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Original Article

MicroRNA-21 depletion by CRISPR/Cas9 in CNE2 nasopharyngeal cells hinders proliferation and induces apoptosis by targeting the PI3K/AKT/MOTOR signaling pathway

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Abstract: Background: We assess the effects exerted by CRISPR/Cas9 mediated *microRNA 21* (*miR-21*) depletion on the biologic characteristics of CNE2 nasopharyngeal carcinoma (NPC) cells and the underlying mechanisms. Methods: The sgRNA was designed targeted at *miR-21* gene, along with the construction of the CRISPR/Cas9 lentivirus system and the detection of editing efficiency through T7EN1 enzyme digestion. Effects of *miR-21* depletion on the biologic characteristics of CNE2 cells were detected through CCK-8, Transwell Invasion Assay and flow cytometry. Mechanistic studies were based on bioinformatic analysis and immunoblotting. Results: A CRISPR/Cas9 system with targeted knockdown of *miR-21* gene was obtained. *miR-21* depletion evidently inhibited the growth, clone formation, and invasion as well as migration abilities of CNE2 cells, thus inducing apoptosis. A total of 28 KEGG were identified through the bioinformatic analysis. Further immunoblotting showed that the expressions of proteins involved in the PI3K/AKT/mTOR signaling pathway were decreased in response to *miR-21* depletion. Conclusions: *miR-21* depletion can suppress the cell growth as well as proliferation and induce apoptosis in CNE2 cells possibly by inhibiting the PI3K/AKT/mTOR signaling pathway.

Keywords: microRNA 21, CRISPR/Cas9, CNE2, nasopharyngeal carcinoma

Introduction

Nasopharyngeal carcinoma (NPC) represents one of the most common head and neck malignancies in Southeast Asian region, whose pathogenic factors include environment, food, genetic factor, exposure to carcinogen, the previous infection of EB virus, as well as the combined action and various effects between oncogenes and tumor suppressor genes, or among signal transduction pathways [1-3]. In recent years, although the three-year local control ratio of NPC patients has jumped to 80-90% with the application of intensity modulated radiation therapy concurrent chemotherapy [4, 5], 22-34% of patients still face treatment failure due to the rapid growth rate and easy metastasis of NPC [6]. Therefore, investigating reliable molecular marker(s) for monitoring the generation as well as development process of NPC, exploring its correlation with the proliferation, invasion, and metastasis of NPC, and expounding the pathogenesis of NPC can contribute to the early diagnosis and prognosis of NPC.

MicroRNA constitutes an RNA micromolecule consisting of 18~25 nucleotides, which inhibits mRNA degradation or translation by combining with the 3'-non-coding area of target mRNA through complementary base pairing [7]. As one of the relatively early discovered miRNAs, *miR-21* is located in the TMEM49 gene coding region of chromosome 17 with a length of 22 nucleotides [8]. It has currently been proven that abnormal expression of *miR-21* can be found in several tumors, including NPC [9-11]. *miR-21* has participated in processes such as proliferation, invasion, blood vessel invasion,

and migration of cancer cells as well as radiotherapy resistance in NPC, making it one of the potential tumor treatment therapeutic targets [12, 13].

Over the past few years, with the development and maturity of the technology of CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/Cas9), this system has been viewed as an "immediate" technology for DNA mutation and editing [14, 15]. Specifically, consisting of three parts, namely, Cas nuclein Cas9 (DS-DNA endonuclease), crRNA, a kind of target complement, and crRNA, an auxiliary transacting factor, the CRISPR/Cas system has made a full use of the feature of short gRNA to target bacterial Cas9 endonuclease and identify genetic locus [14]. Because the gRNA can provide specificity, thus it only needs to change the design of gRNA sequence coding when changing the target [14]. In this paper, we target and knock down the *miR-21* gene in CNE2 gene through CRISPR/Cas9, and evaluate the gene targeting editing effect of CRISPR/Cas system on NPC cell lines through detecting the proliferation, clone formation, and apoptosis of CNE2 cells.

Materials and methods

Cell culture

The human CNE2 cell line was preserved in this experiment and incubated in RPMI-1640 culture medium containing 10% FBS with an incubator at 37°C and 5% CO_2. The researcher changed the culture medium every 2 to 3 days and the cells generated every 5 to 7 days.

