European Review for Medical and Pharmacological Sciences 2019; 23: 3270-3276

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MiR-182-5p inhibited proliferation and migration of ovarian cancer cells by targeting BNIP3

X.-N. JIA, S.-D. YIN, Y. WEI, L. CHEN

Department of Obstetrics and Gynecology, Navy General Hospital, Be[illegible]hin[illegible]

Abstract. – OBJECTIVE: To investigate the potential effect of microRNA-182-5p (miR-182-5p) on the development of ovarian cancer (OC) and the relevant mechanism.

PATIENTS AND METHODS: The expression levels of miR-182-5p in OC tissues and paracancerous normal tissues were detected. The miR-182-5p expression in OC cells and ovarian epithelial cells was also determined. Through online prediction (TargetScan, miRDB), the potential target of miR-182-5p was screened and further confirmed by the Luciferase rep[illegible]ter gene assay. The effects of the miR-182-5p[illegible] man ovarian serous papillary cystadeno[illegible]ino ma cell line (SKOV3) cells were determine[illegible] *in vitro* experiments.

RESULTS: The low expression of miR-18[illegible] in OC was confirmed by quantitative Reve[illegible] Transcription-Polymerase Chai[illegible]tion (qR[illegible] PCR) assay. BCL2/adenovir[illegible]Da protein-interacting protein 3 ([illegible]P3) wa[illegible]entified as a direct target of miR-[illegible]5p. Subs[illegible]uent experiments showed that [illegible]NIP3 resulting from the u[illegible]regu[illegible] inhibited cell prolif[illegible]tion, clo[illegible]mation and migration ability o[illegible]C cells.

CONCLUSION[illegible] research sh[illegible] the inhibitory functi[illegible]o[illegible]82-5p in OC by targeting BNIP3, th[illegible]provid[illegible] experimental basis for the trea[illegible]ment of OC.

Key W[illegible]

Mi[illegible]NA-182-[illegible] (miR-182-5p), Ovarian cancer (OC[illegible]2/ade[illegible]rus E1B 19 kDa protein-interacting prote[illegible] Prolifera[illegible], Migration.

[illegible]oduction

O[illegible]er (OC), one of the three major [illegible]ignan[illegible] in the female reproductive sy[illegible] in the world, has the highest mortality rate a[illegible]gynecological malignant tumors. It is esti[illegible]ed that in 2018 there will be 22,240 new cases of O[illegible]erica and 14,0[illegible] death cases, account[illegible] for [illegible]f all cancer-related deaths. Moreover, 1/2,5[illegible]t-menopausal women hav[illegible]oped OC[1].

[illegible]espite the progress in surgical techniques and [illegible] improvement of chemotherapy regimens over [illegible] past four de[illegible]s[2], the majority of OC patients [illegible] diagnosed [illegible]dvanced stage due to the lack o[illegible]tive earl[illegible]creening methods. Many OC patie[illegible]otherapy resistance and relapse after che[illegible]therapies. Additionally, the high he[illegible]ogeneity of OC makes the diagnosis and treat[illegible]complicated. To improve the diagnosis [illegible]d t[illegible]ment of OC, it is necessary to further understand the molecular mechanism of OC onset. As science progresses, the roles of micro-ribonucleic acid (miRNA) in malignancies and related targeted drugs have become the study focuses in medical community nowadays.

In 1993[3] miRNAs were initially discovered in *Caenorhabditis elegans*, and they exist in the genomes of most eukaryotes, including humans[4]. Mature miRNAs are a class of small endogenous, non-coding, single-stranded RNAs composed of 20-22 nucleotides. They participate in forming RNA-induced silencing complexes and act as the post-transcriptional regulators of gene expressions[5]. MiRNAs have been identified in many basic life activities, and they are abnormally expressed under various pathological conditions, including cancers as oncogenes or tumor-suppressor genes[6]. The overexpression of miRNAs may exert a tumor-promoting effect *via* inhibiting tumor-suppressor genes. MiRNAs downregulation might play a tumor-inhibiting effect by negatively regulating oncogenes[7,8]. The potential association between miRNAs and cancers was first detected in chronic lymphocytic leukemia[9]. Extensive studies[10-12] have gradually revealed different miRNAs that are abnormally expressed in human

Corresponding Author: Lei Chen, Ph.D; e-mail: 56462063@qq.com

malignancies. Surprisingly, approximately 50% of human miRNAs with annotations are related to cancers[13].

