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The role of NR2C2 in the prolactinomas

NR2C2 targeted by miR-129-5p in prolactinomas

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Abstract: Prolactinomas are the most frequently observed pituitary adenomas. Prolactinomas invasion is a key risk factor associated with operation results, and it is highly correlated with clinical prognosis. Nuclear receptor subfamily 2 group C member 2 (NR2C2) first cloned from testis is involved in the invasion and metastasis of several human tumors. In 46 patients with prolactinamas, the expression levels of CCNB1, Notch2, and NR2C2 was determined with tissue micro-array (TMA). The association between NR2C2 levels and clinical parameters was established with univariate analysis. The levels of Notch2 and CCNB1 were analyzed by RT-PCR and western blot techniques. The average methylation levels of the NR2C2 promoter were 0.505 and 0.825 in invasive prolactinomas (IPA) and non-IPA groups, respectively (p = 0.013). Univariate analysis also showed that there is a significant relationship between high NR2C2 expression and invasion ($x^2 = 7.043$, p = 0.008), prolactin granules $(x^2 = 8.712, p = 0.003)$, and tumor size $(x^2 = 4.261, p =$ 0.039). With the knockdown of NR2C2, cell proliferation was inhibited. Genes related to epithelial-mesenchymal

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transition (EMT) induced the apoptosis in MMQ cells. In addition, the level of Notch2 and CCNB1 were down-regulated with the knockdown of NR2C2. Moreover, miR-129-5p reduced mRNA levels of NR2C2, and they inhibited cell proliferation by inducing apoptosis levels of MMQ cells. Our findings proved NR2C2 played the important role in tumorigenesis tumor invasion of prolactinomas; moreover, NR2C2 is identified as a potential target.

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Keywords: Prolactinomas; NR2C2; Invasion; miR-129-5p; Notch pathway.

1 Introduction

Prolactinomas account for approximately 40% of all pituitary adenomas (PAs). The objectives of therapy are as follows: normalization of prolactin levels, restoration of eugonadal fuction, and reduction of tumor mass. Most subjects respond well to conventional treatment, which includes dopamine agonists (DAs) [1]. WHO Guidelines suggest that treatment may be withdrawn after two years if certain criteria are met, but most patients require longterm therapy [2]. For patients treated with cabergoline (CAB), the success rate of drug withdrawal was 41.2% (95% CI 32.3-50.4%). When patients were administered CAB over a period of 24 months, the success rate of drug withdrawal was 48.7% (95% CI 38.9-58.5%). For patients with idiopathic hyperprolactinemia, the success rate of drug withdrawal was 73.2% when treated with CAB. (95% CI 55.6–87.7%) [3]. In general, morphological alterations caused by DAs are rapidly reversible with the discontinuation of medication. However, long-lasting effects may be sometimes produced in scattered cells [4]. However, the main limitations of DAs therapy are drug intolerance and resistance. In patients with pituitary adenomas, the most reliable marker of biological behavior was Ki-67 proliferation index [5,6]. Out of 199 drug resistant-prolactinomas, the recurrence rate was 27.27%

and 8.47% for the two respective groups: Ki-67 labeling index (LI) >3% and Ki-67 LI < 3% [7]. It may be noted that Ki-67 LI may not always predict the recurrence risk of pituitary adenomas; however, it may be a useful predictor of the progression risk of pituitary adenomas in tumor remnants [8]. Markers of invasiveness are used in patient management, especially while administering adjuvant radiotherapy.