Design, synthesis, screening and verification of gRNA oligonucleotide sequence

sgRNA was designed via an online tool (http://crispr.mit.edu/) and the Blast was employed to rule out sgRNA possibly leading to off-target effects. The preverified sgRNA sequences were: sgRNA-miR-21, Guide# 5'-TCATGGCAACACCAGTCGAT-3'. The oligonucleotide sequence was synthesized by Shanghai Genechem Co., LTD., and each pair of oligonucleotides was transformed into DS-DNA fragments through annealing and then linked onto the Cas9-Easy-lentivirus vector. The CNE2 cells were collected after 72 h of infection, along with the application of mammalian genomic DNA extraction kit to extract genome DNA and T7EN1 (NEB, USA; M0302S) to detect the editing efficiency of gRNAs. The whole genomic DNA was extracted by utilizing the DNA Extraction kit (Beyotime, Shaihai, China) reported previously [16]. The primer sequences for miR-21 in genome were forward, 5'-AGAATAGAATTGG-3'; reverse 5'-GCCACCAGACAGAA-3'. The amplifications were performed in a Mastercycler gradient thermocycler (Applied Biosystems, USA) and the reaction conditions were documented in our published studies [16].

CCK-8 test

Cells in the logarithmic phase were inoculated into 100 μl 96-well culture plate with a concentration of 5×10^3. A sgRNA-miR-21 and a sgRNA-control group with 5 double wells in each group were included in the experiment. After being cultured in RPMI-1640 for 12, 24, 36, 48, 72 h, each well was added to 10 μl CCK-8 and cultured for another 3 h, along with the detection of the OD value of absorbance at 450 nm and 630 nm dual-wave length. The experiment was repeated three times.

Clone forming assay

Monolayer cells in the logarithmic phase were selected and digested by 0.25% trypsin and transformed into single cells. The cell suspension was inoculated into the six-well plate with a concentration of 800 cells/well where the saturation humidity condition was 37°C with 5% CO_2. The culture solution was changed every 2 to 3 days. After 12 to 14 d, cells were rinsed by PBS twice and then fixed by 2 mL of methyl alcohol. The 0.1% crystal violet staining solution was applied to stain the cells under indoor temperature and the number of clone formation cells was counted.

Wound healing migration assay

To each well was added around 5×10^5 cells and this was incubated overnight, with the fusion rate over 90%. On day 2, a pipettor of 200 μl was utilized to make a scratch on each well with a straight ruler. Cells were rinsed for 3 times by PBS to remove unnecessary cells, and then photographed on 0, 24^{th}, 36^{th} d, separately to count the number of migrated cells.

Transwell invasion assay

A polycarbonate membrane with a bore diameter of 8 μm was employed to seal the bottom of

the Transwell chamber, and 50 μl Matrigel was added from one side and kept at 37°C for 2 h. A detailed protocol was documented in our published study [17].

Apoptosis analyses

DNA ladder and caspase-3 activity analyses were respectively performed utilizing the commercial DNA Ladder Extraction Kit and Caspase 3 Activity Assay Kit (Beyotime, Shanghai, China) according to the protocols. The 4', 6-diamidino-2-phenylindole (DAPI) dye for staining was also from Beyotime (Shanghai, China). For more specific information about operations, see our previously published study [16].

Immunoblotting

Total cell protein was extracted through the RIPA lysate and quantified by the BCA Protein Quantitation Kit. Phosphorylated proteins were extracted using a commercial Kit (BC3730, Solarbio, Beijing, China). The weighted proteins underwent electrophoretic separation at constant 80 V, and then transferred onto the PVDF membrane at constant 250 mA, along with the application of the TBST with 5% skim milk powder to seal cells overnight. Rabbit anti-human polyclonal primary antibody PI3K (Abcam, ab154598, 1:500), PI3K (phospho Y607) (Abcam, ab182651, 1:500), rabbit anti human monoclonal primary antibody AKT (Abcam, EPR16798, 1:500), AKT (phospho T308) (Abcam, ab38449, 1:300), rabbit polyclonal to mTOR (Abcam, ab2732, 1:500) and mouse anti-human β-actin monoclonal primary antibody (Beyotime, Shanghai, China; 1:1000) were added and incubated for 4 h. Goat anti rat/rabbit IgG (1:10000) secondary antibody solution marked by horse radish peroxidase was added and cultured under room temperature for 2 h, and the ECL luminous fluid was added for developing and photography.

