Exosomal CircMFN2 Enhances the Progression of Pituitary Adenoma Via The MiR-146a-3p/TRAF6/NF-κB Pathway

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Abstract:
Background: Pituitary adenoma (PA) is a common intracranial endocrine cancer, but no precise target has been found for the effective prediction and treatment of PA. Methods: qRT-PCR analysis showed that circMFN2 could affect the expression of miR-146a-3p in PA samples. Moreover, we used Western blotting to evaluate the expression levels of TRAF6 and NF-κB markers. The EdU assay, scratch wound-healing assay, and matrigel invasion assay were performed to assess the potential function of this pathway in PA cells. Based on the bioinformatics analysis including KEGG, GO analysis and microarray analysis, we evaluated the efficacy of circMFN2 as a potential biomarker for diagnosing PA, and we aimed to determine its mechanism of action in PA cells. Results: Our findings indicate that there is a significant increase in the expression of circMFN2 in tissues, serum, and exosomes in the invasive group compared to the non-invasive and normal groups. Furthermore, this difference was statistically significant both preoperatively and postoperatively. To clarify its function, we downregulated this gene, and the experimental results suggested that the motility and proliferative capacity were reduced in vitro. In addition, rescue assays showed that miR-146a-3p could successfully reverse the inhibitory effect of circMFN2 knockdown on motility and proliferation in PA cells. Moreover, down-regulation of circMFN2 and miR-146a-3p significantly changed the expression of TRAF6 and NF-κB. Conclusion: This study identified that circMFN2 regulates miR-146a-3p to promote adenoma development partially via the TRAF6/NF-κB pathway and may be a potential therapeutic target for pituitary adenoma.

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Exosomal CircMFN2 Enhances the Progression of Pituitary Adenoma Via The MiR-146a-3p/TRAF6/NF-κB Pathway

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ABSTRACT

Background: Pituitary adenoma (PA) is a common intracranial endocrine cancer, but no precise target has been found for the effective prediction and treatment of PA. Methods: qRT‒PCR analysis showed that circMFN2 could affect the expression of miR-146a-3p in PA samples. Moreover, we used Western blotting to evaluate the expression levels of TRAF6 and NF-κB markers. The EdU assay, scratch wound-healing assay, and matrigel invasion assay were performed to assess the potential function of this pathway in PA cells. Based on the bioinformatics analysis including KEGG, GO analysis and microarray analysis, we evaluated the efficacy of circMFN2 as a potential biomarker for diagnosing PA, and we aimed to determine it mechanism of action in PA cells. Results: Our findings indicate that there is a significant increase in the expression of circMFN2 in tissues, serum, and exosomes in the invasive group compared to the non-invasive and normal groups. Furthermore, this difference was statistically significant both preoperatively and postoperatively. To clarify its function, we downregulated this gene,
and the experimental results suggested that the motility and proliferative capacity were reduced in vitro. In addition, rescue assays showed that miR-146a-3p could successfully reverse the inhibitory effect of circMFN2 knockdown on motility and proliferation in PA cells. Moreover, downregulation of circMFN2 and miR-146a-3p significantly changed the expression of TRAF6 and NF-κB. Conclusion: this study identified that circMFN2 regulates miR-146a-3p to promote adenoma development partially via the TRAF6/NF-κB pathway and may be a potential therapeutic target for pituitary adenoma.

KEYWORDS
Pituitary adenoma, circMFN2, miRNA, exosome, biomarker

Nomenclature
PA    Pituitary Adenoma
IPA   Invasive Pituitary Adenoma
siRNA Small Interfering RNA
NF-KB Nuclear Factor Kappa-B
TRAF6 TNF receptor associated factor 6
TEM   Transmission Electron Microscope
NTA   Nanoparticle Tracking Analysis
MA    Meningioma

1 Introduction

Pituitary adenoma (PA) is a common neuroendocrine tumor originating from pituitary cells in the sella turcica and is second only to glioma and meningioma in terms of incidence. Almost 25–55% of PAs show invasive growth [1], manifested by local infiltration that causes the destruction of adjacent tissues, such as the cavernous sinus, saddle base, and hypothalamus; these tumors are called invasive PAs [2].
Invasive PAs are challenging to diagnose, and almost 50% of patients have varying
degrees of residual tumor after surgery, which may lead to recurrence and a poor
prognosis. Molecular markers that can accurately predict the prognosis of PA patients
have been unsatisfactory [3]. Consequently, it is necessary to study the detailed
mechanism underlying invasive PA development in depth, identify target genes for
the treatment of invasive PA and provide a new experimental basis for the targeted
therapy of PA.
CircRNAs are noncoding RNAs. The presence of a large number of circRNAs greatly
increases the complexity of the human transcriptome. These molecular molecules
form a heterogeneous class of RNAs that lack protein-coding function. CircRNAs
play key roles in the regulation of transcriptional and posttranscriptional processes,
including X chromosome inactivation, epigenetic regulation, genomic imprinting, and
mRNA splicing [4]. In 2010, with the advent of high-throughput sequencing and
bioinformatics analysis of the non-polyadenylated transcriptome, thousands of
circRNAs were discovered and found to be diverse and genetically and structurally
stable throughout the genome [5-8]. There is growing evidence that circRNAs are
associated with the occurrence of different diseases [9]. Most circular RNAs
(circRNAs) exert regulatory functions by competitively binding with microRNAs
(miRNAs). Studies have reported that circ_0001368 can promote the proliferative
ability of gastric cancer cells through the miRNA-6506-5p/FOXO3 pathway [10].
This study investigates the role of circMFN2, a newly identified circRNA in the high-
throughput microarray of pituitary adenoma, in the diagnosis and mechanism of
action of PA. The study evaluates circMFN2 expression in serum, exosomes and tumor tissues of PA patients and found it to be a potential molecular marker for the diagnosis of pituitary adenoma. The study also explores and verifies that circMFN2 could regulate miR-146a-3p to increase the proliferation and invasive function of PA cells, partly through TRAF6/NF-κB pathway. This study provides valuable insights into the role of circMFN2 in the diagnosis and mechanism of action of pituitary adenoma. This finding also showed that circMFN2 is involved in the progression of PA and can act as a critical molecular marker for PA diagnosis.