Nuclear receptor subfamily 2 group C member 2 (NR2C2), which is also known as testicular receptor 4 (TR4), was first cloned in 1994. It belongs to nuclear hormone-receptor family, which acted as ligand-activated transcription factors and governed following biological processes: development, cellular differentiation, and homeostasis [9,10]. It has been established that NR2C2 transcriptionally regulates the oncogene Enhancer of Zeste 2 Polycomb Repressive Complex 2 subunit (EZH2) by binding with its 5' promoter region. To suppress EZH2 expression, NR2C2 was targeted. The expression of its downstream key metastasis-related genes, including NOTCH1, Transforming Growth Factor beta 1 (TGFb1), Snail Family Transcriptional Repressor 2 (SLUG), and Matrix Metallopeptidase 9 (MMP9), was suppressed [11]. Moreover, NR2C2 increased the invasion of C4-2 and PC3 cells by decreasing the expression of miR-373-3p, resulting in the activation of TGFβR2/p-Smad3 signals [12]. The expression of NR2C2 was detected in the pituitary gland of rat and mouse. A potential NR2C2-binding site was identified in proopiomelanocortin (POMC), which is a precursor protein [13]. In patients with Cushing's disease, NR2C2 would simultaneously reduce the secretion of Adreno-Cortico-Tropic-Hormone (ACTH) and corticotroph tumor growth [14]. Furthermore, NR2C2 binds with glucocorticoid receptor (GR), which plays an important role in regulating the expression of POMC in glucocorticoid-directed corticotroph tumor. Moreover, NR2C2 also modulates glucocorticoid actions on other GR targets [15].

The epigenetic activation or inactivation of genes plays a critical role in the pathogenesis of many important diseases in humans, especially cancer. The expression of NR2C2 was remarkably higher in 46 patients with prolactinomas, which was confirmed by performing tissue microarray assays (TMA). To determine the extent to which miR-129-5p targeted NR2C2 in MMQ cells, the methylation status of NR2C2 promoter was analyzed by MassARRAY system.

2 Materials and methods

2.1 Patients, tissue specimens, and cells

At the Department of Neurosurgery, Beijing Tian Tan Hospital, Capital Medical University, China, files were searched to identify cases of PAs for the period extending from May 2008 to July 2014. Histologically, 46 prolactinomas cases were diagnosed according to the histological classification of PAs, which was laid down by the World Health Organization in 2017 [4]. The diagnostic criteria for invasive PAs are as follows: 1) Knosp classification grade III-IV tumors and Hardy classification invasive adenomas; 2) tumor cells were confirmed via pathology as invading sellar bone or adjacent dura mater; and 3) tumor cells invaded sphenoid sinus cavity or peripheral vascular nerve. A new tumor was detected and confirmed histologically in eight patients, indicating the recurrence of PAs. Tissue samples were divided into two portions: the first portion was frozen in liquid nitrogen for isolating mRNA and proteins, and the second portion was used for pathological examination. Surgery was performed in two cases: i) patients with drug-resistant tumors and ii) patients that could not tolerate drug sideeffects, such as headache, nausea, and constipation. In patients with drug-resistant tumors, a daily dose of 15 mg Bromocriptine was administered for at least three months but serum prolactin levels (PRL) were abnormally high. Three specimens of normal pituitary gland were obtained from an organ donation program. The protocols were approved by the Internal Review Board (IRB) of Beijing Tiantan Hospital, which is affiliated to Capital Medical University in China. The analysis was conducted according to the principles expressed in the Declaration of Helsinki. Tumor volume was calculated with the following formula: $V = a \times b^2/2$, where a and b represented the length and width of tumor, respectively.

In this study, MMQ cells were cultured in F12 culture medium. This was supplemented with 2.5% (v/v) fetal bovine serum and 10% (v/v) horse serum in a humidified incubator at 37°C in 5% (v/v) $\rm CO_2$. The sequences of pGFP-C-shLenti plasmid (NR2C2) are as follows: A: ACCACAGATAGTGCYYCACCTCCAAGCCT, B: GGATTCTTGTCCGCCTGCCGACACTCAGG, C: CACTTACAGAACAGCATCCAGGAAGATAA, D: AGAGACATCCAGTGCCAAGCAGCTCATAT.

Using electroporation, cells were transfected with miR-129-5p mimic (Mi), mimic control (Mc), inhibitor (In), and inhibitor negative control (Inc) (Beijing Genomics

Institute, Shenzhen, Guangdong, China) in SF cell line Table 1: The clinicopathological data in 46 prolactinomas cases. solution box (Lonza).