Mechanistic studies

miR-21 related signaling pathways in NPC were predicted by conducting bioinformatic analysis. Briefly, the RNA-seq data at level 3 was downloaded from TCGA database, whose gene name was converted from ensemble ID into the matrix of gene symbol by the database of Ensemble. The correlation test of the gene was conducted through R language to find out genes with the co-expression relations with miR-21, where the screening condition was set as (cor<-0.2 & P<0.001). Based on the biologic pathway database (http://www.genome.jp/) of Kyoto Encyclopedia of Genes and Genomes (KEGG), the biologic pathway enrichment analysis was performed on the target gene set, along with the selection of the intersection on the most related biologic signaling pathways. Immunoblotting was employed for verification.

Statistical analysis

Measurement data were expressed as mean ± SD (standard deviation) and analyzed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA) software. Student's t-test, or non-parametric test was adopted to compare differences between groups. The test level was set at $\alpha=0.05$, and $P<0.05$ was accepted as statistical significance.

Results

Analysis of the miR-21 gene knockdown efficiency

After the cleavage of target gene locus through the CRISPR/Cas9 system, insertion or deletion mutations would be generated in the locus based on the recovery mechanism of the cells. Genes at the target site were amplified and denatured and annealed to form not fully matched DNA double-strands. The sgRNA-miR-21 targeting site is shown in **Figure 1A**. The T7EN1 assay was employed to recognize and cleave these mismatched DNAs. As shown in **Figure 1B**, the multiple site-specific bands were detected in gRNA-miR-21group, demonstrating the existence of miR-21 editing. PCR assay demonstrated that mature miR-21 was markedly reduced in sgRNA-miR-21 group (**Figure 1C** and **1D**).

miR-21 depletion weakening the growth, invasion and migration capacity of CNE2 cells

CCK-8 assay showed that the cell numbers were reduced markedly after 27 h of infection, and cell numbers were lower in sgRNA-miR-21 group in comparison with the control group, indicating an inhibition of cell growth ability (**Figure 2A**). Clone formation testing revealed that the cloned CNE2 cells in sgRNA-miR-21 group were evidently less than that in the con-