2 Methods

2.1 Pituitary adenoma microarray analysis

CircRNA microarray assays were performed on five pairs of non-functioning pituitary adenomas and normal tissues and used to identify differentially expressed non-coding RNAs. All microarray data was downloaded from the GEO database (http://www.ncbi.nlm.nih.gov/geo). The raw data were downloaded as MINiML files. The raw sequencing data underwent pre-processing using FastQC and cutadapt software. The resulting clean reads were then mapped to hg38 and circBase using Tophat and MapSplice software. Using the limma package in the R software to study the differentially expressed circRNA and miRNA. Differential expression analysis of RNAs was conducted using the R package EdgeR (http://www.R-project.org) with criteria of |Fold change|>2 and P value <0.01. All statistical analyses and graphics were performed using R. ClusterProfiler package (version: 3.19.0) in R was employed to analyze the GO function of potential targets and enrich the KEGG
pathway; the R software pheatmap package was used to draw heatmap. The statistical method for gene expression comparisons was the wilcox-tests method.

2.2 Collection of patients’ PA Tissues and Serum

We enrolled 50 serum samples obtained from patients of The First Affiliated Hospital of Medical College of Shihezi (Shihezi, China). Prior to the research, none of the patients underwent surgery or therapy. Healthy human serum samples were collected from 50 volunteers without underlying diseases in the department. Fresh tissue specimens were frozen in a -80°C refrigerator for quantitative reverse transcription polymerase chain reaction (qRT‒PCR) analysis. Table S2 shows the clinicopathological parameters of the patients. We used MRI+ Knosp Grades to determine the invasiveness of pituitary adenomas. All patients’ blood and tissue were collected with written signed consent and validated by the Ethics Committee of The First Affiliated Hospital of Medical College of Shihezi (KJ2021-076-02).

2.3 Serum Exosome Isolation

The samples were subjected to centrifugation at 3,000 × g for 30 minutes to separate serum and blood cells. The resulting supernatant was then subjected to further centrifugation at 10,000 × g for 30 minutes, followed by an additional round of centrifugation at 10000 × g for 70 minutes at 4°C. The aqueous layer was filtered using a 0.22 µm filter to separate it from the solid layer, and the resulting exosome pellet was resuspended in phosphate-buffered saline (PBS).

2.4 TEM
The exosome suspension was diluted to 0.5 mg/mL with PBS, and then spotted onto a glow-discharged copper grid placed on a filter paper and dried for 10 min by exposure to infrared light. Next, the exosome samples were stained with one drop of phosphotungstic acid (1% aqueous solution) for 5 min and dried for 20 min by exposure to infrared light. Finally, the exosomes were visualized under a transmission electron microscope (Thermo Fisher Scientific, Waltham, USA) at 100 keV.

2.5 NTA

The collected exosomes were suspended in a PBS solution and thoroughly mixed. A portion of the exosomes were filtered through a millipore, while the remaining solution was diluted to ensure that the exosomes were clearly visible in the solution alone. The exosomes were then assayed using NanoSight N300 (Worcestershire, UK).

2.6 Cell Culture and Transfection

The PA cell lines, including GH3 and MMQ, were sourced from Procell (Wuhan, China). The cells were placed in the incubator at 37°C with 5% CO₂ and seeded on a special medium for GH3 cells, including a mixture of Ham's F-12K, 15% HS, 2.5% FBS, and 1% P/S. The medium was changed every 2-3 days, and 1 ml of special medium was added during each change. Experiments were conducted until the cells reached 80% density. The cell growth cycle was approximately 5-7 days. PA cells were transfected with siRNA using LipofectamineTM 3000, encompassing RNAi and NC (Genepharma, Shanghai, China). In our study, we utilized a 6-well plate for transfection procedures. Specifically, we added 8 μl of LipofectamineTM 3000 and 4 μl of RNAi to each well. Then, the cells were incubated for 24-48 h until the
constructed plasmid was in the cells and si-RNA was downregulated in the cells. qRT-PCR was used to validate the transfection efficiency of siRNA. The successful transfection was determined based on achieving a change of approximately 60%-80% in the expression of the experimental group compared to the NC group.

