Research Article

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miRNA-199a-5p functions as a tumor suppressor in prolactinomas

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Abstract: Prolactinomas are the most frequently observed pituitary adenomas (PAs), and 5%–18% tumors were resistant to the dopamine agonists (DAs). MicroRNAs (miRNAs) dysfunction play a key role in tumorigenesis. Agilent miRNA and an expression chip were used for six prolactinomas and three normal pituitary specimens. Differentially expressed genes were confirmed by RT-qPCR. The level of DDR1 and SAT1 was determined with tissue micro-array (TMA) and western blot. A MMQ cell line was used for functional experiments. We have identified 5-miRNA and 12 target gene signatures of prolactinomas through gene ontology analysis. miRNA-199a-5p was selected for experiments that integrated the results from prolactinomas specimens and a rat prolactinoma model induced by 17- β -estradiol. Tumors with low miRNA-199a-5p had a significantly invasive behavior and a higher tumor volume ($p < 0.05$). DDR1 and SAT1, target genes of miRNA-199a-5p, had higher H-scores in the invasive group than those of the non-invasive group through TMA. An overexpression of miRNA-199a-5p suppressed the PRL secretion and the cell viability through upregulated the apoptosis level in MMQ cells ($p < 0.01$). Furthermore, we found the target genes expression of DDR1 and SAT1

were affected by miRNA-199a-5p regardless of mRNA levels or protein levels. This study provided evidence that downregulation of miRNA-199a-5p may contribute to prolactinoma tumorigenesis.

Keywords: Prolactinomas, miRNA-199a-5p, tumorigenesis, DDR1, SAT1.

1 Introduction

Pituitary adenomas (PAs) are the second most common intracranial tumors, and prolactinomas are the most common subtype. Most prolactinomas respond well to DAs. DAs are the first choice to treat hyperprolactinemia [1]. Guidelines suggest that treatment withdrawal may be considered after 2 years if certain criteria are met, but many patients require long-term therapy [2]. However, 5–18% prolactinomas do not respond to the treatment, even at high doses of DAs [3]. The main shortcoming of DAs is the long-term medication compared to surgery. Cabergoline is generally favored as the treatment of choice, although this drug may have the same increased risk of clinically relevant cardiac valvulopathy in prolactinomas as it does in Parkinson's disease [4]. Bromocriptine-resistant tumors are more frequently invasive than bromocriptine-responsive tumors, with a high Ki-67 index that is a predictive sign of poor prognosis in women with resistant prolactinoma [5, 6]. The molecular mechanisms underlying DA-resistant prolactinomas have not been fully explained.

MicroRNAs (miRNAs) play a key role in tumorigenesis and tumor progression and are differentially expressed among normal tissues and cancers [7]. miRNAs bind to the 3'-untranslated regions (3'-UTR) of target mRNAs, blocking translation or inducing mRNA degradation [8]. The differential expression of miRNA plays an important role in the human tumorigenesis. miRNAs can induce the disorder of the cell cycle which is related to the initiation and development of PAs, indicating that dysregulation of

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miRNA expression is involved in the etiology of PAs [9]. For example, miRNA-1, miRNA-195 and miRNA-206 are complementary to cyclin D1 (CCND1) and to upregulate CCND1 expression, which causes the tumorigenesis and malignant transformation of PA [10, 11]. miRNA-9 represses the dopamine D2 receptor (D2R) gene and its spliced variant and increases the synthesis and secretion of prolactin (PRL) in MMQ cells [12]. There are nine upregulated miRNAs and three downregulated miRNAs in drug-resistant prolactinoma patients compared to drug-sensitive patients [13]. miRNA-26b and miRNA-128 play the antagonistic role in the colony formation experiment and transwell experiment of AtT-20 cell line [14]. Finally, miRNA-34b, miRNA-326, miRNA-432, miRNA-548c-3p, miRNA-570 and miRNA-603, for which overexpression and/or activation of their target genes play a key role in pituitary tumorigenesis, were drastically and consistently downregulated in somatotroph adenomas [15].