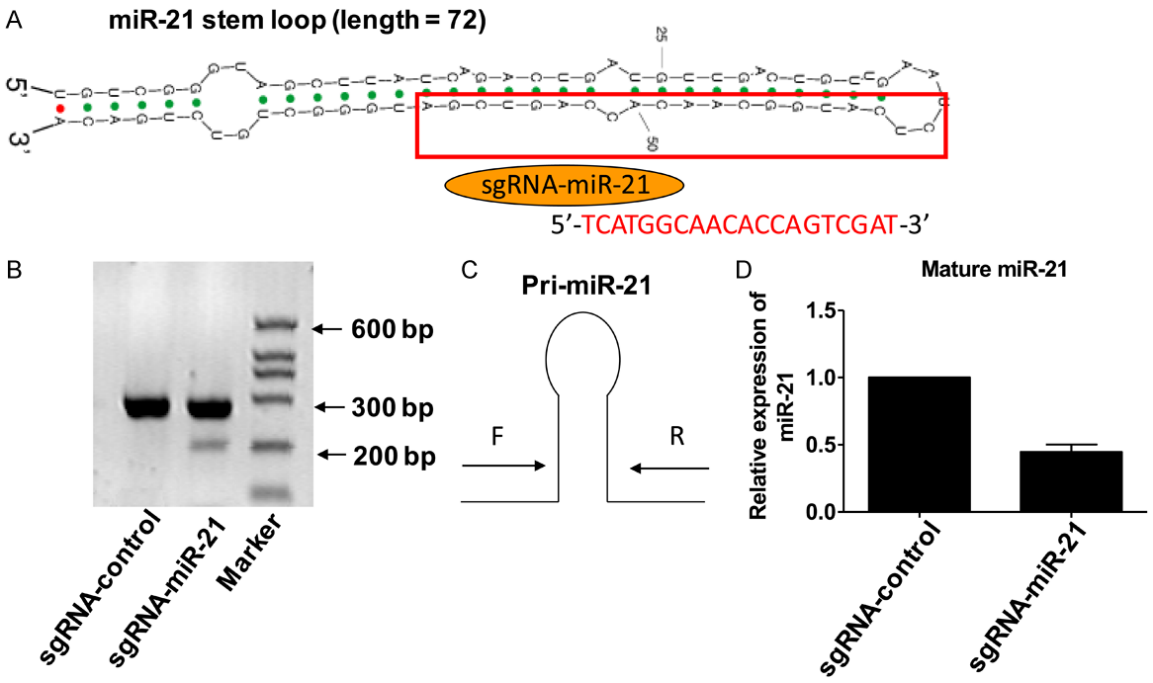

Figure 1. CRISPR/cas9 can significantly downregulate the expression of *miR-21* in CNE2 cells. A. Schematic diagram of the design of sgRNAs for *miR-21*. B. DNA cleavage by CRISPR/cas9 is detected by T7EN1 assay. C. Design of the primers of miR-21 in the genome. D. Expression of mature *miR-21* following CRISPR/cas9 editing by PCR.

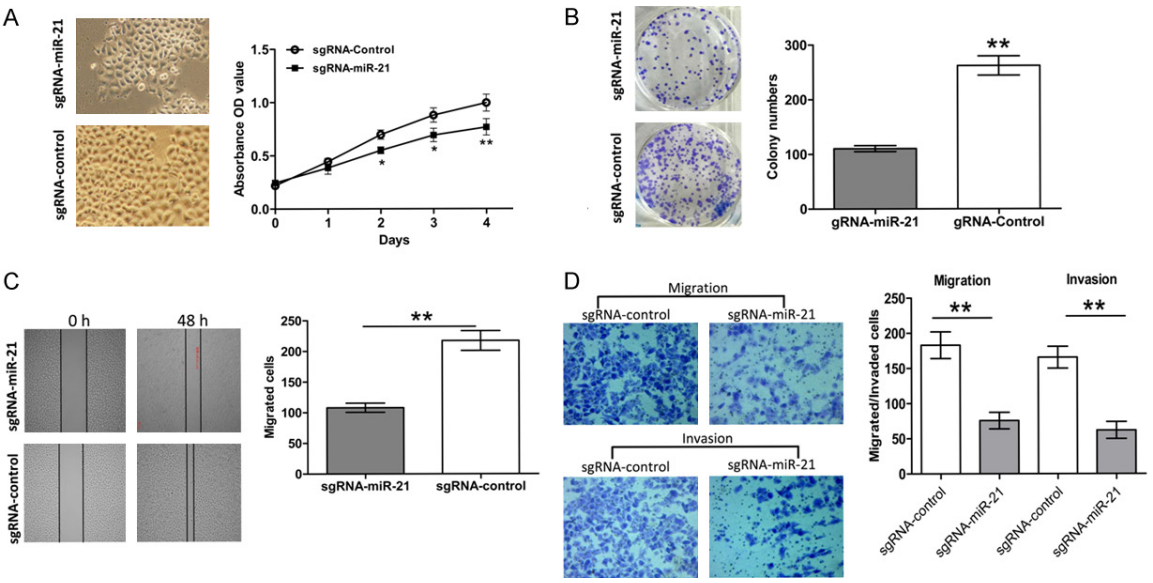

Figure 2. *miR-21* depletion suppresses the growth, proliferation, invasion, and migration of CNE2 cells. A. Cell viability assay using CCK-8. B. Colony formation assay. C. Wound healing migration assay. Migrated cells were counted 48 h after transfection. D. Transwell invasion assay. **P*<0.05, ***P*<0.01.

trol group (**Figure 2B**). The wound healing migration assay showed that migrated cells in the sgRNA-miR-21 group werelower than those in the mock control group, thus implying the decrease of cell migration ability following miR-21 knockdown (**Figure 2C**). Moreover, Transwell