2.7 Cell Proliferation Assay

According to the instructions provided by the APExBIO® EdU In Vitro Imaging Kit, the EdU assay was carried out (APExBIO, Houston, USA). Briefly, 20 μM EdU was incubated with the treated cells in 6-well plates for one night. Afterward, the cells were fixed with 4% paraformaldehyde and washed with PBS. After that, each well received 1 mL of PBS blended with 5 μL of Triton® X-100, which was incubated in a cell incubator for 20 minutes. Then, a click reaction solution was configured for the click reaction. Each well was washed with 1 ml PBS to remove the washing solution before being stained with 1 mL 1× Hoechst 33342 and incubated for 30 minutes. Using a fluorescence microscope, pictures were taken (Olympus, Tokyo, Japan). The proliferation rate was determined as the proportion of EdU-positive cells. In 96-well plates, we cultured the transfected cells and incubated them for 24 h. Then, 10 μL of CCK-8 reagent was added to each well in a 96-well plate and incubated for 4 hours. At 450 nm, we collected results using THERMO Multiskan GO (THERMO, Suzhou, China) each 2 h.

2.8 Matrigel Invasion Assay

The Transwell chamber system (Costar, Corning, NY, USA) was used to fulfill perform the Matrigel invasion assay. The system is equipped with a polycarbonnic
membrane. Single PA cells were suspended in serum-free medium at a density of $1 \times 10^5$ cells/mL. Then, we added PA cells into the upper compartment containing 200 μl of serum-free medium with Matrigel. We pipetted 600 μl of culture medium for pituitary tumor cells consisting of 15% HS into the bottom 24-well plate. After standing in the incubator for 48 h, the cells penetrated the membrane and grew on the lower surface of the chamber with Matrigel. The top surface of the membrane was cleaned with cotton swabs to remove any nonmigrating cells. After 4% paraformaldehyde fixation and crystal violet staining, stained cells were photographed using Olympus fluorescence microscope. Three photos of each area of invasive cells were taken, and the number of stained cells was calculated independently.

2.9 Wound Healing Assay

PA cells were transfected and cultured in six-well plates until their numbers reached $1 \times 10^6$. They were starved for 24 h using DMEM. A 200 μl pipette tip was used to create a wound (time 0), the detached cells were washed off, and then serum-free DMEM was used for culture. After 48 h, photographs of five nonoverlapping fields were taken.

2.10 Total RNA Isolation and qRT‒PCR Analysis

A plasmid Midi Kit (QIAGEN, Hilden, Germany) was used to isolate serum total RNA. RNA extraction, including tissues and cells, was performed using the TRIzol method according to the reagent manufacturer's instructions. Nanodrop (Thermo Scientific, Waltham, USA) was used to check the RNA purity and concentration just like OD260/OD280 in the range of 1.8-2.1 and RNA concentration more than
900ng/μL. For reverse transcription, the purified RNA was subjected to the TIANScript II RT kit (TIANGEN, Beijing, China). Next, FastReal qPCR PreMix (SYBR Green) was used to conduct qRT–PCR experiments. qRT-PCR experiments were performed using the 7500 fast system, Please refer to the supplementary materials for specific reaction conditions. We used U6 and β-actin as reference genes for PCR experiments (Table S1). Each experiment was performed in triplicate.

2.11 Western Blot

To extract the protein, 1 mL of RIPA buffer was added to the cells, wait for 15 min. This process should be done on ice. The protein solution (approximately 15 ml) was subjected to 10% SDS-polyacrylamide gel electrophoresis. The power was turned on and the voltage level was initially set to 100V. After 20 minutes, the voltage was adjusted to 220V and electrophoresis was continued for 60 minutes. Subsequently, the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane. Next, pretreat the PVDF membrane in anhydrous ethanol for 2 hours. Turn on the electro-transfer switch and maintain the current at 300mA for 90 minutes. Once the electro-revolution is complete, turn off the power. Submerge the PVDF membrane in the containment solution for 1 hour. Place the primary antibody and the PVDF membrane together in the antibody tube and incubate them overnight on a low-temperature shaker. The PVDF membrane was placed in sealing liquid for 1 h. The PVDF membrane in the overnight antibody tube was put into the tank of TBST, washed for five minutes, added into the antibody tube of the second antibody, and incubated for one hour. The membrane strip was placed in the water channel again and washed for 5
minutes. The color solution was added to the dish where the film strip was placed, and the darkroom exposure system was used for imaging and analysis. GAPDH served as a loading control.

2.12 Target prediction

TargetScan and Circbase databases can be used to predict the potential targets of circMFN2. This prediction was performed for miRNA targets in human tissues and rat cells. For subsequent validation experiments, the top three were combined.

2.13 Statistical analysis

GraphPad Prism software (GraphPad Software, San Diego, CA, USA) was used for all experimental data analyses. Correlation analysis of genes and statistical analysis of clinical data between different groups were performed using the t-test. The diagnostic efficacy evaluation of circMFN2 was analyzed by ROC curves. P < 0.05 was considered statistically significant. Spearman’s analysis was performed for analysis of correlation between circMFN2, miR-146a, TRAF6, NF-κB. The difference between the two sides p<0.05 was statistically significant.

3 Results

3.1 Identification of differentially expressed circRNAs in PA

We conducted high-throughput circRNA microarray assays on five pairs of pituitary samples from patients with pituitary adenoma (PA) and normal subjects. We utilized a volcano plot and heatmap to display genes with significant expression differences. We found 6656 up-regulated genes and 2156 down-regulated ncRNA genes by testing (Fig. 1A), and Fig. 1B shows the Heat map of these differentially
expressed genes. We screened the signaling pathway RNAs related to adenoma growth and invasion by doing KEGG pathway and GO analysis [11-15] on the above high-throughput microarray (Fig. 1C). In our study, we compared the top 50 expressed circRNAs and found that circMFN2 satisfied the aforementioned conditions (p ≤ 0.05, adj.P ≤ 0.05, FC = 2). To validate its role in other chips, we selected the GSE51618 and grouped individuals as invasive, non-invasive, and normal. After conducting the assay, we observed a statistically significant difference in the expression of circMFN2 in the pituitary gland of the invasive and non-invasive groups (G1 group) compared to normal individuals (G2 group), as shown in Fig. 1D.