In this study, 5-miRNA and 12 target genes signature of prolactinomas was identified compared to those of normal pituitary according to microarray hybridization. Gene ontology analysis revealed that the suppression of miRNA-199a-5p suppressed the cell differentiation and invasive behavior of pituitary tumor cells. We determined that miRNA-199a-5p controlled the MMQ cell proliferation through inhibiting the expression of the discoidin domain receptor tyrosine kinase (DDR1) and spermidine/spermine N1-acetyltransferase 1 (SAT1).

2 Materials and methods

2.1 Patients and tissue specimens

The files of the Department of Neurosurgery, Beijing Tian Tan Hospital, Capital Medical University, from May 2008 through July 2014 were examined for PA cases. Histologically, 42 prolactinoma cases were diagnosed according to the endocrine-related tumor classification of 2017 World Health Organization [16]. The standard of invasion and recurrence was adopted as described previously [17]. There were seven patients that showed recurrence according to the standard. The study was agreed upon by the Ethics Committee of Beijing Tiantan Hospital affiliated with Capital Medical University. All subjects signed the informed consent.

2.2 Animal and Cell culture

Adult Fisher 344 rats were housed at 20°C–23°C in this study. A previously reported method [17] was used to develop prolactinomas. MMQ cells were purchased from China Institute of Cell Line Resources. Cells were cultured with F12 culture (2.5% fetal bovine serum and 10% horse serum). According to the manufacturer's protocol, pEGFP-N1-miRNA199a-5p or pEGFP-N1-miRNA199a-5p-NC (BGI, Beijing, China) was transfected in 1×10^6 MMQ cells.

2.3 Microarray hybridization

Total RNA was isolated and purified using TRIzol reagent (Ambion, Thermo Fisher Scientific, USA) and assayed using an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Samples with a 28S to 18S rRNA ratio ≥ 0.7 number and 2100 RNA integrity (RIN) ≥ 7.0 were used to generate labeled targets. According to the protocol, total RNA was amplified and labeled using a One-Color Low Input Quick Amp Labeling Kit (Agilent). RNeasy Mini Kit (QIAGEN) was used for purifying labeled complementary RNA (cRNA). Each slide was hybridized with 1.65 μ g Cy3-labeled cRNA using a Gene Expression Hybridization Kit (Agilent Technologies) and a hybridization oven (Agilent Technologies). After 17 hours of hybridization, the slides were washed with a Gene Expression Wash Buffer Kit (Agilent Technologies) and scanned on a Microarray Scanner (Agilent Technologies).

The miRNA molecules were labeled through the miRNA Complete Labeling and Hyb Kit (Agilent Technologies). Then each slide was hybridized with 100 ng Cy3-labeled RNA at 55 °C, 20 rpm for 20 h. The slides were washed with a Gene Expression Wash Buffer Kit (Agilent Technologies) and scanned on a Microarray Scanner (Agilent Technologies). The significance threshold criteria were a false discovery rate (FDR) less than 0.05 and a fold change greater than 2.

2.4 Real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from frozen PAs (~10 mg) using the RNeasy® Mini Kit (Qiagen) and synthesized the first strand cDNA. PCR and RT-PCR was performed as described previously [18], using the Applied Bio-systems 7500 Fast System (Life Technologies). The fold-change in differential expressions for each gene was calculated

using the comparative CT method (also known as the $2^{-\Delta\Delta CT}$ method) [18].

2.5 Immunohistochemistry (IHC) Staining

Tissue microarray (TMA) construction and staining protocol were performed as described method. Primary antibodies used were D2R (1:1000, Abcam), DDR1 (1:1000, Thermo Fisher) and SAT1 (1:800, Thermo Fisher). The primary antibody concentration was based on pre-experimental results. The slides were scanned with Aperio Scanscope software using Aperio AT2. Staining value of slides (0-300) was evaluated as describes previously [19].