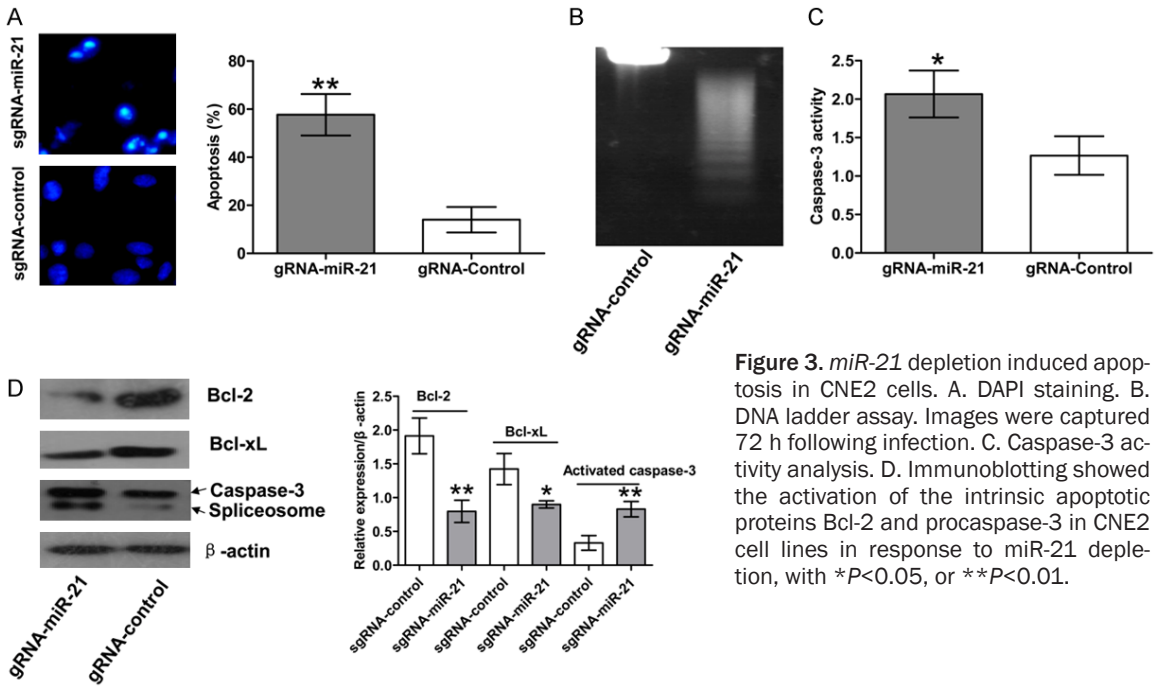

Figure 3. *miR-21* depletion induced apoptosis in CNE2 cells. A. DAPI staining. B. DNA ladder assay. Images were captured 72 h following infection. C. Caspase-3 activity analysis. D. Immunoblotting showed the activation of the intrinsic apoptotic proteins Bcl-2 and procaspase-3 in CNE2 cell lines in response to miR-21 depletion, with *P<0.05, or **P<0.01.

Assay demonstrated that both migrated and invaded cells in the sgRNA-miR-21 group were different from those in the controls (**Figure 2D**).

Targeted knockdown of miR-21 inducing CNE2 cell apoptosis

The visual DAPI staining showed increased apoptotic cells in sgRNA-miR-21 group corresponding to miR-21 knockdown (**Figure 3A**). Correspondingly, results of a DNA ladder also exhibited a relatively obvious DNA degraded fragment in the sgRNA-miR-21 group after 72 h following infection (**Figure 3B**). Moreover, caspase-3 activity analysis further confirmed the induced apoptosis in cells of the sgRNA-miR-21 group (**Figure 3C**). Immunoblotting assay indicated that the expressions of Bcl-2 and Bcl-L decreased, and Caspase-3 was activated (**Figure 3D**). These results imply that targeted knockdown of miR-21 is capable of inducing apoptosis in CNE2 cells.

miR-21 depletion targeting the PI3K/AKT/mTOR signaling pathway

Based on online searching and KEGG databases, a biological pathway enrichment analysis was conducted on target gene set (**Figure 4A**), and 28 KEGG was found (**Figure 4B**). Furthermore, the intersection among such signaling pathways was analyzed, along with the identification of the PI3K/AKT/mTOR signaling pathway that may be implicated in miR-21 related signaling pathways. Further immublotting demonstrated that the expressions of PI3K, AKT, mTOR as well as pPI3K and pAKT in cells of the sgRNA-miR-21 group were also altered. Statistically, miR-21 depletion could inhibit the PI3K/AKT/mTOR signaling pathway.