Figure 1

3.2 circMFN2 expression in PA patients and ROC curve analysis

We confirmed the presence of circMFN2, which is located on chromosome 1. This circRNA is derived from an exonic region of the MFN2 gene and has a length of 315 nucleotides. The confirmation was done using circbank (http://www.circbank.cn). To investigate the correlation between the expression of circMFN2 and PA pathogenesis, we selected 28 cases from a sample of 50 serum samples collected for the experiment. Meanwhile we collected the tissue specimens of 8 PA patients and MA patients, to investigate the difference in expression of circMFN2 in pituitary adenomas and meningiomas. First, we divided the patients into invasive and noninvasive groups. In the invasive group, serum circMFN2 expression was significantly upregulated before surgery compared to that after surgery and overall exhibited higher expression levels compared to those in the healthy control group (Table. S1 and Fig. 2A). Compared
with the noninvasive group, the invasive group exhibited significantly upregulated serum circMFN2 expression before surgery, and there was no significant differential expression between the healthy controls and either of the groups after surgery (Fig. 2B). Moreover, circMFN2 expression was not significantly different between the invasive and noninvasive groups after surgery. In both tissues and serum, we found that the expression levels of circMFN2 was significantly higher in the invasive group than in the noninvasive group (Figs. 2C and 2D). The statistical analysis of the patient data showed that the expression of circMFN2 was closely related to the invasiveness of PA (Table S2 and Table S3). Second, extensive deposition of circMFN2 was observed in PA tissues compared to that in other brain tumor tissues, such as meningioma (MA). The Mantel‒Cox test revealed that the expression of circMFN2 in MA tissues was downregulated compared to that in PA tissues (Fig. 2E). The purpose of this part was to investigate the differential expression of circMFN2 in pituitary adenoma compared to other common intracranial tumors. Finally, we used circMFN2 as a test variable, normal and adenoma groups as the status scalars, and MRI+ pathological diagnosis as the gold standard to construct the ROC curve. The ROC curve area of circMFN2 was 0.802 (Fig. 2F), and the model could distinguish healthy individuals from PA patients.

3.3 Exosomal circMFN2 expression in PA patients

We utilized ultracentrifugation to isolate exosomes from the serum of patients with pituitary adenoma. Our results, which were obtained through the use of WB, NTA,
and TEM, indicated the presence of exosomes with a mean particle size of approximately 150 nm and positive surface markers (Fig. 3A, 3B and 3C). Then we used qRT-PCR to detect the expression of circFMN2 in serum exosomes of 50 patients with pituitary adenoma and 50 normal people (Table S4). The study found that the expression of circFMN2 in serum exosomes was significantly higher in patients with invasive pituitary adenoma before surgery compared to those with non-invasive pituitary adenoma and normal individuals. Similarly, the expression of circFMN2 was also elevated in serum exosomes of patients with non-invasive pituitary adenoma before surgery as compared to normal individuals (Fig. 3D). The content of circFMN2 in serum exosomes of patients with pituitary adenoma after surgery was higher than that in serum exosomes of normal people. Compared with the normal group, circFMN2 was higher in serum exosomes of patients with invasive pituitary adenoma after surgery. There was no significant difference in the expression of circFMN2 in serum exosomes between non-invasive pituitary adenoma group and normal human serum exosomes after surgery (Fig. 3E). The data presented are consistent with the findings of circMFN2 in the sera of patients with pituitary adenoma, suggesting that circMFN2 has potential as a molecular marker for diagnosing pituitary adenoma in both serum and exosomes.

Figure 3

3.4 CircMFN2 promotes motility and tumorigenesis in PA cells

To investigate the mechanism of action of circMFN2 in PA cell proliferation and development, we transfected siRNA to downregulate the expression of circMFN2 in
GH3 and MMQ cells. The transfection results were determined using qRT-PCR (Fig.
4A). After siRNA transfection, EdU staining and CCK-8 assays were used to evaluate
whether cell proliferation was decreased. As shown in Fig. 4B and 4C, the
proliferation rate of the si-circMFN2 group was significantly lower than that of the si-
NC group. Transfection with the siRNA effectively inhibited the growth of PA cells.

Wound healing and Matrigel invasion assays were conducted to evaluate migration
and invasion. There was a significant difference between the si-circMFN2 and si-NC
groups. The mobility of the si-circMFN2 group was markedly less than that of the si-
NC group. Downregulated circMFN2 lowered cell motility (Fig. 4D and 4E). Overall,
the results demonstrated that circMFN2 knockdown could limit the proliferation,
migration, and invasion of PA cells.

