

Novel Biomarkers for Non-functioning Invasive Pituitary Adenomas were Identified by Using Analysis of microRNAs Expression Profile

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Abstract The microRNAs (miRNAs) are involved in multiple pathological processes among various types of tumors. However, the functions of miRNAs in benign brain tumors are largely unexplored. In order to explore the pathogenesis of the invasiveness in non-functional pituitary adenoma (NFPA), the miRNAs expression profile was analyzed between invasive and non-invasive non-functional pituitary adenoma by miRNAs microarray. Six most significant differentially expressed miRNAs were identified including four upregulated miRNAs hsa-miR-181b-5p, hsa-miR-181d, hsa-miR-191-3p, and hsa-miR-598 and two downregulated miRNAs hsa-miR-3676-5p and hsa-miR-383. The functions and corresponding signaling pathways of differentially expressed miRNAs were investigated by bioinformatics techniques, including Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. The result of GO analysis indicates regulation of voltage-gated potassium channel activity, positive regulation of sodium ion transport, positive regulation of GTPase activity, negative regulation of Notch signaling pathway, etc. KEGG pathway reveals a series of biological processes, including prolactin signaling pathway, endocrine and other factor-regulated calcium reabsorption, fatty acid metabolism, neuroactive ligand-receptor interaction, etc. The miRNAs hsa-miR-181a-5p was verified by quantitative real-time PCR, and the expression level was in accordance with the microarray result. Our result can

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provide the evidence on featured miRNAs which play a prominent role in pituitary adenoma as effective biomarkers and therapeutic targets in the future.

Keywords microRNAs · Expression profile · Invasive pituitary adenoma · Non-functioning pituitary adenoma · Pathogenic mechanism

Introduction

Pituitary adenoma (PA) is the most frequent tumor in the sellar area, with the incidence rate after glioma and meningioma. The prevalence of pituitary adenoma in the general population is approximately 17% (Molitch 2017; Fernandez et al. 2010). Pituitary adenomas can be divided into three types according to their biological function: benign adenoma, invasive pituitary adenoma, and pituitary carcinoma. Invasive pituitary adenoma (IPA) is a type of pituitary tumor between the benign adenoma and carcinoma (Raverot et al. 2014), which shows the growth characteristics of malignant tumors such as violation of the sellar base, dura mater, and cavernous sinus, although they are mostly benign in histology (Ostrom et al. 2013). Therefore, the clinical diagnosis of IPA is mainly by using Computed Tomography (CT) and Magnetic Resonance Imaging (MRI) imaging combined with Wilson-Hardy and Knosp classification and staging criteria (Knosp et al. 1993; Wilson 1984). The pathogenesis of IPA is mainly indistinct, which is the primary reason for poor overall therapeutic effect of IPA, resulting in difficulties in effective clinical treatment of IPA and prevention of recurrence. Although molecular markers of pituitary adenomas have been identified in histology, early prediction of IPA recurrence remains considerably arduous.

Because of the complicated pathogenesis of pituitary adenoma, it was believed that the risk factors include genetic events, hormonal stimulation, and growth factors in general; these three factors can promote tumor cell growth and proliferation, in which hereditary factors are the main factors (Ezzat and Asa 2006). The miRNAs are the small RNA molecules which are involved in regulation of cell proliferation, differentiation, and other physiological processes in the genetic factors (Krol et al. 2010; Hata and Lieberman 2015). There are classes of single-stranded, non-coding small RNAs consisting of 19–25 nucleotides, which are usually found regulating target genes expression after transcription (Ameres and Zamore 2013). They are bound to the 3'UTR, 5'UTR, or coding sequence of the target gene mRNAs by either complete or incomplete base pairing, by either degrading mRNAs, or interfering mRNAs translation process to suppress target gene expression, which negatively modulates the expression level of the corresponding proteins participating in most physiological processes like body growth and development (Bartel 2009).

The miRNAs are designated to play critical roles in the pathophysiological processes of a wide range of diseases, such as cardiovascular diseases, respiratory diseases, infectious diseases, and various types of tumor (Bottoni et al. 2007; Dumache et al. 2015; Horhat et al. 2017). The differential expression of miRNAs plays a comprehensive part in the occurrence and suppression of tumor and the early

prevention and prediction of cancer (Weber et al. 2010; Nitu et al. 2017). However, the functions of miRNAs in pituitary adenomas remain elusive. In a recent study, the miRNAs have been proposed as an ideal biomarker which contributes to the primary diagnosis, prognosis, postoperative follow-up, and early prediction of postoperative recurrence for pituitary adenoma (Melmed 2011). Weber et al. have indicated a definite correlation between miRNAs expression levels and clinical features such as tumor sizes, hormone secretion types, efficacy of drug treatment, and recurrence of pituitary adenoma (Weber et al. 2010). It was also suggested that miRNAs could be closely related to the invasive growth of IPA, despite the fact that the underlying mechanism is not completely understood.