Discussion

Representing a a regulatory gene, *miR-21* is a single stranded microRNA consisting of 22 nucleotides, which plays an essential role in the growth, migration, invasion as well as the diagnosis, treatment and other physiopathologic processes of various cancers [9]. Overexpression of *miR-21* has been witnessed in many cancers, including NPC [10, 11]. The CRISPR/Cas9 system is a recently developed target gene editing technology, which is characterized by simple design, multiple gene editing and high targeting efficiency compared with traditional gene editing tools [14, 15]. Moreover, CRISPR/Cas9 system can also be applied in genetic engineering of immune cells and tumor immunotherapy [18-20].

In the first section, a CRISPR/Cas9 lentiviral vector system targeting *miR-21* hasbeen estab-

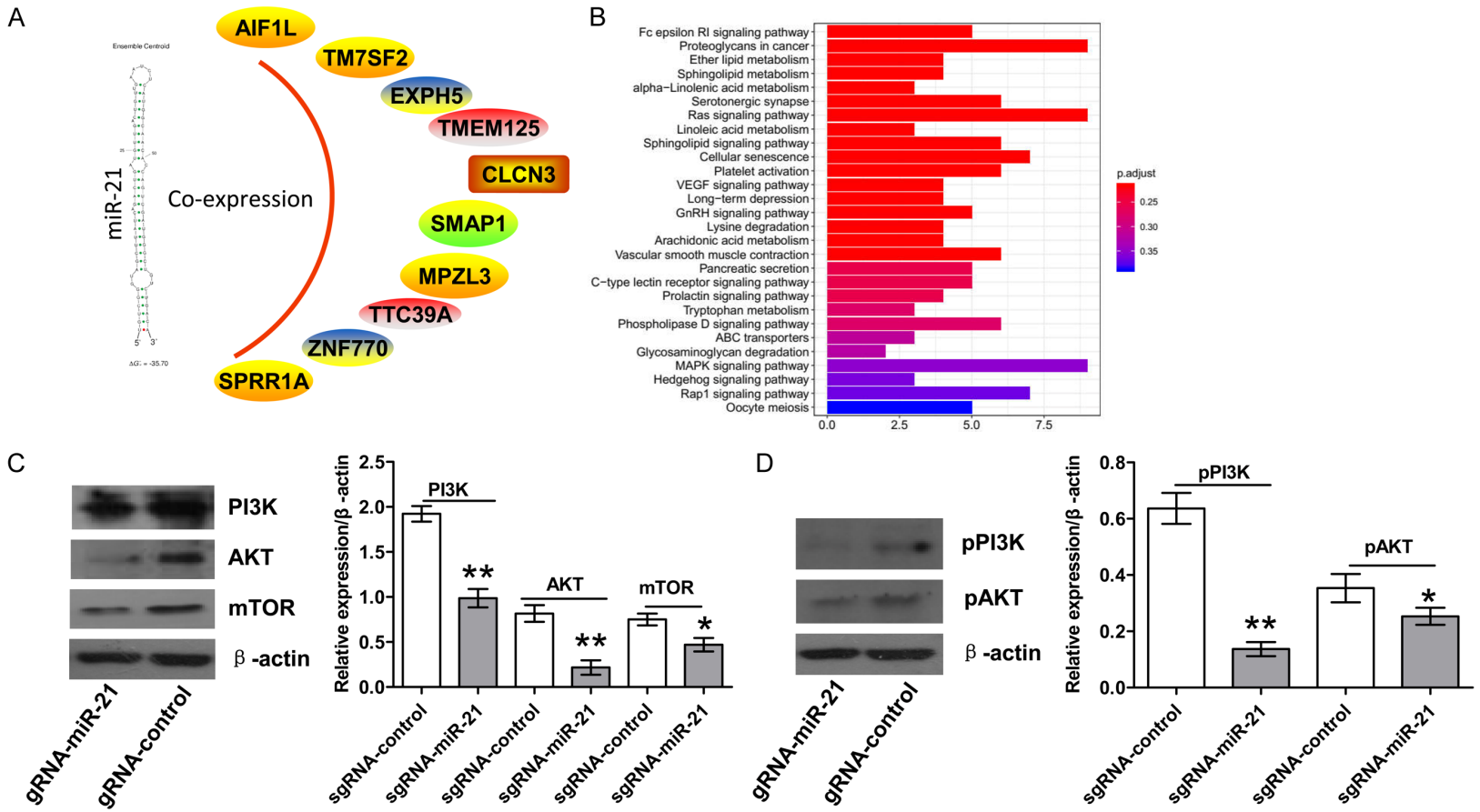

Figure 4. Signal Pathway Prediction of *miR-21* in NPC and Verification. A. Predicted co-expression genes of *miR-21*. B. Predicted signal pathways. C. Expression of PI3K, AKT, and mTOR detected by immunoblotting. D. Expression of phosphorylated PI3K and AKT detected by immunoblotting. **P*<0.05, or ***P*<0.01.

lished, and the knockdown level of mature *miR-21* level in CNE2 cells approached about 50% through the T7EN1 digestion detection, indicating the CRISPR/Cas9 plasmid system can conduct cleavage at the target site. Our work further confirmed that *miR-21* depletion could suppress the growth, proliferation, clone formation, invasion, and migration of CNE2 cells. It is generally acknowledged that *miR-21* has been reported as an oncogene implicated in the tumorigenesis of various cancers [21, 22], which prompted us to hypothesize that *miR-21* depletion in CNE2 cells may induce apoptosis. As guessed, further apoptosis analysis demonstrated that *miR-21* knockdown could induce the apoptosis of CNE2 cells. It was also indirectly proven in another finding that *miR-21* malfunction could inhibit the growth and proliferation of colorectal carcinoma cells [23]. More specifically, when CNE2 cells modified by CRISPR/Cas9 underwent single-cell monoclonal culture, the cell mass that proliferated from single CNE2 cells were