MicroRNA-182-5p (miR-182-5p) is an important component of the microRNA regulatory network. Lately, it has been widely certified that miR-182-5p exerted critical regulatory effects on many diseases[14-17], but the effects of miR-182-5p on the OC have not been researched yet. This work aimed to detect the functions of miR-182-5p in the occurrence and progression of OC, to provide new ideas and theoretical basis for the clinical treatment and prevention of OC.

Patients and Methods

Ovarian Cancer Cases and Cells

A total of 100 pairs of OC tissues and adjacent normal ovarian tissues surgically resected in the Obstetrics and Gynecology Department in Navy General Hospital from July 2016 to September 2017 were collected as the study groups. The enrolled patients were aged 22-65 with 55 years on average. None of the enrolled patients underwent preoperative chemotherapy and radiotherapy, and were definitely diagnosed with OC after the operation. The present study was approved by the Medical Ethics Committee of the hospital. After being resected, the tumor tissue samples were immediately put into liquid nitrogen.

Human ovarian serous papillary cystadenocarcinoma cell line (SKOV3) and ovarian epithelial cell line (OEC) were purchased from the [illegible] Library of Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, Logan, UT, USA) supplemented with [illegible]% fetal bovine serum (FBS), 100 μg/mL streptomycin and [illegible] IU/mL penicillin (Gibco, Grand Island, NY, USA).

Luciferase Reporter Assays

Through searching TargetScan, miRDB and micro[illegible]sites, it was found that BCL2/adenovirus E[illegible]kDa protein-interacting protein 3 (BNIP3) was [illegible] gene of miR-182-5p. The binding sequence of miR-182-5p at the 3'UTR of BNIP3 was mutated using a point mutation kit (A[illegible]logies, Santa Clara, CA, USA), [illegible] the mutated BNIP3 (Mut-type) and wild-type BNIP3 (WT-type) were inserted into the p[illegible]Basic Luciferase reporter vector (Promega, Madison, WI, USA). SKOV3 cells were inoculated in a 24-well plate and co-transfected with miR-182-5p mimics or negative contr[illegible]type BNIP3 or WT-type BNIP3, respectively. [illegible] Luciferase activity was determined using the Luciferase Reporter Gene Assay [illegible] Biotech, Encinitas, CA, USA).

Transfection

MiR-182-5p mimics [illegible] LV-BNIP3 were [illegible]sfected into SKOV3 cells, with reference to the instruction of Lipo[illegible]ine [illegible]000 (Invitrogen, Carlsbad, CA, USA). A[illegible]s cultur[illegible] for 48 h, they were subjected to re[illegible]s. Three group were established to ana[illegible]logical function of m[illegible]: the NC group (negative control), miR-182-5p [illegible]s (SKOV3 cell transfected with miR-182-5p mi[illegible] and mimics + BNIP3 (SKOV3 cell transfected [illegible] miR-182-5p mimics a[illegible]V-BNIP3). Transfection plasmids were purchased from RiboBio (Guangzhou, China).

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) Analysis

SKOV3 cells were inoculated into a 6-well plate [illegible] the density of 1×10^6 cells/L. After 36 h of culture, [illegible] RNA was extracted from the cells in accordance with the instruction in TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and the concentration and purity of RNA were measured using an ultraviolet spectrophotometer. The complementary Deoxyribose Nucleic Acid (cDNA) diluted in SsoFast EvaGreen reagent (1:10) was utilized for qPCR. The relative expressions were normalized with the corresponding reference genes. QRT-PCR conditions were 37°C for 15 min and 98°C for 5 min. Afterward, PCR was performed according to the instruction of the kit. The expression level of mRNA was calculated by $RQ=2^{-\Delta\Delta CT}$. Primer sequences used in this study were as follows: miR-182-5p, F: 5'-TTAGGAACCCTCCTCTCTC-3', R: 5'-CGGTGATGTGAAGAAGGA-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'.