2.6 SDS-PAGE gel electrophoresis and western blot analyses

The methods of protein extraction and the concentration measure were previously described [19]. 40 μ g protein/lane was loaded onto 4–12% the gels. The antibodies against DDR1 (1:1000, Thermo Fisher), Bax (1:2000, Abcam), Bcl 2 (1:2000, Abcam), SAT1 (1:1000, Thermo Fisher) and GAPDH (1:8000, Sigma) were incubated overnight. Blots were visualized by chemiluminescence (Santa Cruz Biotechnology), and image was performed by the Amersham Imager 600.

2.7 Cell proliferation and apoptosis assay

miRNA-199a-5p or miRNA-199a-5p-NC (30nM) was transfected in 1×10^6 MMQ cells. MMQ cells were adjusted to a density of 1×10^5 cells/ml after 24h transfection. A 100 μ L volume of cell suspension was plated into each well of 96-well plates and cultured. After 24h, 48h and 72h, we added 20 μ L of 3-(4,5-diethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium an inner salt solution to each well and incubated it for an additional 4 h. The absorbance at 490 nm in each well was measured using an ELISA plate reader (Thermo). Apoptosis was determined with the Annexin V & PI Apoptosis Detection Kit (Roche Diagnostics). MMQ cell was transfected with miRNA 199a-5p or miRNA199a-5p-NC, incubated for 72 h and analyzed using a flow cytometry (Mark II, Merk), following the kit instructions.

2.8 Statistical analysis

χ^2 tests were used for the significance of clinicopathological data. One-way ANOVA tests were used to analyze the differential expression of DDR1, Bcl2 and Bax in MMQ cells. *P*-values are two-sided and 0.05 was adopted as the significance level.

3 Results

3.1 Patient characteristics of the cohort

42 prolactinomas specimens were retrospectively studied which had sufficient material for standard hematoxylin & eosin staining and IHC, including samples from 12 males and 30 females (median age 41 years, range 14–62 years). The tumor sizes ranged from 0.06–15.66 cm^3 (mean $\pm$ SD = $3.05 \pm 4.26 \text{ cm}^3$). Based on the Knosp classification, the samples were divided into 21 invasive PA (IPA) cases and 21 non-invasive PA (nIPA) cases. Patient characteristics are provided in Table 1. High D2R expressing specimens (H-Score>150) consisted of 12/21 IPA cases and 19/21 nIPA cases ($\chi^2 = 6.035$, $p = 0.014$). High ER expressing specimens (H-Score>150) consisted of 6/21 and 19/21 IPA and nIPA cases, respectively ($\chi^2 = 16.70$, $p = 0.001$). Recurrence was found in 5/21 IPA cases and 2/21 nIPA cases ($\chi^2 = 1.543$, $p = 0.214$).

3.2 Expression profile of prolactinomas

Compared to normal pituitary tissue, 4,225 genes and 151 miRNAs were abnormally expressed ($p < 0.05$ and FDR both < 0.01 , with fold change > 2.0 or < 0.5) in prolactinoma specimens. Among the significant differentially expressed RNAs, 581 displayed a fold-change > 10 , including 330 upregulated mRNAs and 251 downregulated mRNAs. Among the differentially expressed miRNAs, 68 displayed a fold-change > 10 , including 22 upregulated miRNAs and 46 downregulated miRNAs. The association of miRNAs and their target genes was verified by databases using MicroT v5.0, MicroT-v4, TargetMiner, miRTarBase, TargetScan, TarBase and MiRDB databases. Gene ontology analysis indicated that the biological functions of differentially expressed proteins were related to an amacrine cell differentiation, regulation of transcription regulatory region DNA binding, cell type specific apoptotic process, neural retina development and cell fate commitment. Gene pathway analysis showed that a calcium signaling

Table 1: The clinicopathological data in 42 prolactinomas cases.