We have identified differential DNA methylome profiling between invasive and non-invasive non-functioning pituitary adenomas (Gu et al. 2016). Furthermore, the aim of this study is to expound the pathogenesis of the invasiveness in pituitary adenomas by investigating the miRNAs expression profile of invasive and non-invasive non-functional pituitary adenoma, to further probe innovative biomarkers for diagnosis of pituitary adenoma, and provide fundamental evidences to guide clinical application for novel drug treatment of pituitary adenoma in combination with surgical treatment and radiotherapy.

Materials and Methods

Clinical Samples

Non-functional pituitary adenoma tissue samples were collected from patients who underwent endoscopic surgery in the Department of Neurosurgery of Affiliated Zhongshan Hospital of Fudan University, from May 2014 to July 2015. All tumor specimens were divided into two groups with ten cases, respectively, including invasive (non-functional) pituitary adenoma and non-invasive (non-functional) pituitary adenoma. Of the 20 PAs, 12 adenomas were included in the discovery cohort, and 8 adenomas were included in an independent cohort. The invasive pituitary adenoma was diagnosed by radiology and pathology if any of following criteria were present: (1) Wilson-Hardy classification III, IV, V, or D, E stage and/or Knosp grade 4; (2) the tumor was confirmed by saddle bone destruction and through violations of adjacent structures observed in the surgery, such as sphenoid sinus cavity and/or cavernous sinus and/or parasellar region, etc. The mean ages of patients were 53.1 (range 31–75 years old) and 58.3 (range 36–81 years old) in invasive and non-invasive pituitary adenoma group, respectively, in which 12 were males and 8 were females. There was no significant difference in age and gender between the two groups. The subtypes of tumor in pathological diagnosis were null cell, silent Growth Hormone (GH), and silent adreno-cortico-tropic-hormone (ACTH) types. The tumor tissues were obtained with informed consent from the patients, and all studies were performed with the approval of the Regional Ethics Committee at Zhongshan Hospital of Fudan University. The detailed information of patients is listed in Table 1.

Table 1 The clinical data of patients and tumor characteristic in this study

Number	Name	Gender	Age	Pituitary function	Diameter (cm)	Wilson-Hardy	Knosp	Pathology	ki-67
1	FSF	F	53	Dysfunction	8.5	C, IV	3	Null cell	1
2	HXH	F	62	Dysfunction	6.8	D, IV	4	Null cell	5
3	ZXB	M	42	Dysfunction	3.3	C, IV	4	Null cell	2
4	YZM	F	67	Normal	4.1	E, IV	4	Null cell	2
5	LKE	F	65	Dysfunction	5.2	E, IV	3	Null cell	2
6	ZXY	F	54	Normal	4.2	E, IV	3	Silent ACTH	10
7	LHS	M	32	Normal	3.2	C, II	4	Null cell	3
8	ZJY	F	75	Normal	3.8	E, IV	3	Null cell	6
9	LN	M	31	Normal	4.4	D, III	4	Null cell	15
10	GX	F	50	Normal	3.5	E, II	4	Null cell	1
11	XLH	F	77	Normal	2	A, II	2	Null cell	20
12	YBG	M	61	Dysfunction	2	A, I	2	Null cell	2
13	WXY	M	36	Dysfunction	2.5	B, II	1	Null cell	2
14	YXZ	F	54	Normal	2.2	0, II	1	Silent ACTH	1
15	SJH	F	59	Normal	1.9	A, II	1	Null cell	2
16	WL	F	55	Normal	1.7	A, II	1	Silent ACTH	1
17	LDT	M	81	Dysfunction	2.8	A, II	1	Null cell	2
18	ZJC	F	59	Normal	3.2	B, II	2	Null cell	1
19	LYF	M	42	Dysfunction	2.5	A, I	2	Silent GH	3
20	MWR	M	59	Normal	2.2	B, II	2	Null cell	5

Patient 1–10 were invasive (non-functional) pituitary adenomas, in which 1–6 were discovery cohort and 7–10 were independent cohort

Patient 11–19 were non-invasive (non-functional) pituitary adenomas, in which 11–16 were discovery cohort and 17–20 were independent cohort

Immunohistochemistry

All tissue specimens were embedded in paraffin for 5 μ m serial sections. Some sections were stained according to the instructions in the SABC kit (Boster, China). The first antibody was anti-human ki-67 rabbit polygonal antibody (Abcam, USA). The others were stained with Hematoxylin/Eosine (HE). The images were captured under a SP5/Leica confocal microscope with LAS AF Lite software.