2.2 Real-time reverse-transcription PCR (RT-qPCR)

Total RNA was extracted from frozen PAs (~10 mg) using RNeasy® Mini Kit (Qiagen). As described previously, RTqPCR was performed using Applied Bio-systems 7500 Fast System (Life Technologies) [16]. A comparative CT method was used to calculate fold-change in the differential expression of each gene. (It is also known as 2-DACT method.) The primer details of genes were described in a supplementary of Table 1.

2.3 Immunohistochemistry (IHC)

A previously described method was used to construct tissue micro-array (TMA) [17]. To assess the content and quality of tumor, all TMA slides were evaluated with hematoxylin (HE) stain. The TMAs were placed in Leica BOND-III equipment (Leica Biosystems), which is a fully automated slide-staining system for performing IHC tests simultaneously. In this machine, IHC protocol F was selected. For heat-induced epitope retrieval (HIER) parameter, epitope retrieval (ER1) was set after three minutes. BondTM Ploymer Refine Detection (Leica Biosystems, DS9800, Germany) system was used for detecting primary antibodies. Slides were scanned into digital pictures using Aperio AT2 (Leica Biosystems). Primary antibodies, CCNB1 (1:1000, Abcam), Notch2 (1:500, Abcam), and NR2C 2 (1:500, Abcam) were used. The optimal titer of primary Abs was determined on the basis of pre-experimental results. The results were calculated using Aperio AT2 (Leica Biosystems) and a digital slide viewing software. The staining intensity was stratified on a scale of 0-3 (0 = no staining, 1 = weak, 2 = veakmoderate, and 3 = strong). An H-score was obtained by multiplying staining intensity with a constant, and the mean was adjusted with the strongest staining [H-score = 1.0 (%weak) + 2.0 (%moderate) + 3.0 (%strong)].

2.4 SDS-PAGE and Western blot analyses

The specimens of PAs were lysed in TNE buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA) (Sigma-Aldrich), containing 1% Nonidet P-40 (Calbiochem) along with protease and phosphatase inhibitor cocktails (Roche).

Category		Prolactinoma		
		Invasive	Non-Invasive	p-Value
Cases		23	23	
Age	Range	14-61	16-57	p=0.140
	Mean±SD	36.3±10.7	42.8±13.2	
Sex	Males	11	5	p=0.063
	Females	12	18	
PRL	(ng/ml)	1423±563	352±153	p<0.05
Tumor size	<5.13cm ³	8	15	p=0.038
(median)	≥5.13cm³	15	8	
Particle	Sparsely	16	8	p=0.018
	Densely	7	15	
Recurrence	Yes	6	2	p=0.120
	No	17	21	

Total extracts were centrifuged at 12000×g for 30 min at 4°C. Protein concentration of the supernatant was determined using a BCA protein assay kit (Pierce Biotechnology). Western blot analysis was performed as follows: 40 µg of lysate per lane was loaded onto 4-12% Bis-Tris SDS-PAGE gels, which were separated electrophoretically and blotted onto polyvinylidene fluoride (PVDF) membranes. Different blots were incubated with following anti-bodies: CCNB1 (1:2000, Abcam), NR2C2 (1:2000, Abcam,), Notch2 (1:2000, Abcam), and GAPDH (1:8000, Sigma-Aldrich). Then, they were incubated with secondary antibodies and tagged with horseradish peroxidase (Santa Cruz Biotechnology). Blots were visualized by enhanced chemiluminescence, and densitometry was performed with Amersham Imager 600. Loading control was determined by analyzing GAPDH levels.

2.5 Cell proliferation and apoptosis assay

The density of MMQ cells was adjusted to 1×10^5 cells/ ml. Cell suspension volume of 100 µL was plated into each well of 96-well plates and cultured for 24 h. Then, 20 µl of (MTS) solution [3-(4,5-diethylthiazol-2-yl)-5-(3carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-etrazolium, inner salt] was added to each well and further incubated for 4 h. Absorbance of each well was measured at 490 nm using an plate reader (Thermo Scientific).