Category		Prolactinoma		
		Non-Invasive	p-Value	
Cases		21	21	
Age	Range	14-57	18-62	p>0.05
	Mean±SD	31.3±10.7	34.8±13.2	
Sex	Males	10	5	p=0.107
	Females	11	16	
PRL	(ng/ml)	1138±237	682±317	p<0.05
Tumor size	<3.05cm3	7	14	p=0.031
	≥3.05cm3	14	7	
D2R	H-Score<150	9	2	p=0.014
	H-Score≥150	12	19	
ERα	H-Score<150	15	2	p=0.000
	H-Score≥150	6	19	
Recurrence	Yes	5	2	p=0.214
	No	16	19	

pathway, melanogenesis and a thyroid hormone signaling pathway were involved in pathogenesis and cell differentiation (Figure 1). After bioinformatics analysis, we found 12 microRNA and 35 target genes involved in the tumor genesis and invasive behavior of prolactinomas.

3.3 Validation of candidate genes using RT-qPCR

5 miRNAs and 12 target genes confirmed the difference of the PCR experiment among 20 prolactinomas and 5 pituitary tissues. miRNA-13p, miRNA-137, miRNA-142-3p, miRNA-146-5p and miRNA-199a-5p were downregulated to 0.068 fold, 0.039 fold, 0.582 fold, 0.532 fold and 0.121 fold, respectively, compared to normal pituitary tissue (Figure 2A). In our animal model, miRNA-13p, miRNA-137 and miRNA-199a-5p were downregulated to 0.143 fold, 0.435 fold and 0.224 fold, respectively, compared to normal rat pituitary (Figure 2B).

Statistically significant differences were observed between miRNA-199a-5p target genes mRNA levels in the invasive groups compared to those in the non-invasive groups (Figure 2C). There were significant statistical differences of the mRNA levels of *DDR1*, *SACS* and *SAT1*

in the rat prolactinoma model compared to those of the sham group (Figure 2D). There were no differences in miRNA-13p target genes between invasive and non-invasive tumors although there were differences between tumors and normal pituitaries.

3.4 The association between miRNA-199a-5p target gene level and clinicopathologic parameters

To ensure the role of *DDR1* and *SAT1* in prolactinomas, we analyzed the relationship between H-scores of *DDR1* and *SAT1* staining and clinicopathologic features (Figure 3A). The number of *DDR1* and *SAT1* elevated cases (H-Score>150) were 18/21 and 12/21 in the IPA group, and 11/21 and 5/21 in the nIPA group ($\chi^2=5.459$, $p=0.019$; $\chi^2=4.842$, $p=0.028$). The levels of *DDR1* and *SAT1* in IPAs were 2.1 fold and 1.9 fold higher, respectively, than in nIPAs by western blot analysis (Figure 3B). In addition, *DDR1* elevated cases were more prevalent in males (13/15) than in females (16/27; $\chi^2=3.389$, $p=0.066$), while *SAT1* high level cases were more frequent in males (9/15) than in females (8/27; $\chi^2=3.692$, $p=0.055$). There was no statistic difference found between tumor

Figure 1: Gene Ontology analysis of prolactinomas. A: Differentially expressed mRNAs (fold change > 2 and false discovery rate $q < 0.1$) between the invasive and non-invasive groups of tissue were analyzed using hierarchical clustering. Each row represents a single mRNA and each column represents an individual sample. Red indicates relatively high expression and green indicates relatively low expression. B: KEGG pathways. KEGG pathway analysis showed the calcium signaling pathway, melanogenesis and the thyroid hormone signaling pathway were involved in pathogenesis and cell differentiation.

Figure 2: A novel miRNA–gene network regulating prolactinoma formation in patients and animals. RT-qPCR analysis of five miRNAs and 12 target genes confirmed changes in mRNA levels. A: Five miRNAs from prolactinoma specimens. B: Similar changes in miRNA-1-3p, miRNA-137 and miRNA-199a-5p in the animal model. C: mRNA levels of miRNA-199a-5p target genes in prolactinomas. D: mRNA levels of miRNA-199a-5p target genes in the animal model.

Figure 3: Analysis of miRNA-199a-5p target genes in prolactinoma specimens. A: IHC analysis of DDR1 and SAT1 expression in prolactinomas. Bar=60 μ M. B: Western blotting analysis of DDR1 and SAT1 expression in prolactinomas.

size and either DDR1 ($\chi^2=2.785$, $p=0.095$) or SAT1 ($\chi^2=2.471$, $p=0.116$) in supplementary Table 1.