Total RNA Extraction, Purification, and Marking

The total RNA was extracted and purified from the tumor tissues (50–100 mg) on RNeasy columns according to the manufacturer's protocols (Qiagen, Germany). The

purity and concentration of RNA were quantified by NanoDrop ND-2000, and the integrity of RNA was determined by denaturing agarose gel electrophoresis in each sample. Then, 50–100 μg of total RNA was isolated by using the Ambion miRNAs Isolation Kit (Thermo Fisher Scientific, USA). CU-cy3 and CU-cy5 (Dharmacon, USA) were labeled with T4 RNA ligase, respectively, and then precipitated with absolute ethanol and dried for chip hybridization.

microRNAs Microarray

Agilent human miRNAs ($8 \times 60 \text{ K}$) V19.0 was used for miRNAs microarray in this study. For detailed probe data of the human genome chip, refer to the website (<http://www.shbio.com>). The chip is based on UniGene v.175 database, using light-controlled in situ synthesis of DNA microarrays patented technology for the development of high-specific design of the photo-etching in situ synthesis of oligonucleotide microarrays and unique PM -MM probe. One square centimeter area can be arranged to probe synthesis area (“point”) from hundreds of thousands to one million. microRNAs microarrays, hybridization scans, and data analyses were performed at the Technical Services Department (Biotechnology Corporation, China). GeneSpring GX (AT, version 11.5) was used to analyze the differentially expressed genes.

Pathway Enrichment Analysis for Differentially Expressed Genes (DEGs)

In order to analyze the DEGs at the functional level, enrichment analysis of Gene Ontology (GO, <http://www.geneontology.org>), Kyoto Encyclopedia of Genes and Genomes (KEGG, <http://www.genome.jp/kegg/>) pathway, and Pathway Commons (<http://www.pathwaycommons.org>) for the interesting genes was performed by DAVID (The Database for Annotation, Visualization and Integrated Discovery), the online software.

Real-Time Quantitative PCR

For validating the results of miRNAs microarray, we selected two upregulated genes (hsa-miR-181a-5p and hsa-miR-191-3p) which were significant differentially expressed in miRNAs chip analysis. Total RNA (100 ng) of the tumor tissue was transcribed into cDNA by TaqMan[®] microRNAs Reverse Transcription Kit (Life technology, USA) according to the instruction. We detected their expression levels in 10 invasive pituitary adenomas (IPA) and 10 non-invasive pituitary adenomas (NIPA) by qRT-PCR (Applied Biosystems 7900HT Fast Real-Time PCR System, USA) using TaqMan[®] microRNAs Assay quantitative PCR kit according to the manual (Applied Biosystems, USA). The fold change of each gene in expression level was calculated by $2^{-\Delta\Delta C_t}$ method. U6 snRNA was served as an internal standard control.

Statistical Analysis

All experimental data were presented as the $x \pm s$. One-way ANOVA was used for SPSS22.0 statistical software and *t* test for independent samples. The difference was statistically significant when $p < 0.05$. We selected $p < 0.01$ for the difference was statistically significant in the bioinformatics pathway enrichment analysis.

Results

Histopathological Examination

The mean diameter of the tumor was 4.7 cm in IPA group and 2.3 cm in NIPA group. The histological type of all tumor specimens was defined according to pathological criterion by staining with Hematoxylin/Eosine and ki-67 (Fig. 1). The ki-67 is a nuclear protein encoded by the MKI-67 gene, which is closely related with tumor differentiation, invasion, metastasis, and prognosis. The mean of the proportion of ki-67-immunopositive cells in the specimen was 4.7% in IPA group and 3.9% in NIPA group. There was no significant difference in the percentage of ki-67-immunopositive cells between the two groups.

Sample Clustering Correlativity Analysis of miRNAs Microarray

After homogenization and log conversion, the original data of a single fluorescent chip were drawn as Box–Whisker plot to assess the distribution and dispersion of chip hybridization data. The dispersion and symmetry of the data distribution were substantially homogeneous in each group (Fig. 2a). We found that non-functional invasive and non-invasive pituitary adenoma samples were roughly separate from miRNAs gene clustering analysis in each group of six samples (Fig. 2b). There are also interspersed samples, suggesting that these samples share certain similar biological functions (Fig. 2c). Nevertheless, the samples in each group were related but completely different; and that the correlation was very low or even absent between the groups, indicating that the sample grouping was considered precise and credible.