To determine apoptosis, flow cytometry was performed with Annexin V&PI Apoptosis Detection Kit (Roche Diagnostics). Furthermore, MMQ cells were transfected with miRNA129a-5p mimic or miRNA129a-5p inhibitor. Then, these cells were incubated for 72 h. Apoptosis was determined by Annexin V&PI Apoptosis Detection Kit (Roche Diagnostics). This assay was based on the following principle: phos-phatidylserine (PS) membrane translocated from the inner lipid bilayer to the outer lipid bilayer as soon as apoptosis was initiated. On the cell surface, PS was easily detected by staining with Annexin V, a fluorescent conjugate of high-affinity PS binding protein.

2.6 Methylation analysis

Deoxyribonucleic acid (15-50 mg) was extracted from 15 invasive NFPAs (fresh-frozen), 15 non-invasive NFPAs, and 5 normal pituitary glands by using Trizol reagent according to manufacturer's protocol (Invitrogen). The concentration and purity of DNA were determined by measuring UV absorbance at wavelengths of 260/280 nm (Nanodrap ND-1000). Bisulfite treatment was performed using EZ-96 DNA Methylation kit (Zymo Research). Two overlapping PCRs were performed to amplify a 1039-bp area (–743 to +294, relative to translation start site) of NR2C2 promoter. For every cleaved CpG site, mass spectra were obtained using MassARRAY Compact MALDI-TOF (Sequenom). The spectral methylation ratios were generated with the help of https://www.baidu.com/?tn=98012088_4_dg&ch=12 (Sequenom).

2.7 Statistical analysis

In this experiment, χ^2 and Fisher's exact tests were used to determine the significance of categorical variables. Oneway ANOVA test was performed to examine the differential expression of CCNB1, Notch2, and NR2C2 in MMQ cells. All p values were two-sided and 0.05 was considered to be statistically significant.

3 Results

3.1 Sample characteristics of cohort

In this study, 46 cases of prolactinomas were retrospectively identified from pathological records. For standard HE staining and IHC, sufficient archival material was collected from 46 samples that included 16 men and 30 women with a median age of 38 years (range:

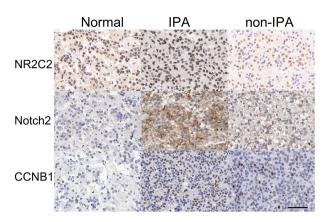


Figure 1: IHC analysis of the NR2C2, Notch2 and CCNB1 in prolactinomas NR2C2 and CCNB1: nuclear staining; Notch2: cytoplasmic staining. bar=60μm

16-61 years). The tumor size was in the range 0.74 to 24.52 cm³ (median = 5.13, 6.42 \pm 7.63). According to the Knosp classification and intraoperative parallelism, there were 23 invasive cases (IPA) and 23 non-invasive cases (non-IPA). Table 1 presents the characteristics of the two categories of cases, which were included in this study. The expression of CCNB1 protein should be optimum for controlling G2/M transition phase of cell cycle, which is associated with maturation-promoting factor and tumor proliferation [18]. According to the median of H-scores, CCNB1 protein level specimens were 16/23 and 7/23 for IPA and non-IPA, respectively (Figure 1) ($x^2 = 7.043$, p = 0.008). The PRL levels were 1423 ± 563 ng/ml and 352 ± 153 ng/ml for IPA and non-IPA groups, respectively (ANOVA, p < 0.01). The recurrence of IPA was 6/23, and the recurrence of non-IPA was 2/23 ($x^2 = 2.421$, p = 0.120). These results indicate that the expression of CCNB1 and PRL was significantly higher for IPA than for non-IPA. For the two groups of patients included in this study, there was no significant difference in the recurrence rate of prolactinomas.

3.2 NR2C2 expression and correlations with clinicopathologic parameters in prolactinomas

Tissue micro-array (TMA) is a recently developed technology that improves the performance of molecular profiling in cancer research studies. With the help of TMA, large-scale studies can be performed rapidly with fewer experimental variables. Moreover, innumerable tissue samples can be conserved with TMA technology. To assess the scattering of data on the expression of NR2C2 and Notch2, TMAs was performed on 46 specimens of prolactinomas and 3 specimens of normal pituitary gland.

