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Expression and Significance of miR-134 in the Tissues of Hypophyseal Adenoma*

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ABSTRACT Objective: To detect the expression of miR-134 in normal pituitary tissue and each pituitary adenomas subtype, and to analyze the correlation between its expression level and the proliferation and invasion of non-functioning pituitary adenoma (NFPA). **Methods:** To collect 104 cases with pituitary adenoma specimens (10 for ACTH adenoma, 18 for GH adenoma, 10 for PRL adenoma, namely, 66 cases of NFPA)and 8 cases of autopsy normal pituitary and clinically relevant information. To use experimental techniques such as immunohistochemistry, quantitative real-time fluorescence PCR (qRT-PCR) and so on to detect the expression level of Ki-67, MEG3 andmiR-134, etc in each specimen, and then analyze the date in combination with clinical data. **Results:** The expression level of miR-134 in NFPA is significantly lower than that of other types of pituitary adenomas and normal pituitary ($P<0.01$) while there exists negative correlation between the expression level of miR-134 and NFPA patients' age, the positive rate of tumor cells Ki-67 as well as tumor invasion ($P<0.01$). **Conclusion:** The down-regulation of miR-134 may be one of the important causes for the ocurrence and growth of NFPA tumors,.miR-134 is expected to be a new target for the treatment of NFPA.

Key words: Nonfunctional pituitary adenoma; miR-134; Proliferation and invasion

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Introduction

According to the epidemiological survey, the incidence rate of pituitary adenoma (PA) is larger than that of meningioma, ranking second among the incidence of intracranial tumors, following glioma, and the incidence rate of population amounts to about 20% among which non-functioning pituitary adenoma (NFPA) is the most common, accounting for more than 30% of the total number of pituitary adenomas[1,2]. Some NFPA can conduct the invasion-like growth toward the surrounding tissue, and surgery, drugs or radiotherapy is less effective, and the recurrence rate of tumor is rather high. Therefore, it is important for clinical and practical value to conduct the research into the molecular mechanisms for NFPA tumor and proliferation so as to find the relevant specific therapeutic targets.

miR-134 is a period of mi-croRNA that is made up of 22 nucleotides, and it is located in the same imprinted gene cluster DLKl/MEG3 maternal allele with the coding sequence of some non-coding RNA.The methylation region of the upstream promoter in this gene cluster can regulate its transcription. The research confirms that MEG3 is a class of non-coding RNA which has anti-tumor function and it is located in the gene cluster. Because of the hyper-methylation of the gene cluster promoter, the specific expression of MEG3 in NFPA is missing or reduced while the expression exists in the normal pituitary and other types of pituitary tumors[3,4]. Clinical findings have showed that the expression level of miR-134 was significantly down-regulated in some tumors[5]; The research showed that miR-134 could induce the stagnation of tumor cells cycle or inactivation of tumor-associated signaling pathways by relatively inhibiting downstream target genes and it affect the ability of tumor cell invasion and migration by inhibiting epithelial-mesenchymal transformation[6-8]. Therefore, since the anti-tumor factor miR-134 and MEG3 are in the same gene cluster, and they are both influenced by the same promoter, is the specific expression of miR-134 in the NF-PA down-regulated just like MEG3? Does the expression level of miR-134 relate to the cells' proliferation and invasion in NFPA? In this study, immunohistochemistry, real-time quantitative PCR(qRT-PCR) and other methods are used to detect the expression of miR-134 in various types of pituitary tumors and normal pituitary tissues,and to analyze the relationship between the expression level of miR-134 and NFPA proliferation and invasion. It is aimed to provide early theoretical basis for the diagnosis and treatment research in terms of NFPA targeting gene.

1 Materials and Methods

1.1 Specimen collection

The surgical specimens were collected in neurosurgery department from January 2011 to December 2012. 104 pituitary adenoma surgical specimens, including 10 cases of ACTH adenoma, 18

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cases of GH adenoma, 10cases of PRL adenoma, namely, 66 cases of NFPA. were collected. And another 8 normal ones which were donated with 4 males and 4 females died without the nervous system disorder diseases. 10% neutral formalin was used to fix some surgical specimens within half an hour in vitro，and, it overnighted with the temperature of 4 ℃, and would be used as later immunohistochemistry experiment while another part was frozen in liquid nitrogen to be used as the late RNA line of qRT-PCR extraction experiment.

All the patients were confirmed by clinical imaging, endocrinology hormone testing, surgery and pathology, and the pituitary hormone immunohistochemistry is used to distinguish different endocrine types. Combined with preoperative imaging test, the one who meets at least one of the following four conditions is regarded as invasive NFPA: (1) Improved Hardy grade level is grade Ⅲ or Ⅳ; (2) Knosp grade is grade 3 or 4; (3) during the surgery, it can be seen that the inner side wall of the cavernous sinus eroded by penetration; the sinus structure is surrounded by tumor or bone of saddle area are destroyed; (4) Pathological diagnosis confirms that there exists infiltration in saddle diaphragm, sphenoid sinus mucosa, or the bottom bone of saddle. See relative basic clinical results information in Table 1. The research was approved by the ethics committee of Beijing Union Medical College Hospital, and all patients signed an informed consent form.