Six Significant DEGs were Identified from Analysis of miRNAs Microarray Results

A total of 2006 differentially expressed miRNAs were detected by microarray analysis from all 12 tumor samples. Fold change and *p* value are calculated via *t*-test analysis between groups from microarray results and drawn into volcano plot. In order to find out the most significant differentially expressed miRNAs between invasive and non-invasive non-functional pituitary adenomas, we first screened 10 differentially expressed miRNAs using the criteria of *p* value < 0.05 and fold change > 2 or < 0.5 from the volcano plot (Fig. 2d). Using more rigorous standards referred in the articles, we finally screened 6 most significant differentially

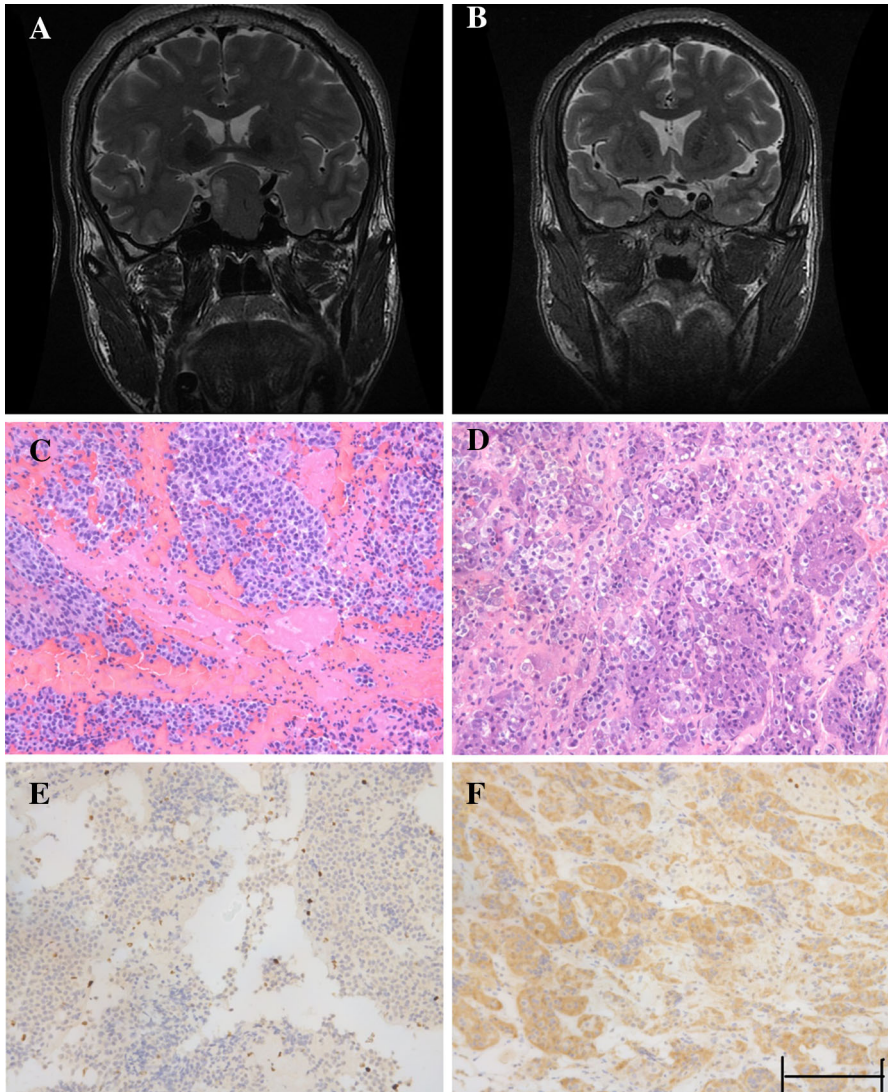


Fig. 1 MRI imaging, HE, and Immunohistochemical characterization of tumor. **a** The MRI imaging of the ninth patient, male, 31 years old. The MRI image of invasive non-functional pituitary adenoma showed that the tumor encroached left cavernous sinus, internal carotid artery, and sphenoid sinus. **b** The MRI imaging of the fifteenth patient, female, 59 years old. The MRI image of non-invasive non-functional pituitary adenoma displayed that the tumor was small and did not encroach the surrounding tissue. **c** and **d** Hematoxylin/Eosine staining of the tumor tissues. Both tumors appeared as nuclear atypism and mitotic figures. The ninth surgical specimen (**c**) had more prominent nuclear atypism and mitosis than the fifteenth surgical specimen (**d**). **e** and **f** Immunohistochemical staining of ki-67 in the ninth (**e**) and 15th (**f**) specimens. Both tumors presented positive staining of ki-67. The proportion of ki-67-immunopositive cells of the ninth specimen was 15%, while the proportion of ki-67-immunopositive cells of the fifteenth specimen was 2%. Bar 300 μ m