As shown in Figure 1, immunoreactivity was primarily observed in the nucleus of tumor cells. In representative samples of PAs, the expression levels of NR2C2 were determined by IHC staining. The expression levels of NR2C2 were estimated from the following H-Scores: 148.4 ± 42.7 and 45.2 ± 28.72 for IPA group and non-IPA group, respectively (p < 0.01). There was no significant correlation between IPA and non-IPA groups for the following parameters: NR2C2 levels, age, gender, and tumor recurrence ($x^2 = 2.421$, p = 0.120). Based on the results of univariate analysis, it can be inferred that high expression of NR2C2 is significantly correlated with the following parameters: invasion ($x^2 = 7.043$, p = 0.008), prolactin granules ($x^2 = 8.712$, p = 0.003), and tumor size $(x^2 = 4.261, p = 0.039)$ (Table 2). Notch pathways are mainly active in the early phase of pituitary organogenesis, and they are essential for the development of somatotrophs, lactotrophs, thyrotrophs, and corticotrophs. These pathways have been implicated in the pathogenesis of pituitary adenomas [19]. Figure 1shows that H-scores of Notch2 and CCNB1 were specifically up-regulated in IPA group than that in non-IPA group (p < 0.05).

By performing RT-PCR and Western blot analysis, NR2C2 and Notch2 expression was determined in 12 specimens of prolactinomas and 3 normal pituitary glands. Compared to normal pituitary specimens and non-IPA group, the expression of NR2C2 and Notch2 were higher in IPA group (Figure 2).

3.3 Knockdown of NR2C2 expression suppresses cell proliferation and genes related with invasion

The efficiency of NR2C2 shRNA was first measured in MMQ cells. By using four different NR2C2 shRNA pairs (Sh-A-D), it was confirmed that results were a direct consequence of NR2C2 knockdown rather than an off-target effect. Figure 3 shows that compared to control cells, NR2C2 protein expression was reduced to 52.3% (Sh-A), 23.4% (Sh-B), 34% (Sh-C) and 41% (Sh-D) after 48 h. Notch pathway conveys anti-tumor or tumor-promoting effects in different tumors types, which depend on micro-environment [16]. Since Notch2 is an oncogene, it should be inhibited for preventing invasive bladder cancer [20]. By performing western blot analysis, it was found that the level of Notch2 and CCNB1 could be down-regulated with the knockdown of NR2C2 (Figure 3) (p < 0.01).

By performing cell viability assay, it was found that NR2C2 significantly promoted cell proliferation and was positively correlated with NR2C2 levels (Figure 4A).

Table 2: Relationship between NR2C2 and various clinical parameters in 46 cases.

Category		NR2C2		
		High	Low	p-Value
Cases		23	23	
Age	<38	14	9	p=0.140
	≥38	9	14	
Sex	Males	10	6	p=0.140
	Females	13	17	
PRL	(ng/ml)	1271±473	433±162	p<0.05
Tumor size	<5.13cm ³	8	15	p=0.039
	≥5.13cm³	15	8	
Invasion	Yes	16	7	p=0.008
	No	7	16	
Granules	Sparsely	17	7	p=0.003
	Densely	6	16	
Recurrence	Yes	6	2	p=0.120
	No	17	21	

Annexin V assay shows that apoptosis was significantly activated in Sh-B group and Sh-C group cells (Figure 4B).

Epithelial-mesenchymal transition (EMT) is a process through which an epithelial cell turns into a more motile mesenchymal cell [21]. E-cadherin, Vimentin, and snail are EMT markers, which can be considered as significant indicators of cystic lesions, tumor progression, bone destruction, and endocrine functions. The migration of endothelial cells and various tumor cells is regulated by signaling extracellular VEGF. By inhibiting intracrine VEGF signaling, CRC cell migration and invasion is strongly inhibited and proteins involved in cell motility are regulated [22]. RP-PCR results indicate that the expression of mRNA levels obviously changes among E-Cad, EGFR and VEGF in Sh-B group compared to control group (fold change > 4 or < 0.25; p < 0.05). As shown in Figure 4C, there was no statistical difference in the expression of MMP9, PTEN, and Vimentin (p > 0.05).