Figure 4

3.5 CircMFN2 and miR-146a-3p may act as molecular sponges that promote the
invasiveness of PA cells.

We performed further analyses to validate that circMFN2 regulates PA cells. We
identified the predicted targets of circRNA and miRNA through TargetScan software.
We screened the downstream genes and selected three miRNAs with high correlation.
The three genes included miR-3084a-5p, miR-509-5p, and miR-146a-3p (Fig. 5A). To
validate the predictions, we performed qRT–PCR in GH3 and MMQ cells. Fig. 5B
and 5C show the expression of each gene in PA cells, and we selected miR-146a-3p,
which was most differentially expressed in the two cell lines, as the experimental
gene. To further validate this finding, we cotransfected GH3 and MMQ cells with
miR-146a-3p mimic and inhibitor and observed circMFN2 expression. The results indicated that the miR-146a-3p mimic downregulated the expression of circMFN2 compared to that in the NC group, and the miR-146a-3p inhibitor upregulated circMFN2 expression in GH3 and MMQ cells (Fig. 5D and 5E). We also analyzed five non-functional pituitary adenoma specimens and five normal human pituitary specimens using bioinformatics tools. The findings revealed a statistically significant decrease in miR-146a expression in non-functional pituitary adenoma specimens compared to normal human pituitary specimens (Fig. 5F). Although miR-3084a-5p and miR-509-5p were not detected, the high-throughput microarray results matched the cellular results, indicating that miR-146a-3p may regulate the expression of circMFN2 in PA cells. Additionally, these two genes are negatively correlated and may function as molecular sponges to promote the progression of PA.

Figure 5

3.6 MiR-146a-3p inhibits motility and proliferation via circMFN2 in PA cells

We conducted several explorations to investigate the mechanism of miR-146a-3p's action as a circMFN2 sponge and its impact on PA cells in PA cell lines. we cultured PA cell lines with upregulated and downregulated miR-146a-3p. We also conducted rescue experiments by simultaneously downregulating circMFN2 and miR-146a-3p in PA cells to determine if the inhibitory effect of si-circMFN2 on cells could be reversed. The results showed that PA cell proliferation and migration were inhibited through the miR-146a-3p mimic. The miR-146a-3p inhibitor promoted motility and tumor growth (Fig. 6A, 6B, 6C and 6D). The experimental results also showed that
the proliferation, migration, and invasion abilities of the group cotransfected with miR-146a-3p inhibitor and si-circMFN2 were enhanced to different degrees compared with those in the group transfected with si-circMFN2 or miR-146a-3p mimic alone. This finding suggested that si-circMFN2 could be partially rescued by the miR-146a-3p inhibitor to attenuate the inhibition of proliferation, invasion and migration of PA cells. Thus, we experimentally observed that miR-146a-3p, as a suppressor gene in PA cells, could regulate the oncological function of PA by downregulating the expression of circMFN2.

Figure 6
3.7 circMFN2 affected PA tumorigenesis by binding to miR-146a-3p to regulate the TRAF6/NF-κB signaling axis.

To further explore the signaling pathway through which circMFN2 regulates the PA pathogenesis, after analyzing high-throughput RNA microarrays from 5 pairs of normal subjects and patients without functional pituitary adenoma, we identified 9000 up-regulated and down-regulated genes. We used Targetscan software to screen the downstream mRNA of miR-146a-3p, the algorithm excluded false-positive specimens and identified TRAF6 and NF-κB as potential downstream mRNA molecules of miR-146a-3p (Fig. 7A and 7B). Our study utilized high-throughput microarrays to analyze the gene expression of TRAF6 and NF-κB. Our findings indicate that these genes were upregulated in non-functional pituitary adenomas, with their expression being lower in normal human controls than in pituitary adenoma controls. To verify consistency between results in pituitary tumor cells and tissues, PA cells were
transfected with si-circMFN2, miR-146a-3p mimic, and miR-146a-3p inhibitor. Western blotting was then used to evaluate TRAF6 and NF-κB protein expression levels in the PA cells. The results indicated that TRAF6 and NF-κB expression levels were significantly higher in the transfected si-circMFN2 group compared to the control group and si-circMFN2 NC group (Fig. 7C and 7D). Moreover, when we knocked down miR-146a-3p, TRAF6 and NF-κB expression levels were higher than those in the NC group. After transfection with the miR-146a-3p mimic, TRAF6 and NF-κB expression levels were significantly downregulated compared to those in the NC group (Fig. 7E and 7F). We concluded through correlation analysis that the expression levels of TRAF6 and NF-κB were positively correlated (Fig. 7G). circMFN2 expression was positively correlated to TRAF6 protein expression (Fig. 7H). The same was true for NF-κB (Fig. 7I). MiR-146a-3p expression was negatively correlated with TRAF6 and NF-κB expression levels (Fig. 7J and 7K). Therefore, circMFN2 may promote the proliferation, migration and invasion of PA by sponging miR-146a-3p via the TRAF6/NF-κB signaling axis.

4 Discussion

Pituitary adenoma accounts for approximately 15% of all intracranial cancers in China. It is the third most common brain cancer in terms of incidence. Almost 25–55% of cases of PA exhibit invasion and local immune cell infiltration, causing destructive changes in adjacent tissues [16]. The pathogenesis of PA is a complex process that involves multiple factors and has not yet been clarified. It has been
theorized that the lack of early biomarkers in many patients with PA is attributed to endocrine disease and nerve compression [17]. Furthermore, PA patients are challenging to identify in current medical diagnoses, and they often experience various adverse reactions during treatment [18]. Since the tumor arises from the pituitary gland, which is resides outside of the blood–brain barrier, serum molecular markers and therapeutic targets may be able to effectively predict the prognosis of PAs [19]. CircRNAs are candidate biomarkers because of their stable structure, tissue specificity, and high expression in human tissues and body fluids [20]. The insensitivity and stability of circRNAs to ribonucleases have made circRNAs a popular research topic [21]. An increasing number of circRNAs associated with multiple diseases are being discovered and verified [22]. In general, encouraging results have been reported by exploring the functions of these circRNAs.