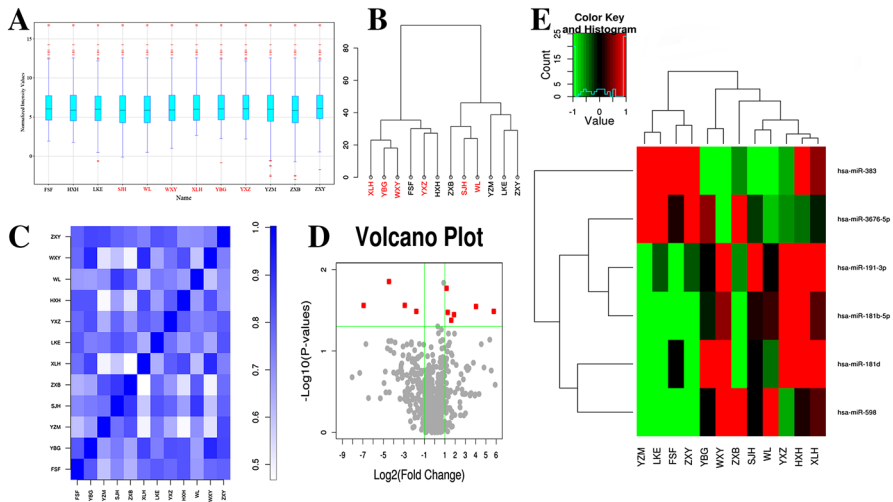


Fig. 2 microRNA chip sample clustering correlativity analysis. **a** The Box–Whisker plot generated by miRNAs microarray results showed that the 12 samples are symmetrical and distributed in a decentralized mode in this experiment, where the Y-axis means the normalized intensity value. **b** Clustering analysis of samples revealed that most samples of the same group appeared in the same or close cluster with similar biological function, in which the invasive samples were abbreviated in *black* and the non-invasive samples were abbreviated in *red*. **c** Linear correlation between the two groups of samples by correlativity analysis with sample correlation coefficients. **d** Volcano plot analysis for microRNAs chip data screening. This figure was plotted on p value and Fold Change values by intergroup t test. Gray data points are all the distribution of differentially expressed miRNAs; *red* data points are significant differentially expressed miRNAs selected under the criteria of $p < 0.05$ and fold change >1 or <-1 . **(e)** Hierarchical clustering map of differentially expressed miRNAs. Six miRNAs with significant difference in expression were selected according to $p < 0.05$ and Fold Change >2 or <0.5 (Color figure online)

expressed miRNAs (including up or down regulation) which were subjected to the condition of $p < 0.05$ and fold change >2 or <0.5 (Table 2). We further analyzed these six most significant differentially expressed miRNAs by hierarchical clustering method (Fig. 2e). We found that miRNAs expression levels showed obvious color distinction and clusters. The results indicated that there were significant differences in the expression of certain groups of miRNAs between the invasive and non-invasive non-functional pituitary adenomas.

Table 2 Significant differential expression of miRNA in IPA

microRNA	P values	Fold change	Active sequence	chr	Regulation
hsa-miR-181b-5p	0.016985	2.243886579	ACCCACCGACAGCA	chr1	Up
hsa-miR-181d	0.041912	3.07968363	ACCCACCGACAACAATG	chr19	Up
hsa-miR-191-3p	0.035749	3.698528557	GGGACGAAATCCAAG	chr3	Up
hsa-miR-3676-5p	0.032659	0.290234102	AACCCAGGATCTCT	chr17	Down
hsa-miR-383	0.027498	0.133212993	AGCCACAATCACCTTCT	chr8	Down
hsa-miR-598	0.033557	2.428911794	TGACGATGACAACGATGAC	chr8	Up

Functional Annotation Analysis of miRNAs

According to GO (Gene Ontology) analysis, the six differentially expressed miRNAs genes were divided into different types of functions including regulation of cell proliferation and differentiation of development and apoptosis process, metabolism, ion channel transport, and other major cellular functions. The functions of these miRNAs are to modulate the activity of voltage-gated K⁺ channels, regulate the transport of Na⁺, regulate the activity of GTPase, negatively regulate Notch signaling pathway and cytochromatosis, etc. (Fig. 3a).

