell line (IOSE80). E and F: Proetin expression of NF-1 in EOC cell lines, including 8910, SKOV3, A2780 cells, and human normal ovarian cell line (IOSE80). **p<0.01, compared with control.

*Western blot*

Protein was extracted using RIPA lysis buffer (Thermo Fisher Scientific, Inc.) and protein concentration was determined by BCA assay (Thermo Fisher Scientific, Inc.). 20 μg protein was loaded and separated with 10% SDS-PAGE. After separation, proteins were transferred onto PVDF membrane (Millipore, MA, USA), and then blocked using 8% non-fat milk. Membranes were incubated overnight at 4°C with primary antibodies (β-actin: 1:500; Atg5: 1:1000; Beclin 1: 1:1000; LC3: 1:1000; mTOR: 1:1000; p-mTOR: 1:1000; P70S6K: 1:1000; p-P70S6K: 1:1000). After washing with TBST, the membranes were incubated with a horseradish peroxidase (GRP)–conjugated secondary antibody (Thermo Scientific, USA) at 37°C for 1 h. Protein bands were visualized with the ECL and captured using BIORAD Imaging Systems (BIORAD, CA, USA).

*Migration assay*

Cell migration abilities of EOC cells were evaluated by wound healing assay. In brief, an artificial wound in EOC cells was created by scrape using a 200-μl pipette tip. Then, cells were incubated with fresh medium containing mitomycin C (5 μg/ml; Applied Biosystems; Thermo Fisher Scientific, Inc.) for 12 h. The fraction of cell coverage across the line was capture using the light microscope.

*Statistical analysis*

The data are shown as the means ± SEM and data analysis was performed using Graphpad software. Significance was analyzed using One-way analysis of variance (ANOVA) followed by Turkey analysis. P<0.05 was considered significant.

## Results

*NF-1 expression was reduced in EOC tissues and cell lines*

EOC and normal ovarian tissues were collected and expression of NF-1 was determined. As shown in **Figure 1A-C**, mRNA and protein level of NF-1 was significantly reduced in EOC tissues, compared with that in normal ovarian tissues. In addition, in all the EOC cell lines, including 8910, SKOV3, A2780 cell lines, mRNA and protein level of NF-1 was markedly lower than that in the human normal ovarian cell line (IOSE80) (**Figure 1D-F**). The results suggested a possible negative role of NF-1 in the development of EOC.

*Overexpression of NF-1 inhibited the proliferation and migration ability in A2780 cells*

In order to evaluate the role of NF-1 in the growth and invasion of EOC, A2780 EOC cells were transfected with pCMV-NF-1. In **Figure 2A**, we confirmed the efficiency of the plasmid