Through a TargetScan database, we found that miRNA-199a-5p binds the 3'UTR of DDR1 and SAT1 (Figure 4A). We measured the DDR1 and SAT1 promoter methylation levels in prolactinomas. The DDR1 promoter (chr6:30860565-30861035) methylation levels were 58.1% in the IPA group and 78.4% in the nIPA group ($p=0.001$). The SAT1 promoter (chrX: 23802619-23803387) methylation levels were 30.6% in the IPA group and 59.6% in the nIPA group ($p=0.04$). We also observed downregulation of DDR1 and SAT1 after miRNA-199a-5p overexpression in MMQ cells whether the mRNA or protein levels (Figure 4B and 4C). We also checked the mRNA level of D2DR and PRL related to the effect of DAs treatment in prolactinomas. The mRNA level of D2DR in miRNA-199a-5p group was 0.18 ± 0.07 folds of that of miRNA-199a-5p-NC group ($p<0.05$). There was no statistical difference of SACS protein after miRNA-199a-5p transfection.

Figure 4: miRNA-199a-5p controlled the expression of DDR1 and SAT1 in MMQ cells. A: miRNA-199a-5p targeted the 3'UTR of DDR1 and SAT1. B: RT-qPCR data from target genes of miRNA-199a-5p and genes related to DAS treatment. C: Western blots of DDR1 and SAT1. * compared to miRNA-199a-5p-NC n=3.

3.5 miRNA-199a-5p could affect cell viability and induce the apoptosis in MMQ cell line

miR-199a-5p plays the antitumor role in esophageal cancer, bladder cancer, head and neck cancer [20–22]. After transfection of miRNA-199a-5p into MMQ cells, assessment of cell viability suggested that miRNA-199a-5p inhibited MMQ cell viability compared to the vector miRNA-199a-5p-NC group (Figure 5A). Significant inhibition of PRL secretion was observed at 72 h (Figure 5B). Annexin positive percent was 13.4% in miRNA-199a-5p group and 3.8% in NC group. PI positive percent was 8.3% in miRNA-199a-5p group and 2.2% in NC group. Statistical assays showed that Annexin V-positive cell was significantly elevated after transfection of miRNA-199a-5p (Figure 5C). Quantitative analysis of Bax and Bcl2 protein

expression was performed using a scanning densitometer. The Bcl2/Bax ratio was increased more than fivefold after transfection of miRNA-199a-5p than that of NC group (Figure 5D).

4 Discussion

The differential miRNA expression modified the phenotypes of cancers. miRNAs played the key role in chemotherapy resistance, and could be manipulated, either alone or in combination to improve the treatment response and the increase in cure rates. The mechanisms of miRNA were involved in apoptosis, proliferation, differentiation, invasion, metabolism and angiogenesis. In this study, we found numerous deregulated miRNAs

Figure 5: miRNA-199a-5p acted as an anti-tumor miRNA in MMQ cells. A: The effect on cell viability by MTS experiment. B: The effect on PRL secretion. C: The effect on apoptosis assessed by the Annexin V & PI apoptosis detection kit with MARK II. Green: GFP; Orange: PI; Red: Annexin V. D: Western blots of Bcl-2 and Bax.

in prolactinomas, and confirmed changes in five miRNAs and 12 target genes at the mRNA level. Gene ontology analysis indicated that the biological functions of differentially expressed proteins were related to amacrine cell differentiation, regulation of transcription regulatory region DNA binding, cell type specific apoptotic process, neural retina development and cell fate commitment.