Western Blot (WB) Analysis

Total proteins were extracted from cells and quantified *via* bicinchoninic acid (BCA; Pierce, Waltham, MA, USA). The total protein was loaded (25 μg/well) for electrophoresis and transferred onto membranes, followed by blocking in 5% skimmed milk powder and inoculation with the primary antibody overnight. After the membranes were washed by Tris-Buffered Saline with

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Tween-20 (TBST) 3 times, the cells were incubated with the corresponding secondary antibody at room temperature for 2 h, and membranes were washed again 3 times, followed by development *via* enhanced chemiluminescence (ECL). Then, ImageLab3.0 (Bio-Rad, Hercules, CA, USA) was employed for analysis, with the ratio between the measured value of target protein and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the relative expression level of proteins. The experiment was repeated 3 times.

Cell Proliferation

Cells were digested and the concentration was adjusted to 5×10^5 cells/mL. After cell adherence in a 96-well plate (100 μL/well), cells were incubated with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) solution (Sigma-Aldrich, St. Louis, MO, USA) to incubate for 4 h at the appointed time point (0, 24, 48 and 72 h). Finally, the optical density (OD) value was measured at 490 nm.

For clone formation, cells were digested, centrifuged and inoculated into a 6-well cell culture plate at 1×10^3/well for 10 days. At 10th da[illegible]ls were washed with Phosphate-Buffere[illegible] (PBS) 3 times, fixed with 1 mL methano[illegible] 15-20 min and with 0.1% crystal violet soluti[illegible] 20-30 min. Finally, the cells were photograp[illegible] counted and analyzed.

Cell Cycle Determinatio[illegible]

After transfection, the [illegible] were di[illegible]ted with trypsin, collected *via* l[illegible]ed ce[illegible] washed with the PBS three [illegible] with 70% ethanol so[illegible]on at 4°C [illegible]ight. Then, cells were washe[illegible]th PBS again [illegible]suspended in about 10[illegible]ase, followed [illegible] incubation *via* wate[illegible]th at [illegible] for about 30 min. 100 μL of propidium iodide ([illegible]&D Systems, Minneapolis, [illegible]N, USA) was ad[illegible] incubation at 4°C fo[illegible]out 30 min in the da[illegible]. The flow cytometr[illegible]as used [illegible]o detect the red fluorescence at an [illegible]tion [illegible]elength of 488 nm, and the data were [illegible]or analy[illegible]

[illegible]Migrat[illegible]ys

[illegible]wound he[illegible]g assay was performed to detect [illegible] effect of miR-182-5p on cell migration and in[illegible] With the cell density adjusted to [illegible] cells/[illegible], the cells were inoculated into a [illegible]ll plate and placed in an incubator containing 5[illegible] for incubation overnight till 100% cell fusio[illegible]he back of each well was marked with a marker pen to ensure the data were obtained from the same places. The plate was scratc[illegible] using the sterilized pipette tip pe[illegible]dicular [illegible] the marked line along the rule[illegible]d the wells were rinsed repeatedly with PB[illegible]e suspension cells were wiped off, and the se[illegible] Dulbecco's Modified Eagle's Me[illegible]m (D[illegible]Gibco, Grand Island, NY, USA)[illegible]s added in[illegible]ture wells for incubati[illegible]n an incubator c[illegible]ning 5% CO_2 at 37°C[illegible]e initia[illegible]stance (0 h) of scratches was meas[illegible]nd [illegible] scratch distance was measure[illegible] after [illegible]llowed [illegible] photography and ca[illegible]ation of c[illegible]rati[illegible] rate.

Statistic[illegible]is

Statis[illegible]l analy[illegible]s performed with the Student's *t*-test or *F*-test. [illegible] values were two-sided and [illegible]5 was conside[illegible]gnificant and analyz[illegible]y [illegible]ism 6.02 software (La Jolla, CA, USA).