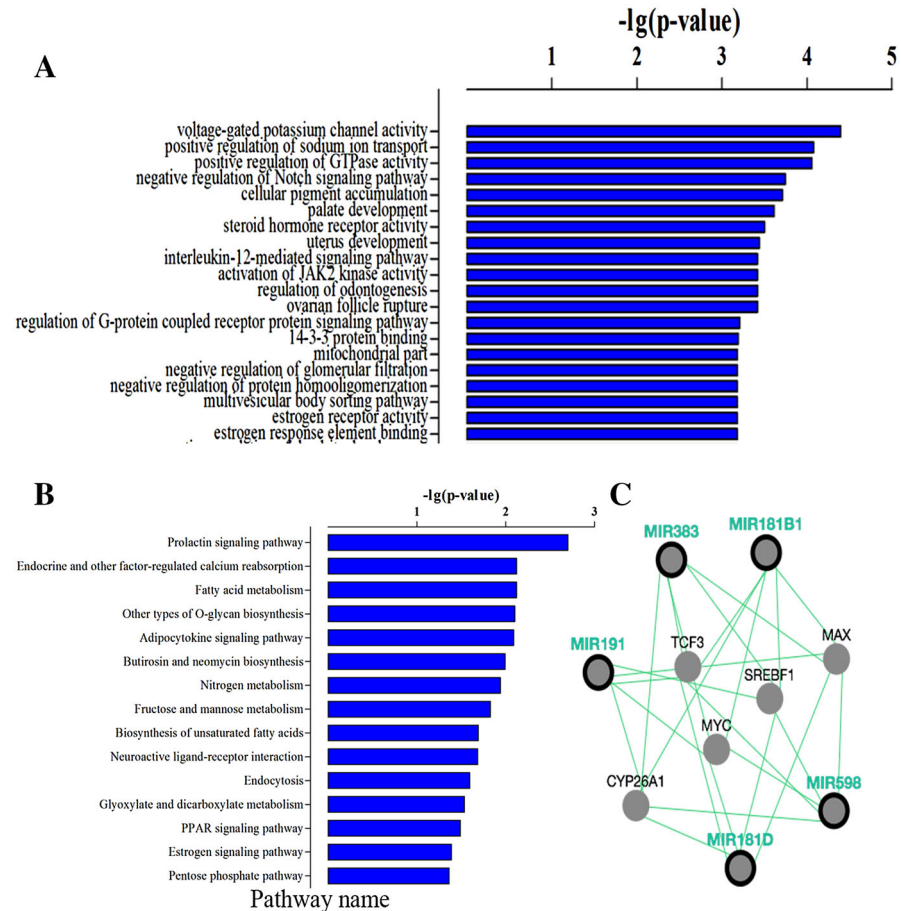


Fig. 3 Functional annotation and pathway analysis of significant differentially expressed miRNAs. **a** Functional annotation analysis for the six significant differentially expressed miRNAs was screened by GO analysis. This showed the most significant ten enriched functions. **b** KEGG signaling pathway analysis of the six significant differentially expressed miRNAs. The 15 most enriched significant signaling pathways were involved. **c** Related network analysis of significant differentially expressed miRNAs by pathway commons. The bone nodes presented those genes closely involved in the significant differentially expressed miRNAs pathways, and the relations between target genes and miRNAs are shown in green lines (Color figure online)

miRNAs Signaling Pathway Analysis

We found that these miRNAs participated in several signal transduction pathways, including prolactin signal transduction pathways, endocrine and other factors regulating calcium reabsorption, fatty acid metabolism, neurotransmitter-ligand interaction by endocrine function, etc. (Fig. 3b). To construct the related network of these miRNAs in invasiveness of pituitary adenomas, the correlation of genes was analyzed by using the Pathway Commons (Fig. 3c). The following six genes TCF3, CYP26A1, MYC, SREBF1, and MAX were found to be involved in the signaling pathways of the selected six most differentially expressed miRNAs between invasive and non-invasive non-functional pituitary adenomas. In addition, the KEGG pathway analysis of these six involved genes showed overlapping results with the pathways of six most differentially expressed miRNAs.

Real-Time Quantitative PCR Validation of Two Significantly Altered DEGs

Among the total 6 most significant differentially expressed miRNAs, hsa-miR-181b-5p, hsa-miR-181d, hsa-miR-191-3p, and hsa-miR-598 were significantly upregulated in non-functional invasive pituitary adenoma. We selected 3 miRNAs (hsa-miR-181b-5p, hsa-miR-181d, hsa-miR-191-3p) from the above 6 miRNAs for further validation. Since we only have miRNA-181a-5p in the miRNAs probe library, which is produced by the same precursor and is highly isogenic to the sequences of hsa-miR-181b-5p and hsa-miR-181d, and also upregulated in invasive non-functional IPA (with $p = 0.014$, fold change = 1.84507654), we chose miRNA-181a-5p instead of hsa-miR-181b-5p and hsa-miR-181d to verify the microarray results. The expression levels of hsa-miR-181a-5p and hsa-miR-191-3p were relatively quantified by qRT-PCR. The relative expression of RT-PCR was calculated as: $\text{miRNAs relative expression difference} = 2^{-\Delta - \{[(\text{invasive group miRNAs})_{\text{ct}} - \text{U6}_{\text{ct}}] - [(\text{non-invasive miRNAs})_{\text{ct}} - \text{U6}_{\text{ct}}]\}}$. According to the validation data (Fig. 4), both hsa-miR-181a-5p and hsa-miR-191-3p showed increased expression level in invasive non-functional pituitary tumors, which is consistent with microarray analysis. The expression level of hsa-miR-181a-5p was significantly different between the two groups.