sent for Sanger sequencing, with results showing that all *miR-21* sequences of the cell masses was of wild type, not the mutant one. Nevertheless, the T7EN1 enzyme digestion and PCR experiments have proved that the knockdown level of CNE2 cells was close to 50%. This phenomenon indicated that it was hard for single CNE2 cell with mutant *miR-21* to clone and proliferate to a cell mass, suggesting that *miR-21* is a crucial gene for stability of the CNE2 cells.

Bioinformatic analysis was further conducted and signal pathway prediction was performed on *miR-21* in NPC, finding a total of 28 KEGG. We identified the PI3K/AKT/mTOR signaling pathway in our analysis, a well-known signaling pathway implicated in cell proliferation and survival. Our work further evidenced the regulation of the PI3K/AKT/mTOR signaling pathway. This implied that *miR-21* depletion could hinder the cell proliferation and induce apoptosis through targeting the PI3K/AKT/mTOR signaling pathway. Yu et al. reported that *miR-21* silencing confers sensitivity to Tamoxifen and Fulvestrant mainly by inhibiting the PI3K/AKT/mTOR signaling pathway in breast cancer cells [24]. Besides, our study also proved the involvement of *miR-21* in the regulation of the PI3K/AKT/mTOR signaling pathway in NPC.

In the present study, there were some limitations. First of all, the knockdown efficiency of *miR-21* based on the CRISPR/Cas9 system didn't reach 100%, making it difficult to obtain the monoclonal CNE2 cell line with a malfunction of *miR-21*, thus leading to a certain degree of hybrid effect in the results. Furthermore, it was uncertain whether CRISPR/Cas9 system had any off-target effects and limitations in verifying editing efficiency through T7EN1 enzyme digestion experiment. Finally, this paper predicted the existence of numerous different signal pathways of *miR-21* in NPC, but we only studied one of them. Thus, it remains unclear whether the knockdown of *miR-21* would activate other signaling pathways, which still needs further exploration.

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Disclosure of conflict of interest

None.

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