3.4 Methylation status of NR2C2 and miRNAs that target NR2C2

Pituitary tumors were initiated by following activities: oncogene activation, tumor suppressor gene inactivation, epigenetic changes, and dysregulation of microRNAs [23]. To inactivate genes epigenetically, methylation of CpG islands is carried out in genome DNA with the help of DNA methyltransferases. In 24 samples of prolactinomas, the

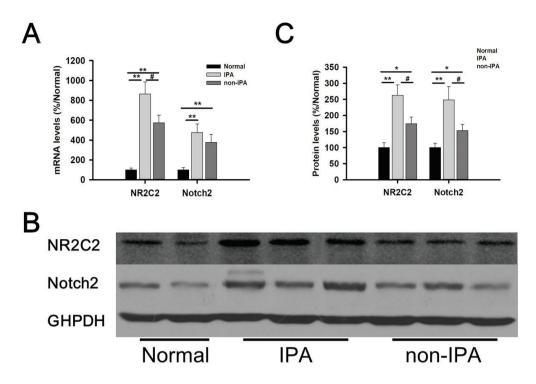


Figure 2: mRNA and protein levels of NR2C2 and Notch2 in prolactinomas. A: The mRNA levels among normal pituitary, IPA and non-IPA. B: Western blot analysis of NR2C2 and Notch2 among normal pituitary, IPA and non-IPA. C: The statistic of western blot. GAPDH was used for normalization. *compared to normal pituitary # compared to IPA, n=4-6.

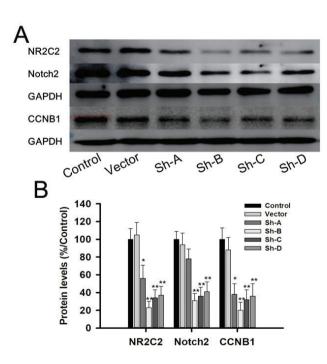


Figure 3: Knockdown of NR2C2 regulates the Notch2 and CCNB1 levels in MMQ cells. A: Western blot analysis show the NR2C2 level in Sh-B group and Sh-C group reduced to 23%, 34% compared to Control group separately. B: The statistic of western blot. GAPDH was used for normalization. n=3.

Table 3: The miRNAs levels targeting NR2C2 in prolactinoams.

Gene	P Value	Folds
miR-16-5p	0.886	0.739
miR-17-5p	0.027	0.44
miR-31-5p	0.353	0.466
miR-93-5p	0.551	0.654
miR-124-3p	0.077	0.269
miR-129-5p	0.023	0.068
miR-140-3p	0.447	0.639
miR-141-3p	0.493	0.837
miR-181a-5p	0.3	1.672
miR-181b-5p	0.029	2.408
miR-181c-5p	0.053	0.353
miR-195-5p	0.071	1.91
miR-196-5p	0.535	0.984
miR-200a-3p	0.023	0.407
miR-219a-5p	0.467	1.18
miR-219b-5p	0.22	0.984
miR-302c-3p	0.455	0.983
miR-424-5p	0.043	0.278
miR-490-3p	0.432	0.983
miR-497-5p	0.067	2.452