Discussion

Pituitary adenoma is the third common intracranial tumor which can cause remarkable morbidity. The vast majority of pituitary adenoma is benign tumor in both pathology and radiology (Theodros et al. 2015). IPA is generally characterized by rapid tumor growth, invasion, and destruction of the surrounding normal tissues in clinic (Theodros et al. 2015). In particular, the patient's condition tends to be delayed owing to the clinical symptoms of non-functioning IPA which are not obvious in the early stage. It is one of the hurdles in neurosurgical diagnosis and

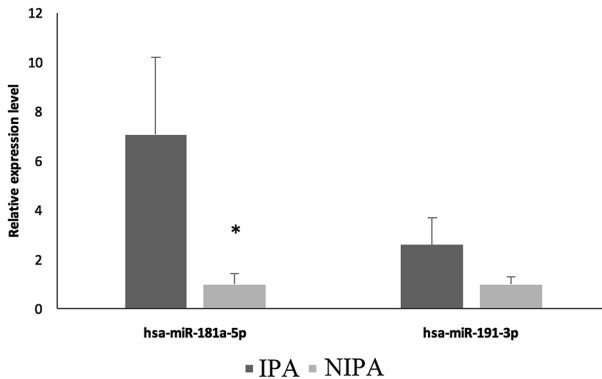


Fig. 4 Validation of differentially expressed miRNAs in the microarray by qRT-PCR. Quantitative RT-PCR results are shown as relative expression levels of the two verified miRNAs between invasive (*dark gray column*) and non-invasive (*light gray column*) pituitary adenomas. The results showed that the both miR-181a-5p and hsa-miR-191-3p miRNAs significantly upregulated in invasive pituitary tumors. The expression level of miR-181a-5p was significantly different between the two groups, and * means $p < 0.05$

treatment because of the low complete tumor resection rate and a high recurrence rate.

The miRNAs affect tumor occurrence by regulating the expression levels of target genes involved in tumor oncogenes or suppressor genes, suggesting that miRNAs may be closely linked to the pathogenesis of pituitary adenomas (Di Ieva et al. 2014). Recent studies have indicated that circulating miRNAs have become novel biomarker candidates of tumor for clinical diagnosis and treatment (Zen and Zhang 2012; Yu et al. 2011; Bartels and Tsongalis 2009). In this miRNAs microarray study, we compared six pairs of non-functioning pituitary adenoma samples that were analyzed, and we identified six significant differentially expressed miRNAs from 2006 differentially expressed miRNAs between invasive and non-invasive groups. Four miRNAs such as hsa-miR-181b-5p, hsa-miR-181d, hsa-miR-191-3p, and hsa-miR-598 were significantly upregulated in the invasive group, which may be involved in the dysregulation of oncogenes during the process of invasion. The downregulation of hsa-miR-3676-5p and hsa-miR-383 may play a significant part in the regulation of tumor suppressor genes in the invasive process of PA.

In order to further comprehend the function of miRNAs, the six significant differentially expressed miRNAs were analyzed by GO and KEGG annotation tools. This study mainly discusses the regulation of voltage-gated K^+ channels and the regulation of Notch signaling pathway in GO. Modulating the activity of voltage-gated potassium channel is the most probable function of those miRNAs predicted by GO analysis. In recent years, several studies have shown that cell membrane ion channels, especially potassium channel, play a vital role in cell signal transduction, proliferation, apoptosis, and regulation of gene expression in tumor (Jimenez-Perez et al. 2016; Kahanovitch et al. 2014). It is presumed that the voltage-gated potassium channels are closely related to the occurrence of pituitary adenoma, but

the specific mechanism is still unclear, and the relationship between tumor-related genes, cell membrane ion channel regulator, and the invasiveness, occurrence, and recurrence of pituitary adenoma needs to be further studied (Xu et al. 1999; Mene and Pirozzi 2010).