CircRNAs, which are non-coding RNAs with a circular structure, have the ability to regulate mRNA stability and gene transcription in cells. They can also act as miRNA sponges to control gene expression. Additionally, CircRNAs are directly involved in the processes of proliferation, apoptosis, and differentiation of neuronal cells and tumor cells [23]. Hsa_circ_0004872, identified as a molecular sponge, has been found to regulate the development of gastric carcinogenesis and gastric cancer by binding to miR-224 [24]. Another study revealed that circ_0009910 is associated with the growth, invasion, and migration of gastric cancer tumors, osteosarcoma, and chronic granulocytic leukemia. [25-27]. Although most circRNAs are non-coding RNAs, a few of them have the ability to encode polypeptides. These circRNAs, which
can encode polypeptides, not only act within their own cells but can also be secreted into the intercellular matrix through exosomes. This allows them to communicate and transmit information to other cells, leading to changes in the biological properties of the surrounding cells [28-29]. Researchers have found that certain circRNAs present in the nucleus can influence their own transcriptional activity by binding to RNA polymerase II. Additionally, they can promote the expression of their parental genes by interacting with the U1 small ribonucleoprotein and the parental gene promoter, thereby enhancing the expression of these genes [30-31]. At the same time, the methylation modification of circRNA has also received widespread attention in the academic community. Modified circRNAs with m6A modification can recruit molecules such as YTHDF3, initiating the protein translation process. Moreover, circRNAs themselves can also engage in translational work. By identifying open reading frames (ORFs) within circRNAs, researchers have associated relevant ribosomal entry sites [31]. Analysis using the RFP dataset revealed that some circRNAs have specific RFP binding sites, indicating a significant connection between circRNAs and translated ribosomes.

However, little research has been done on the pattern of circRNA expression and its clinical importance in PA [32]. Especially for pituitary adenoma related targets and gene prediction, there is no good database to provide data support. Our study revealed that circMFN2 exhibited significant upregulation in aggressive pituitary adenomas and demonstrated a positive correlation with tumor growth, cellular transport, and cellular morphology. This was determined through the analysis of invasive and non-
invasive adenoma samples by retrieving pituitary adenoma-related high-throughput microarrays using GEO and KEGG methods. According to our findings, there appears to be a correlation between the expression of circMFN2 and the presence of invasion, as well as the need for surgery. Specifically, the invasive group had higher levels of circMFN2 expression compared to the noninvasive group. Based on testing of the clinical samples, including serum, exosome and tissue. CircMFN2 expression was significantly lower before and after surgery in the same patients. The current experimental results demonstrate that the removal of adenomas through surgery can lead to changes in the level of circMFN2 in the blood of patients. It is important to note that the majority of circRNA originates from the nucleus and is subsequently secreted into the cytoplasm. The resection of tumor cells in adenomas results in a reduction in the level of circMFN2, which plays a role in regulating cell growth and invasion. This reduction in adenoma cells consequently impacts the level of circMFN2 in the serum. Additionally, the altered levels of hormones secreted by pituitary tumors in humans after adenoma removal can affect the protein-mRNA-circRNA alterations, thereby influencing the levels of circMFN2. More importantly, the efficacy of circMFN2 for the possible diagnosis of PA was analyzed using ROC curves, and the results showed that circMFN2 has a promising ability to serve as an independent diagnostic indicator and molecular marker for the diagnosis of PA. These findings imply that the pathophysiology and development of PA may be influenced by the expression of circMFN2.
It is equally important to elaborate on the function of circMFN2 in PA cells. We explored its effects on tumorigenesis and motility by knocking down the target gene and completing in vitro experiments. Our results demonstrated that circMFN2 downregulation could effectively inhibit the biological function of PA cells, such as proliferation and migration. To further validate the circMFN2 mechanism in PA, we identified the miRNAs downstream of circMFN2 in several databases using bioinformatics analysis and qRT–PCR and found that miR-146a-3p may play a biological role as a molecular sponge of circMFN2 in PA cells. We performed transfection experiments using miR-146a-3p mimics and an miR-146a-3p inhibitor and rescue experiments using si-circMFN2 to verify the ability of circMFN2 to regulate proliferation and migration of PA cells through miR-146a-3p. The result found that miR-146a-3p acts as an anti-oncogene by inhibiting their proliferation and motility. It was also observed that circFMN2 and miR-146a-3p have a competitive binding at the 3'UTR site. When circFMN2 and miR-146a-3p were simultaneously knocked down, the effect on pituitary adenoma cells was successfully rescued. These findings suggested that circMFN2 may regulate the tumorigenic process of pituitary adenomas through its interaction with miR-146a-3p.