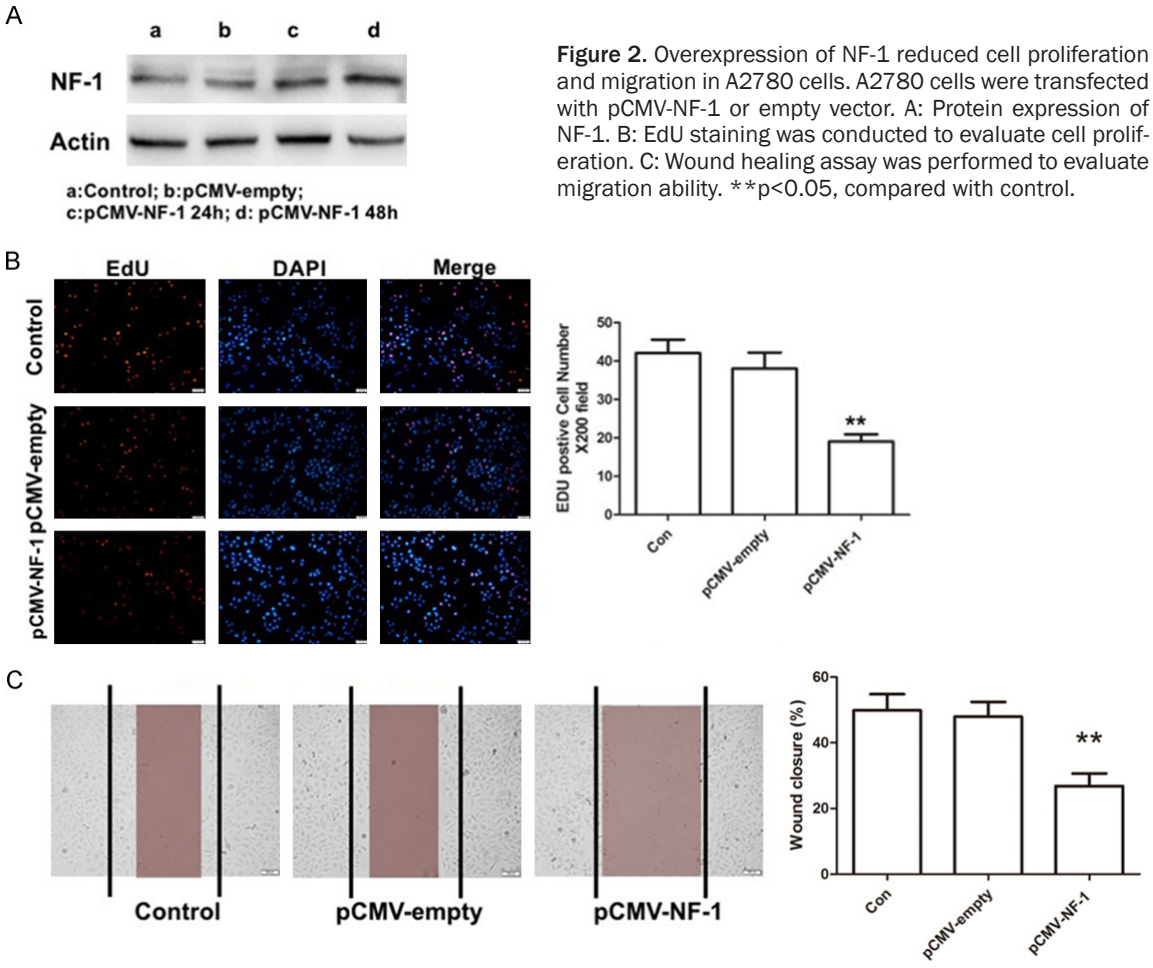


**Figure 2.** Overexpression of NF-1 reduced cell proliferation and migration in A2780 cells. A2780 cells were transfected with pCMV-NF-1 or empty vector. A: Protein expression of NF-1. B: EdU staining was conducted to evaluate cell proliferation. C: Wound healing assay was performed to evaluate migration ability. **p<0.05, compared with control.

showing that NF-1 protein expression was significantly increased after the transfection. The cell proliferation was evaluated by EdU staining. As shown in **Figure 2B**, EdU staining was markedly reduced by overexpression of NF-1, indicating the decrease of cell proliferation. In addition, cell migration ability was assessed by wound healing assay. In **Figure 2C**, we found that the migration of cells transfected with pCMV-NF-1 was remarkably decreased compared with control cells. The results showed that overexpression of NF-1 reduced cell proliferation and migration, suggesting an antitumor role of NF-1 in EOC.

*Overexpression of NF-1 induced autophagy in A2780 cells*

To examine the mechanism of NF-1-mediated inhibition of cell proliferation and invasion, we evaluated the effect of overexpression of NF-1 on autophagy in A2780 cells. The results showed that overexpression of NF-1 significantly increased the protein level of Atg 5, Beclin 1 and LC3 (**Figure 3**), indicating the increase of autophagy in EOC cells. The results demonstrated that induction of autophagy may play a role in NF-1-resulted inhibition of EOC cell proliferation and invasion.

*Overexpression of NF-1 inhibited mTOR/P70S6K signaling in A2780 cells*

Furthermore, we tested the molecular mechanism of NF-1-induced regulation of autophagy. The protein expression of mTOR, p-mTOR, P70S6K, and p-P70S6K was determined. We found that total expression level of mTOR and P70S6K was not significantly altered (**Figure 4**). The phosphorylation of mTOR and P70S6K was markedly reduced by overexpression of NF-1 (**Figure 4**). The data suggested that down-regulation of mTOR/P70S6K signaling may play a role in NF-1-induced autophagy and the

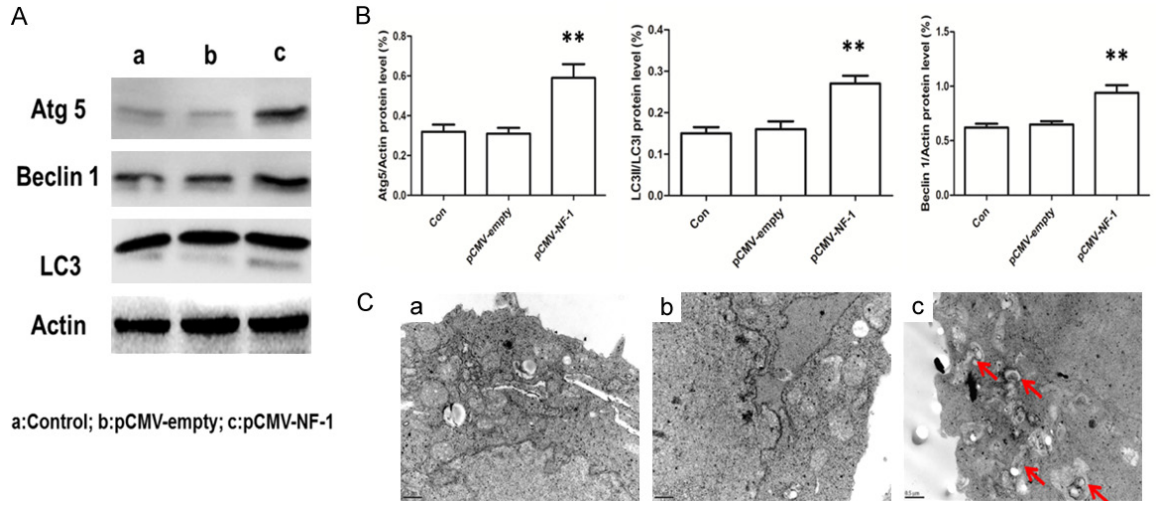


**Figure 3.** Overexpression of NF-1 increased autophagy in A2780 cells. A2780 cells were transfected with pCMV-NF-1 or empty vector. A: Protein expression of Atg 5, Beclin 1 and LC3 was determined to evaluate autophagy. B: Statistical results of protein expression. C: TEM shows the autophagy (n=3, Scale bar indicates 0.5 μm). **p<0.05, compared with control.