Notch signaling pathway is a signal transduction system. Notch receptors play a significant role in signal transduction, regulation of cell proliferation, differentiation, and apoptosis after binding to their ligands. There is poor research on the relationship between Notch signaling pathway and pathogenesis of pituitary adenoma. Moreno et al. found that Notch3 activation can maintain the undifferentiated state of tumor cells, thus more likely to induce tumor cells to diversify (Moreno et al. 2005). Miao et al. have shown that mRNAs and protein levels of Notch3 in NFPA are higher than normal pituitary (Miao et al. 2012). It can be possible to conclude that the high expression level of Notch pathway plays a momentous role in the development of non-functional pituitary adenoma.

Regarding the results from KEGG pathway analysis of the six miRNAs, several studies have found that the gland hormone signaling pathway in the peripheral circulation and pituitary paracrine factor disorder can lead to pituitary cell proliferation, which develops into tumors (Beck-Peccoz et al. 2013). LeRiche et al. reported that various types of pituitary adenomas expressed higher level of EGF and EGFR than the normal pituitary tissues, particularly in the GH-secreting and non-functional invasive pituitary adenomas, suggesting that EGF–EGFR interaction may promote the growth of invasive pituitary adenoma (LeRiche et al. 1996). Recent studies on TGF α have shown that transgenic mice overexpressing TGF α led to the development of PRL adenomas (Ozkaya et al. 2016). These findings suggest that TGF α may be involved in the development of pituitary adenomas that are indirectly induced by hormones. The miRNAs in this study are also considered to be involved in the prolactin signal transduction pathway. Little research has been done on the relationship between prolactin signal transduction pathway and tumor. One recent study on the relationship between prolactin and human breast cancer has shown that prolactin-related signaling pathways are involved in maintaining tumor differentiation and prognosis of patients (Hachim et al. 2016). It is needed to be further verified by numerous experiments due to the deficiency of investigations in the relationship between the prolactin signaling pathway and non-functional invasive pituitary adenoma.

In order to testify to the reliability of the miRNAs microarray results, we selected two miRNAs, hsa-miR-181a-5p (instead of hsa-miR-181b-5p and hsa-miR-181d) and hsa-miR-191-3p from the six miRNAs with most significant differential expression level, for further validation by quantitative real-time PCR. The upgraded expression levels of the two miRNAs in the invasive group verified by real-time PCR are in accordance with the results of miRNAs microarray. The expression level of hsa-miR-181a-5p was markedly different between the two groups. Chen et al. found that miR-181a-5p may be involved in regulating cell proliferation and apoptosis pathway in gastric cancer (Chen et al. 2013). Jianwei et al. revealed that miR-181a played the role of oncogene which can promote cancer cell proliferation and invasion, and inhibition of cancer cell apoptosis in osteosarcoma (Jianwei et al. 2013). It was hypothesized that hsa-miR-181a-5p was expressed differently in various malignancies, but both were

related to cell proliferation and invasive growth. In this study, hsa-miR-181a-5p was linked to the invasive growth of pituitary adenoma by verifying by RT-PCR. However, some miRNAs are more intricate and involute. The hsa-miR-191-3p is located in chromosome 3, which is the third largest chromosome in the human body and accounts for 1/15 in the human genome, which mainly carries high-density cancer-related genes (Muzny et al. 2006). Some scholars have found that expression of miR-191-3 is upregulated in breast cancer and lymph node metastasis, but the function of this miRNA has not further clarified (Wang et al. 2014). In addition, Taguchi and Murakami have found that hsa-miR-191 is expected to become a biomarker for diagnosis of diseases in the blood circulation due to its high sensitivity and stability (Taguchi and Murakami 2013). However, it still needs a large-scale study. In this study, we revealed that hsa-miR-191-3p is an upregulated expression in our samples of invasive non-functioning IPAs compared to the non-invasive group. In general, the same kind of miRNAs can be expressed differently (up or down) in different tumors, all of which are related to its dysregulated oncogenes or tumor suppressor genes. Thus, it is considerably arduous to expound the current situation of miRNAs expression profile.

In summary, the occurrence and development of pituitary adenoma are the complicated biological processes, including multiple genes, extracellular and intracellular signal transduction, dysregulation of signal transduction pathways, and a series of mutual interaction. The functions of miRNAs identified were predicted by bioinformatics, involved in the Notch and the prolactin signal transduction pathway in this study. These findings suggest that miRNAs are functional complex and may be involved in the development of non-functioning invasive pituitary adenomas. More samples and function-related research need to be further expanded. The differentially expressed miRNAs may provide a new insight for the ideal biomarkers on diagnosis of pituitary adenoma in the future by deeply investigating the role of miRNAs in the pathogenesis of pituitary adenomas.

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Compliance with Ethical Standards

Conflicts of interest The authors declare that no conflicts of interest exist.

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