Within the miRNA-mRNA regulatory network, we used bioinformatics techniques to identify downstream molecules linked to tumor growth and motility. The study found that an individual mRNA can bind with multiple miRNAs to produce regulatory effects. The TRAF6/NF-κB pathway was identified as the most suitable pathway for our theory which binds to miR-146a-3p. TARF6/NF-κB, which is present
in almost all human cells and associated with p50 and p52, can be activated by various stimuli and plays a crucial role in regulating apoptosis, cell differentiation, and cellular inflammation. To verify this hypothesis, we detected changes in TRAF6 and NF-κB when circMFN2 and miR-146a-3p were upregulated or downregulated. The results showed that knocking down circMFN2 downregulated TRAF6/NF-κB expression. However, the miR-146a-3p knockdown results were opposite of the circMFN2 knockdown results. Thus, miR-146a-3p was negatively correlated with TRAF6/NF-κB. Several pieces of evidence have also suggested that TNF-α can induce the development and progression of EMT via TRAF6/NF-κB [33]. In combination with the study of Ma, they found that CCL3 promotes proliferation of colorectal cancer related with TRAF6/NF-κB molecular pathway [34], and Deng’s study found that TRAF6/NF-κB pathway was associated with early brain injury after subarachnoid hemorrhage [35]. According to Elisabetta Vergani et al., miR-146a-5p impairs melanoma resistance to kinase inhibitors by targeting COX2 and regulating NF-κB-mediated inflammatory mediators [36]. Based on these studies, we constructed the following conclusion. circMFN2 affected cell proliferation in PA cells by partially regulating the TRAF6/NF-κB axis through miR-146a. The cause of cell proliferation may be that the TRAF6/NF-κB is a key player in regulating cell growth and gene transcription. When the signaling factor TNF binds to the cell membrane receptor, it triggers a series of events that activate TRAF6/NF-κB. The conformational change in the receptor allows it to transmit the signal to IKK kinase (IκB kinase), which then phosphorylates the IκB protein. This phosphorylation leads
to the dissociation of IkB from the trimeric complex, freeing TRAF6/NF-κB to act, which allows it to rapidly move from the cytoplasm into the nucleus. In the nucleus, TRAF6/NF-κB binds to specific sequences on nuclear DNA, promoting the transcription of genes involved in cell growth and proliferation, such as CyclinD1, c-Myc, MMP-9, and VEGF [37-40]. It is important to note that sustained activation of this pathway can lead to uncontrolled cell growth. The reason of cell migration and invasion may be TRAF6/NF-κB. The change of TRAF6/NF-κB expression affects the expression of E-cadherin and N-cadherin protein, loosens the connection between cells, promotes the EMT process of cells, and then increases the migration and invasion ability of PA cells [41].

To summarize, we investigated whether circMFN2 can be used as a molecular marker to distinguish PA patients from healthy individuals. Our study first provided evidence that blockade of circMFN2 could inhibit tumorigenesis in PA cells partially via the miR-146a-3p/TRAF6/NF-κB axis. Therefore, a novel discovered regulatory feedback loop constituted by circMFN2/miR-146a/TRAF6/NF-κB in PA was exposed by us. But based on the current study in this paper, we still need to improve the animal experiments and multi-center collection of patient data for statistical analysis to produce more accurate results. At present, circRNA-RBP is still a research hotspot in the academic field, but there is no research on the downstream RBP of circMFN2, so we will further study its downstream RBP to clarify its detailed mechanism of action, and after clarifying the mechanism, we will be able to target the inhibition of
circMFN2, and carry out a large-scale clinical trial to explore the potential of its potential for the treatment of pituitary adenomas.

5 Conclusion

We identified that circMFN2, as a potential oncogene acting through miR-146a-3p in PA cells, may affect the pathogenesis of PA via the TRAF6/NF-κB axis. CircMFN2 has a specific reference value as a molecular marker for diagnosing PA serum, exosome and tissue. By unearthing this remarkable association, we offered new ideas and targets for the diagnosis of PA.

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Author Contributions: Qi Liu contributed to project management and manuscript editing; Haitong Wan completed the manuscript writing and data analysis and performed an experiment; Xiang Gao was involved in clinical sample collection; Zexu Yang assisted in the investigation; Leiguo Wei and Yufei Qu designed the experiment. All authors reviewed the article.

Availability of Data and Materials: We guarantee the authenticity and validity of all data and results. Open up some of the raw data uploads as supplementary files.

Ethics Approval: All patients’ blood and tissue were collected with written signed consent and validated by the Ethics Committee of The First Affiliated Hospital of Medical College of Shihezi (KJ2021-076-02).

Conflicts of Interest: The authors declare that they have no competing interests.

Supplementary Materials: Supplementary Material File

References


qRT-PCR system reaction settings

1. Settings
   ① Sample Volume (μL) 20
   ② Run Mode Standard 7500
   ③ Data collection Stage 2, Step 2 (61.0@0:34)

2. Thermal Cycler Protocol

Thermal Profile
STAGE1 Reps:1 95.0°C 15min
STAGE2 Reps:40 94°C 20sec - 61°C 34sec
STAGE3 Reps:1 95°C 15sec - 60°C 1min - 95°C 15sec - 60°C 15sec
<table>
<thead>
<tr>
<th>gene</th>
<th>Sequence(5’-3’) or Assay ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>circMFN2(human)</td>
<td>CGCAATGTCCCTGCTCTTCTC</td>
</tr>
<tr>
<td>rat actin f</td>
<td>TCAGGTCACTACATCGGCA</td>
</tr>
<tr>
<td>rat actin r</td>
<td>AAAGAAAGGGTGAAAACG</td>
</tr>
<tr>
<td>human actin f</td>
<td>CTCCATCGCCCTCGCTGT</td>
</tr>
<tr>
<td>human actin r</td>
<td>GCTGTCACCTCCACCGTTCC</td>
</tr>
<tr>
<td>circMFN2(Rat)</td>
<td>CCTGAACACCGCTGGGAT</td>
</tr>
<tr>
<td>miR-146a-3p</td>
<td>ACCUGUGAAGUUCAGUUCU</td>
</tr>
<tr>
<td>U6</td>
<td>001973(Applied biosystems)</td>
</tr>
</tbody>
</table>
Table S2: the clinicopathological parameters of patients with PA enrolled in this study