[illegible]Results

Mi[illegible]pression was Reduced in Both T[illegible] and Cells of OC

[illegible]QRT-PCR was used to detect the expression of [illegible]p. This study enrolled 100 OC patients. [illegible]e [illegible]ession level of miR-182-5p in OC tissue samples was (0.54±0.13), which was significantly lower than that of matched adjacent tissues (1.12±0.07), and the difference was statistically significant ($p<0.01$) (Figure 1A). In ovarian cancer cell line (SKOV3) and ovarian epithelial cells (OEC), the relative expression of miR-182-5p was (0.69±0.08) and (1.03±0.16), respectively. The difference was statistically significant ($p<0.001$) (Figure 1B). MiR-182-5p was lowly expressed in OC.

BNIP3 was a Direct Target of MiR-182-5p in OC Cell

The potential target of miR-182-5p was checked in three publicly available algorithms: TargetScan, miRDB and microRNA. BNIP3 was found to have a pairing sequence with the miR-182-5p at the 3'UTR (Figure 2A). The anti-apoptotic ability of BNIP3 in malignant tumors has been widely reported[18-22], so we tried to verify whether BNIP3 had a correlation with miR-182-5p in OC. The transfection efficiency was first confirmed by qRT-PCR (Figure 2B). We established Luciferase reporter vectors containing the wild-type or mutant-type miR-182-5p sequences of the BNIP3. The overexpression of miR-182-5p resulted in the decrease of the Luciferase activity of the wi-

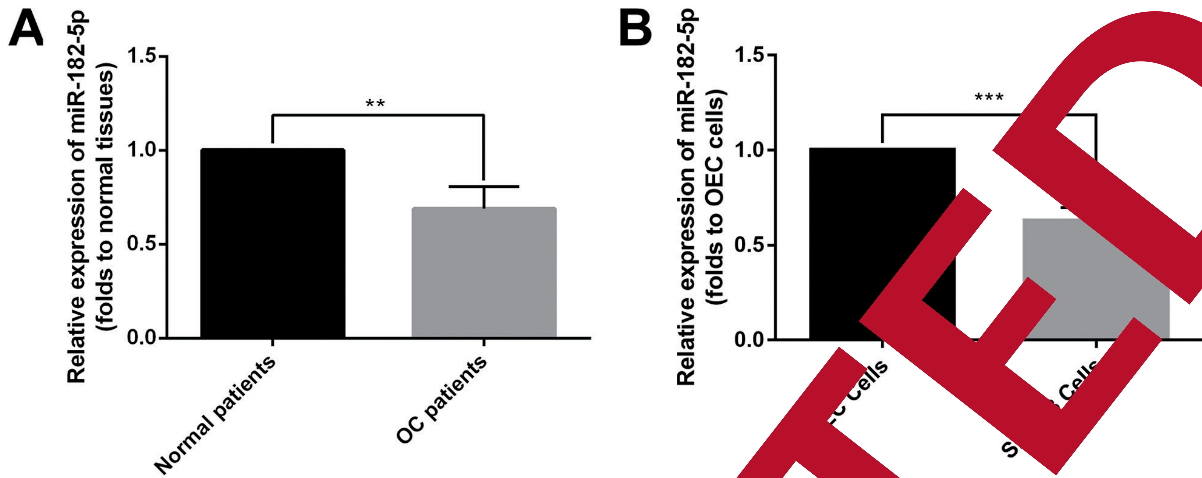


Figure 1. Expressions of miR-182-5p in ovarian cancer tissue samples and ovarian cancer [illegible] Difference in the expression of miR-182-5p between OC tissues and corresponding adjacent normal ovarian tissues (**p<[illegible]. Expression of miR-182-5p in OC cell line (SKOV3) and ovarian epithelial cell line (OEC) (**[illegible]).