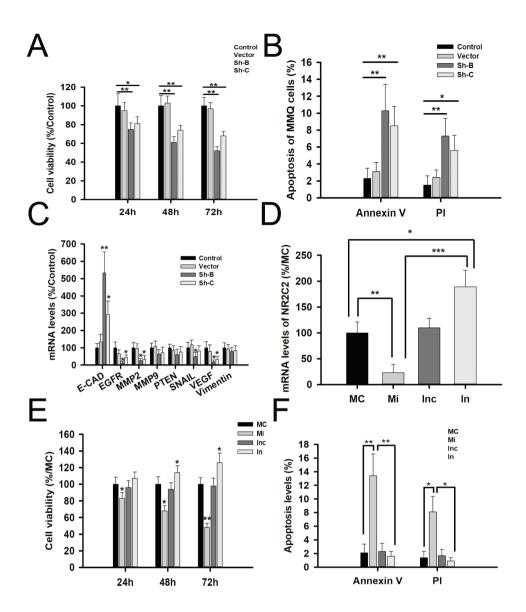


Figure 4: NR2C2 and miR-129-5p regulated the cell proliferation through inducing the apoptosis of MMQ cells. A: MTS experiment showed knockdown of NR2C2 suppressed the cell viability of MMQ cells. B: Knockdown of NR2C2 increased the apoptosis levels of MMQ cells. C: Knockdown of NR2C2 affected the mRNA levels of genes related with EMT in MMQ cells. D: miR-129-5p mimic downreguted the mRNA levels of NR2C2 in MMQ cells. E: MTS experiment showed miR-129-5p mimic inhibited the cell proliferation of MMQ cells. F: miR-129-5p mimic increased the apoptosis levels of MMQ cells. n=3.

methylation status of NR2C2 promoter was investigated by performing Sequenom's EpiTYPER assay. The average methylation was 0.405 and 0.825 in IPA group and non-IPA group, respectively (p = 0.013). Active transcription was probably inactivated with the methylation of NR2C2. Targetscan is a bioinformatics approach, which was used to identify following miRNA binding sites in NR2C23' UTR: miR-16-5p, miR-17-5p, miR-31-5p, miR-93-5p, miR-124-3p, miR-129-5p, miR-140-3p, miR-141-3p, miR-181a-5p, miR-181b-5p, miR-181c-5p, miR-195-5p, miR-196-5p, miR-200a-3p, miR-219a-5p, miR-219b-5p, miR-302c-3p, miR-424-5p, miR-

490-3p, and miR-1-5p. The expression of aforementioned miRNAs was measured by performing RT-PCR (Table 3). Five miRNAs (p < 0.05, fold change >2.0 or <0.5) of IPA specimens were statistically different from those of non-IPA group. Compared to non-IPA group, IPA specimens showed a fold-change>10 for miR-129-5p(p = 0.023). For the next functional experiment, we selected miRNA-129a-5p as the candidate miRNA, targeting NR2C2 gene in MMQ cells.

3.5 miR-129-5p reduces mRNA level of NR2C2 and cell proliferation of MMQ cells

In patients with biliary atresia, miR-129-5p effectively prevented EMT by regulating the expression of EMT pathway-related proteins [24]. Synthetic miR-129-5p mimic (M_i), mimic control (M_c), inhibitor (I_n), and inhibitor negative control (I_{nc}) were transfected into MMQ cell line. By performing RT-PCR, it was found that the expression of *NR2C2* was down-regulated in Mi group while the expression of *NR2C2* was up-regulated in I_n group (Figure 4D). Compared to M_c group, cell viability was significantly inhibited in M_i group (p < 0.01) but slightly elevated in I_n group (p < 0.05) (Figure 4E). Annexin V assay shows that apoptosis was significantly activated after transfection of miR-129a-5p (Figure 4F) (p < 0.05). In all, miRNA-129-5p inhibited pituitary cell proliferation by regulating the expression of NR2C2.

4 Discussion

Most prolactinomas are benign in nature; however, some prolactinoma patients show severe resistance toward DA. Surgery, especially trans-sphenoidal surgery, might be a more rational treatment strategy for treating such cases [25]. A high Ki-67 score is a predictive indicator of poor prognosis and resistance to adjuvant therapy in prolactinomas patients, which show severe resistance to DA therapy. The prognosis is poor in pituitary adenomas, which are severely aggressive. These pituitary adenomas invade surrounding nervous tissues and vascular structures [26]. In this study, the expression of NR2C2 was explored in 46 prolactinomas cases. It was found that NR2C2 was strongly expressed in invasive prolactinomas. When the expression of NR2C2 was high in prolactinomas patients, it was difficult to remove tumors with surgical resection. In prolactinomas specimens, the expression of NR2C2 was positively correlated with tumor aggressive behavior. A novel miRNA-gene network was used for regulating the formation of prolactinomas.