Functional studies showed that miRNA-199a-5p inhibited cell proliferation and activated the apoptosis in the MMQ cell line, suggesting a critical role in prolactinomas tumorigenesis. The miRNA-199 family members were encoded within introns of the dynamin gene in the opposite orientation to the host gene [23]. As tumor suppressors, miRNA-199a-3p significantly reduced the level of CD44 in osteosarcoma [24]. miRNA-199a-3p could inhibit the level of Smad1 which played the antagonistic role to miRNA-199a-3p on prostate cancer cells [25]. miRNA-199a-3p played the inhibitor role of metastasis, migration and angiogenesis of hepatocellular carcinoma through reducing the VEGF secretion and VEGFR1/2 levels of interstitial cells [26]. In the current study, *DDR1*, *KLHL29*, *SACS* and *SAT1* were verified as target genes of miRNA-199a-5p including MicroT V5.0, MicroT-v4, TargetMiner, miRTarBase, TargetScan, TarBase and MiRDB. The mRNA levels of miRNA-199a-5p's target genes were further investigated between patient specimens and animal model specimens. In the current study we found *DDR1*-positive cases in 18/21 of IPA patients and in 11/21 of nIPA patients. Compared to normal pituitary, there were

statistical differences between *DDR1* and *SAT1* levels in both patient prolactinomas and animal models according to RT-PCR and western-blot experiment. We speculated the main target genes of miRNA-199a-5p were *DDR1* and *SAT1*, partly in prolactinomas.

DDRs, comprised of *DDR1* and *DDR2*, are receptor tyrosine kinases and regulate important aspects of cellular processes, for example, cell proliferation, cell migration, adhesion and extracellular matrix remodeling [27,28]. *DDR1* was first shown in *Dictyostelium discoideum* to be activated by various types of collagen [29]. As murine leukemia kinase inhibitors, some of them were found to inhibit *DDR* kinase activity [30]. Hypoxia increases the level of *DDR1* and the secretion metalloproteinase 2 (*MMP-2*) and *MMP-9*, then promotes PA cell proliferation and cell invasion [31, 32]. miRNA-199a-5p overexpression down regulates the levels of *DDR1*, *MMP2*, *N-cadherin* and *vimentin* and down regulates the level of *E-cadherin* in colorectal cancer cell lines [33]. In our experiment, miRNA-199a-5p obviously reduced cell viability and induced the reversal of *Bcl2/Bax* rate in MMQ cells. Flow cytology showed that the Annexin V positive cell rate increased more than twofold after miRNA-199a-5p overexpression. miRNA-199a-5p also down regulated the protein level of *DDR1* of MMQ cells. We hypothesized that miRNA-199a-5p would inhibit the protein synthesis of *DDR1* by targeting the 3'UTR of *DDR1* mRNA and increasing the degradation of *DDR1* mRNA according to the TargetScan database. We also found *SAT1* was consistently associated

with the invasiveness of prolactinomas. miRNA-199a-5p overexpression inhibiting the protein levels of DDR1 in MMQ cells.

Reduction of dopamine receptor subtype 2 (DRD2) may cause the DA resistance in prolactinomas [34]. The miRNA-9 expression showed a negative correlation with the DRD2 gene and its short isoform which led to an increase in PRL synthesis and secretion [35]. SACS was rich between the central nervous system and skeletal muscles, however poor in the pancreas. Mutations of the SACS gene were associated with Charlevoix-Saguenay syndrome [36]. SACS impair the function of intermediate filaments in many cell lines [37]. SAT1, a limiting enzyme of polyamine catabolism, possessed the tumor-suppressive properties in H1299 cells xenograft [38], growth arrest of HeLa cells [39] and apoptotic cell death in glioblastoma cells [40]. We also found the SAT1 was associated consistently with the invasiveness of prolactinomas. Our result indicated that there was a negative correlation between SAT1 and apoptosis because miRNA-199a-5p could inhibit SAT1 expression and induce the apoptosis of MMQ cells.

In conclusion, miRNAs play a key role in prolactinoma tumorigenesis, progression and aggressiveness. Dysregulation of miRNA-199a-5p results in aberrant overexpression of target genes such as DDR1 and SAT1 which could induce the hyperproliferation of pituitary cells. However, much remains to be learned in order to fully understand the mechanisms of the onset, progression and drug resistance of prolactinomas.

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