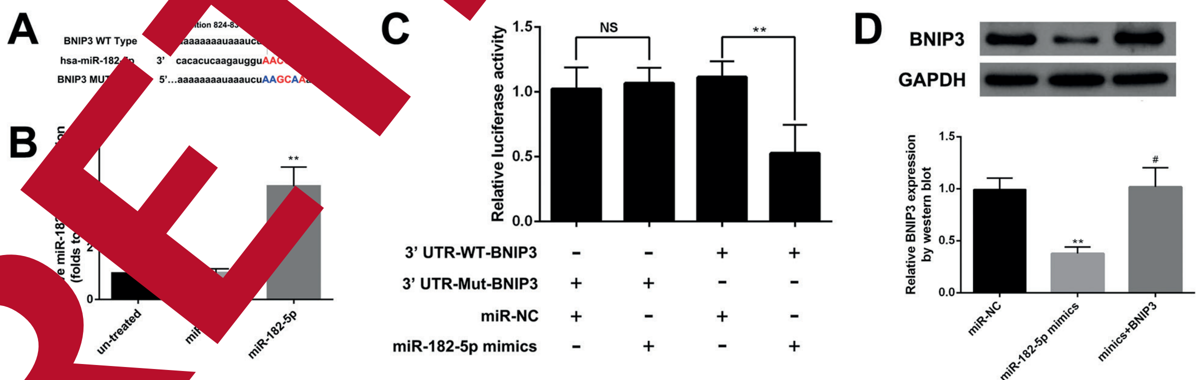
de-type BNIP3 reporter gene, but it had no effect on the Luciferase activity of mutant-type group (Figure 2C). The experimental results of Luciferase reporter assay were encouraging; ho[illegible] the specific interference method needs [illegible] experimental confirmation.

MiR-182-5p Decreased the Expression [illegible] Level of BNIP3

Western blot assay was u[illegible] detect th[illegible] expression of BNIP3 in the [illegible] gro[illegible] negative control), miR-182-5p mim[illegible] (SKOV[illegible] cell transfected with miR-182-5p [illegible]cs) a[illegible] BNIP3 (SKOV3 cell t[illegible]ansfe[illegible] [illegible]ics and LV-[illegible]IP3). The relative expression le[illegible] BNIP3 i[illegible] three groups was (0.98±0.18), (0.3[illegible] 1.02±0.25), respectively, and the difference was statistically significant (p<0.05). [illegible]se data evidenced the regulation of miR-182-[illegible]P3 expression (Figure 2D).

MiR-182-5p Suppressed Proliferation of OC Cell

MTT assay results showed that the overexpression of miR-182-5p inhibited the proliferation of SKOV3 cells. On the third day of the MTT assay, the cell proliferation status began to show significant differences (p<0.05, relative to the negative



[illegible]ure 2. BNIP3 is a direct and functional target of miR-182-5p. ***A,*** Diagram of putative miR-182-5p binding sites of BNIP3. [illegible]sfection efficiency detection by qRT-PCR (**p<0.01). ***C,*** Relative activities of Luciferase reporters (**p<0.01). ***D,*** The ex[illegible]s of BNIP3 in OC cells after different treatment. Data were presented as means ± standard deviations (**p<0.01 *vs.* NC g[illegible], #p<0.05 *vs.* Mimics group).