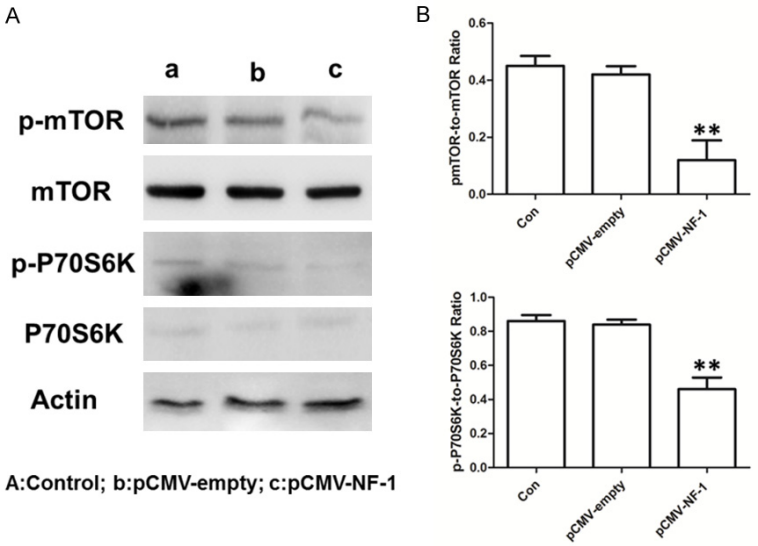


**Figure 4.** Overexpression of NF-1 inhibited mTOR/P70S6K signaling in A2780 cells. A2780 cells were transfected with pCMV-NF-1 or empty vector. A: Protein expression of mTOR, p-mTOR, P70S6K, and p-P70S6K was determined. B: Statistical results of the phosphorylation of mTOR and P70S6K. **p<0.05, compared with control.

subsequent inhibition of cell proliferation and invasion.

## Discussion

In the present study, we investigated the role of NF-1 in EOC cell proliferation and invasion and elucidated the possible molecular mechanisms. It is well-known that NF-1 functions as a tumor suppresser gene in various types of tumors [14-17]. In the present study, we, for the first time, explored the role of NF-1 in EOC cell proliferation and invasion. Our results showed that NF-1 expression was reduced in EOC tissues and cell lines, indicating a negative role of NF-1. Furthermore, overexpression of NF-1 reduced EOC cell proliferation and invasion, as evidenced by decreased EdU staining and migration ability in A2780 cells. The results demonstrated that NF-1 exhibited an inhibitory effect on tumor growth and metastasis.

Autophagy is an essential degradation system of the cell's own lysosomes that facilitates the breakdown of intracellular proteins and organelles. It is usually considered that autophagy provides a protective mechanism, important for the removal of damaged proteins and organelles and conferring stress tolerance and enhancing cell viability under adverse conditions [18]. However, under specific conditions, autophagy can also functions as a pro-death pathway [19, 20]. The process of autophagy is complicated which can be

divided into nucleation, elongation, and formation of autophagosome and subsequent membrane fusing events. The autophagy-related (Atg) protein family is central for the autophagic machinery through regulation of autophagosome formation [21-24], such as Atg 5, Beclin 1 and LC3. Autophagy-mediated increase of amino acid level results in the activation of mechanistic/mammalian target of rapamycin complex 1 (mTORC1) at the cytosolic side. The Akt/mTOR/p70 ribosomal protein S6 kinase (p70S6K) signaling pathway is known to negatively regulate autophagy [19, 25-29]. We showed that overexpression of NF-1 inhibited the phosphorylation of mTOR and p70S6K and increased the expression of Atg 5, Beclin 1 and LC3, providing an evidence for the regulation of autophagy by NF-1. The results demonstrated that enhancement of autophagy may be involved in the anti-tumor role of NF-1 in EOC.

Iyengar et al. studied the differential expression of two isoforms of NF-1 in EOC cells [30]. The found a significant decrease in Type II isoform expression and increase in Type I expression in ovarian cancer cells and tumor tissue relative to HOSE cells. They also demonstrated an increase in Type II:Type I ratio, and a decrease in cell proliferation rate in three ovarian cancer cell lines on treatment with retinoic acid [30]. Based on these results and our findings, we speculated that tumor suppressor role of NF-1 may be mainly attributed to Type II isoform. Further studies are needed to test the hypothesis.

Overall, we found that NF-1 inhibited EOC cell proliferation and migration through increase of autophagy. Our findings identify NF-1 as a novel therapeutic target for the inhibition of EOC growth.

## Disclosure of conflict of interest

None.

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