Previous studies have established that EMT is a potential marker of invasion and metastasis in patients with pituitary adenomas. Notch signaling is an important pathway that regulates following functions: neuroendocrine differentiation, proliferation, cell adhesion, EMT, and chemo-resistance [27]. In gonadotropin and growth hormone secreting adenomas (GHomas), synergistic interactions occur between Notch1 and DLL4. A negative correlation exists between the expression of Notch1 and DLL3, indicating the presence of

a negative feedback loop [28]. The expression of EMT was restrained by the knockdown of Notch2; moreover, the inhibiting effects of miR-146a-5p were partially abrogated with the progression of EMT in oesophageal squamous cell cancer (ESCC) cells [29]. With the up-regulated expression of Notch4 and Hey1, the expression of E-cadherin decreased while the expression of Vimentin, Fibronectin, TWIST1, and SOX2 increased in cases of head and neck squamous cell carcinoma (HNSCC) [30]. Furthermore, NR2C2 may still retain its DNA damage repair function; moreover, NR2C2 gains the ability to increase stem cell population and to alter EMT in prostate cancer cases. With the knockdown of NR2C2, only the level of Notch2 was down-regulated in MMQ cells. Notch1 was subordinate status because of the low level of Notch1 expression in patients with prolactinomas [28].

As a key transcription factor in cancer, NR2C2 may have multiple ways to either modulate or be modulated by miRNAs. Mature miRNAs negatively regulated gene expression or blocked protein translation, depending on the degree of complementarity between miRNAs and its targets or by inducing targeted mRNA cleavage [31]. In this study, 20 miRNAs that regulate the expression of NR2C2 levels were screened. There was statistically significant difference between IPA and non-IPA groups with respect to the biological targets identified by Targetscan: miR-17-5p, miR-129-5p, miR-181b-5p, miR-200a-3p, and miR-424-5p. In subsequent experiments, miR-129-5p was selected as the candidate miRNA that targets NR2C2 gene according to p value and fold change. It was reported that miR-129 played the role of tumor suppressor in many cancers, such as breast cancer, lung cancer, prostate cancer, and hepatocellular cancer [32-34]. To determine whether miR-129-5p was involved in the regulation of NR2C2 expression, the 3'UTR sequence of human NR2C2 was scanned using TargetScan database. It was found that miR-129-5p is a predicted marker that binds with NR2C23'UTR in humans. By enhancing miR-129-5p function, cell viability was significantly down-regulated in MMQ cells; however, miR-129-5p inhibitor mildly increased cell viability. Interestingly, it was found that miR-129a-5p functions as a suppressor to inhibit NR2C2 expression. We also found that miR-129-5p inhibits the expression of mRNA levels, which are related to invasion in MMQ cells.

Based on X-chromosome inactivation studies, it can be inferred that pituitary adenomas are of monoclonal origin [35]. Somatic mutations of GNAS and USP8 were identified as high frequency mutations of pituitary adenomas [36, 37]. Prolactinomas have never shown high frequency mutation till date. DNA methylation is a stable modification that leads to chromatin remodeling, resulting

in transcriptional silencing without gene mutation. Experimental results indicate that tumorigenesis of prolactinomas is caused by hypermethylation of NR2C2 promoter, which down-regulates the expression of NR2C2 and enables preneoplastic cells to escape from stressinduced senescence and accumulate mutations such that they advance into pituitary adenomas. Furthermore, miR-129-5p blocks the autophagy of human nucleus pulposus cell by directly inhibiting Beclin-1, a process that is dependent on the methylation of miR-129-5p promoter [38]. An increased expression of miR-129-5p may be mediated by demethylation, inhibiting the migration and invasion of osteosarcoma cells. For this purpose, valosin-containing protein was targeted in patients with osteosarcoma [39].

This is the first study to prove that the aggressiveness of prolactinomas can be correlated with upregulated expression of NR2C2. This implies that NR2C2 is a biomarker for the aggressiveness of prolactinomas. The expression of NR2C2 is reduced with the hypermethylation of NR2C2 promoter. Differentially expressed miRNAs were most likely to be involved in the tumorigenesis of prolactinomas, which regulated developmental pathways. Moreover, miRNA-129-5p plays an important role in Notch signaling pathway that affects the expression of NR2C2.

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