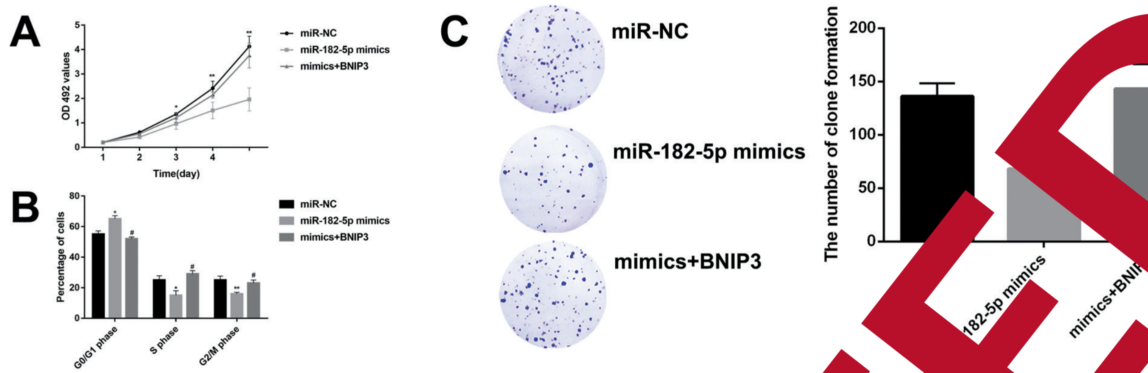


Figure 3. ***A,*** MiR-182-5p inhibited the proliferation of OC cell (*p<0.05, **p<0.0 . NC group). ll e phases of OC cell analyzed using flow cytometry (*p<0.05, **p<0.01 *vs.* NC group; #p< Mimics group). ent of colony formation (**p<0.01 *vs.* NC group; ##p<0.01 *vs.* Mimics group). All data wer s means ± standa deviations.

control group). The proliferation curve of SKOV3 cells in the miR-182-5p overexpression group showed significant inhibition of proliferation (p<0.01, relative to the negative control group). However, the proliferation ability of SKOV3 cells in the mimics+BNIP3 group was not much different from that of the negative control p (p>0.05, relative to the negative contro (Figure 3A).

In the clone formation experiment, th ll proliferation inhibition of miR-182-5p was si taneously demonstrated. The overexpression miR-182-5p led to an approxi 5-fold re duction in clone formation p rties ure 3C).

To explore the mechani f miR-1 5p in the inhibited proliferation o V3 ce the flow cytometry (CM) cell cycle progressi We foun miR-182-5p markedly arreste C cells in the phase. However, SKO verexpressing P3 did not find this nom suggesting that miR-182-5p regulated the pro n of SKOV3 cells by target BNIP3 (Figure

MiR 2-5p I hibited Migration of ells

Scr showe at miR-182-5p overexpressi ifica inhibited the confluence OV3 c es, and the quantitative resu the scrat area of the cells at each time poi ere compared. The relative percentage of wo f the negative control group at 0 d 48 e (0.95±0.04) and (0.37±0.09), whi ere (0.94±0.05) and (0.43±0.01) at 0 h and 48 miR-182-5p mimics group. The wound clos of miR-182-5p mimics group was signifi-

c ger than that of th miR-NC group at 48 p<0.05). However, we found that in the mimic-BNIP3 group relative percentage of wound ure of SKO ells at 48 h was (0.33±0.08), a differen was not statistically significant (p> to the negative control group) (Figure 4).

Discussion

OC is one of the most common malignant tumors in women. Although new biological markers and treatment strategies have been advanced in the past several decades, the survival rate of OC women is still not substantially improved. It is essential to raise the awareness of the molecular mechanism of OC onset to discover highly specific and sensitive biological markers for OC screening and early detection. The development of potential therapeutic targets contributes to improving the survival rate and clinical outcomes of OC patients.

The occurrence of tumors is a complex process involving multi-stage processes. The control of cell proliferation, the balance between cell survival and programmed cell death/apoptosis, the communication with adjacent cells and extracellular matrix, the induction of tumor angiogenesis and the invasion and migration of tumor cells are of great significance. The major biological characteristics of malignancies include the abnormal proliferation, apoptosis, invasion and migration of tumor cells and angiogenesis. MiRNAs are involved in various processes, including the occurrence and development of tumors, and affect

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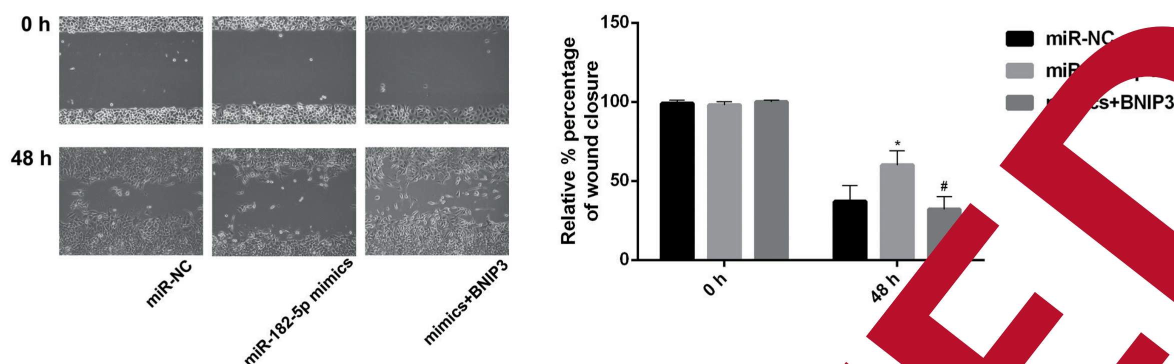