<table>
<thead>
<tr>
<th>Clinicopathological parameters</th>
<th>Total Cases</th>
<th>Circ_0009910 expression</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Age [years]</td>
<td>28</td>
<td>12</td>
<td>16</td>
</tr>
<tr>
<td>≤55</td>
<td></td>
<td>12</td>
<td>5</td>
</tr>
<tr>
<td>≥55</td>
<td>16</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td>15</td>
<td>9</td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td>13</td>
<td>6</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td>17</td>
<td>12</td>
</tr>
<tr>
<td>Invasive</td>
<td></td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td>Non-invasive</td>
<td></td>
<td>28</td>
<td>8</td>
</tr>
<tr>
<td>Ki-67</td>
<td></td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>≥2%</td>
<td></td>
<td>20</td>
<td>12</td>
</tr>
<tr>
<td>≤2%</td>
<td></td>
<td>28</td>
<td>8</td>
</tr>
</tbody>
</table>

Note: Chi-square test was used for tabular statistics, and all values were counted data used X±S
Table S3: Univariate logistic regression analysis of variables to predict invasive behavior

<table>
<thead>
<tr>
<th>Variables</th>
<th>B</th>
<th>S.E.</th>
<th>Wald</th>
<th>OR(95%CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age ≤45</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-.102</td>
<td>1.183</td>
<td>0.111</td>
<td>0.093(0.089- 9.169)</td>
<td>0.946</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.931</td>
<td></td>
</tr>
<tr>
<td>≥65</td>
<td>-.452</td>
<td>1.415</td>
<td>0.102</td>
<td>0.636(0.040- 10.182)</td>
<td>0.749</td>
</tr>
<tr>
<td>≥65</td>
<td></td>
<td></td>
<td></td>
<td>0.749</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td>1.122</td>
<td>1.132</td>
<td>0.983</td>
<td>3.071(0.334- 28.220)</td>
<td>0.321</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.321</td>
<td></td>
</tr>
<tr>
<td>Circ_0009910</td>
<td>2.888</td>
<td>1.165</td>
<td>6.144</td>
<td>17.958(1.830- 176.192)</td>
<td>0.013</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.013</td>
<td></td>
</tr>
<tr>
<td>Ki-67</td>
<td>1.604</td>
<td>1.245</td>
<td>1.661</td>
<td>4.972(0.434- 56.996)</td>
<td>0.198</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.198</td>
<td></td>
</tr>
</tbody>
</table>

Note: Chi-square test was used for tabular statistics, and all values were counted data used X±S.
Table S4: Comparison of general data

<table>
<thead>
<tr>
<th></th>
<th>Invasion group (n=35)</th>
<th>Non-invasive group (n=15)</th>
<th>Normal group (n=50)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>53.9±2.2</td>
<td>55.1±3.3</td>
<td>48.9±1.6</td>
<td>0.162</td>
</tr>
<tr>
<td>Gender (male)</td>
<td>19 (54.3%)</td>
<td>8 (53.3%)</td>
<td>27 (54.0%)</td>
<td>0.998</td>
</tr>
<tr>
<td>Drink (n%)</td>
<td>3 (8.6%)</td>
<td>0 (0.0%)</td>
<td>7 (14%)</td>
<td>0.132</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>167.00</td>
<td>168.00</td>
<td>169.50</td>
<td>0.541</td>
</tr>
<tr>
<td></td>
<td>160.0-175.0</td>
<td>160.0-173.0</td>
<td>163.0-177.2</td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>70.00</td>
<td>67.00</td>
<td>71.50</td>
<td>0.617</td>
</tr>
<tr>
<td></td>
<td>66.0-82.0</td>
<td>60.0-67.0</td>
<td>62.0-81.2</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.78</td>
<td>26.17</td>
<td>24.55</td>
<td>0.143</td>
</tr>
<tr>
<td></td>
<td>24.2-28.5</td>
<td>22.6-30.1</td>
<td>22.9-27.0</td>
<td></td>
</tr>
<tr>
<td>Hypertension (n%)</td>
<td>13 (37.1%)</td>
<td>3 (20.0%)</td>
<td>19 (38.0%)</td>
<td>0.416</td>
</tr>
</tbody>
</table>

Note: n is the number of cases, P < 0.05 the difference was statistically significant.
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**Author contributions**

Qi Liu contributed to project management and manuscript editing; Haitong Wan completed the manuscript writing and data analysis and performed an experiment; Xiang Gao was involved in clinical sample collection; Zexu Yang assisted in the investigation; Leiguo Wei and Yufei Qu designed the experiment. All authors reviewed the article.

**Competing interests**

The authors declare that they have no competing interests.

**Informed consent**

Informed consent was obtained from all individuals included in this study.

**Ethical approval**

All patients' blood and tissue were collected with written signed consent and validated by the Ethics Committee of The First Affiliated Hospital of Medical College of Shihezi (KJ2021-076-02).