Figure 4. The migration of OC cells was analyzed using wound healing assay a[illegible]etected by [illegible]cope [illegible]00). Data were presented as means ± standard deviations (*$p<0.05$ *vs.* NC group; #$p<0.05$ [illegible]imics group).

many characteristics of tumor cells[10,23]. Moreover, miRNAs are differentially expressed in multiple tumors, and may serve as crucial biological markers for tumor treatment. Scholars[12,24] pointed out that multiple miRNAs could be detected in the early stage of OC and are correlated with the prognosis of the disease. Therefore, miRNA is a key molecule with high specificity and sensitivity in the diagnosis and treatment of OC.

In 1994, BNIP3, a kind of apoptosis-i[illegible] protein, was first discovered and named by [illegible]d et al[25] in the screening proteins of the yeast t[illegible]-brid system. It is a member of the BH3-only s[illegible]mily in the Bcl-2 family and able to interact w[illegible] adenovirus EIB 19 kD proteins[illegible]ene, wit[illegible] the full length of 1,535 bp, [illegible]ocate[illegible] human chromosome 10q26.3 and [illegible] encode[illegible]4 amino acids. In different types [illegible] and [illegible]ts, it participates in ex[illegible]ensiv[illegible]esses [illegible]s essential for the nor[illegible] growth, [illegible]opment and metabolism of or[illegible]ms. Moreove[illegible]ld form homodimers or [illegible]imers with ot[illegible] apoptosis suppresso[illegible] the [illegible] family to inhibit the anti-apoptotic effect of t[illegible]er[26]. The findings of publis[illegible] studies[27,28] sho[illegible]at abnormally express[illegible]BNIP3 is closely rela[illegible] to the pathogenesis [illegible]many m[illegible]ignancies mentioned above.

[illegible]h se[illegible]l studies have revealed that miR-182-5p [illegible]ated wit[illegible]e occurrence, development, inv[illegible]nd m[illegible]tion of multiple cancers, [illegible] of the[illegible] focused on its specific effec[illegible] mechan[illegible] the invasion and migration of [illegible] This work demonstrated that, compared wi[illegible]OC tissues, a substantially decre[illegible] miR-1[illegible]p expression level was determined [illegible]racancerous tissues, suggesting that miR-182-5p [illegible]as a tumor suppressor in OC. Additionally, BNI[illegible]was identified as a functional target of miR-182-5p in OC. In the [illegible]nal *in vitro* experiments, q[illegible]R was adopte[illegible]etermine the relative e[illegible] level of miR-18[illegible]p in SKOV3 cells at [illegible]h after the transfection with miR-182-5p mimics, [illegible]d it was signif[illegible]ly raised compared with that in [illegible]egative cont[illegible]group. Subsequently, the biolog[illegible]ffects of m[illegible]182-5p on the proliferation and mig[illegible]V3 cells were studied to further verify w[illegible] miR-182-5p acted as the tumor-sup[illegible]ssor gene of OC. According to the results of the [illegible] formation and flow cytometry assay, [illegible]essed miR-182-5p could effectively repress the proliferation of SKOV3 cells. It was also discovered through *in vitro* wound healing assay that the expression level of miR-182-5p was negatively correlated with the migration ability of SKOV3 cells.

Conclusions

We showed that miR-182-5p/BNIP3, as a target for the treatment of OC, may become a feasible and new method of tumor treatment. The study of the miRNA regulatory mechanism provided a new perspective for the research and treatment of OC.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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