Table 1 Basic clinical information of 104 cases

Classification	Case	Gender(M/F)	Age(year)	Invasion(Y/N)
				38/28
				6/12
NFPA	66	26/40	39.02± 5.83	2/8
				0/10
				46/58
GH	18	8/10	45.26± 6.51	
PRL	10	7/4	40.48± 5.77	
ACTH	10	4/6	28.02± 4.68	
Total	104	44/60	43.19± 7.46	

1.2 Reagents and Materials

(1) Trizol (Invitrogen, Shanghai); (2) cDNA synthesis kit: a. SuperScript III First Strand Synthesis SuperMix kit (Invitrogen, Shanghai, Item :11752-050), for MEG3 and GAPDH; b. NCode VILo miRNA cDNA synthesis kit (Invitrogen, Shanghai, Item: A11193-052), for miR-134 and SNORD44; (3) qRT-PCR kit: a. Super-Script III Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen, Shanghai, Item :11746-100), for MEG3 and GAPDH; b. NCode EXPRESS SYBR GreenER miRNA qRT-PCR kit (Invitrogen, Shanghai, Item: A11193-052), for miR-134 and SNORD44; (4) Immunohistochemistry reagents: rabbit anti-human IgG monoclonal antibody Ki-67 working solution, goat anti-rabbit secondary antibody, DAB display agent (Beijing Zhongshan Golden Bridge biotechnology Co., Ltd.).

1.3 Approaches

1.3.1 RNA extraction and RT－PCR The relative primers in accordance with miR Base database and human MEG3 and miR-134 gene sequences.

MEG3 forward primer: 5'-ATCATCCGTCCACCTCCTTGTCTTC-3';

MEG3 reverse primer: 5'-GTATGAGCA TAGCAAAGGTC-.AGGGC-3';

GAPDH forward primer: 5'-AATGC CTCCTGCACCACCAAC-3';

GAPDH reverse primer: 5'-AAGGCCATGCCAGTGAGCTTC-3';

hsa-miR-134 forward primer sequence: 5'-TGTGACTGGTTGACCA-3';

NORD44 forward primer sequence: 5'-CCTGGATGATGATAGCAAATGCTG-3',

Reverse sequences were provided by the universal reverse primer sequences kit(A11193 one 052) .

RT-PCR: (1) conduct the total RNA extraction in accordance with the Trizol instructions and determine the content and purity of RNA; (2) the synthesis of cDNA: Respectively use the corresponding kit to transcribe 1g total RNARC reversely into related cDNA; (3) PCR reactions: Respectively use the corresponding kit, use synthesized cDNA as a template, and conduct the PCR amplification reaction in ABl7300 real-time PCR instrument. Amplification conditions: 50℃ 2 min; 95℃ 10 min; 95℃ 15 s, 60℃ 1 min, 40 cycles; 60℃ 1 min. The formula of target gene expression level: $2^{-\triangle\alpha}$, $\triangle Ct=Ct_{\text{target gene}}-Ct_{\text{internal reference}}$, to use the corresponding expression level in the four cases of the normal pituitary tissue as the correcting base to correct each expression value of target gene in various types of pituitary adenomas. The above experiments are repeated at least three times.

1.3.2 Immunohistochemistry Use Immunohistochemical approach to determine the percentage of tumor Ki-67 positive cells, and make paraffin tissue blocks out of the tumor tissue fixed in 10% neutral formalin, slices, each 4um in thickness, placed on the

slides undergoing the anti-off process; Dewaxing, ethanol +3% hydrogen peroxide (9:1) dropwise sheet min, closed endogenous peroxidase; After the hot fix, dropping anti-working fluid Ki-67, 4℃ overnight incubation; secondary antibodies incubation at room temperature for 2 h, DAB chromogenic; After the termination of color, hematoxylin, dehydrated, mounted. Select at random five 400 × magnification, take photos of micrograph, using Image J image software to analyze, and then read the average value of Ki-67-positive cells.

1.4 Statistical analysis

SNPSS 17.0 statistical software to conduct data processing, measurement data is expressed in the form of mean standard deviation ($x^2 \pm s$), the normality of Kolmogorov-Smirnov test data. The results indicate that the research data is in line with normal distribution. The single-factor analysis of variance is adopted when making comparisons in expression differences between miR-314 and MEG3 of each group, and because there is no significant homogeneity of variance, Dunnett's test is adopted when pairwise comparisons are conducted in the NFPA and other groups in terms of average value; Pearson correlation test is adopted when respectively detecting the correlation among the expression value of NFPA in miR-134 and that of MEG3 and the ratio of Ki67 positive cells. The independent sample T test is conducted when comparing the expression levels of miR 134 between two groups in NFPA various clinical parameters. P<0.05 indicates that the difference is considered statistically significant.

2 Results

2.1 Expressions of miR−134 and MEG3 in different groups

The application of RT-PCR detection showed that expression of miR-134 and MEG3 in NFPA significantly lower than those of the normal pituitary and other types of pituitary adenomas, of which the expression level of miR-134 in comparison with that of NFPA, the normal pituitary gland was 27 times as miR-134, GH gland aneurysm 84 times, PRL adenoma its 38 times, and ACTH adenoma 25 times, and differences were statistically significant (P<0.01); In terms of the expression of MEG3, compared with the NFPA, the normal pituitary was 38 times as high as MEG3, GH adenoma 30 times, PRL adenoma 18 times, ACTH adenoma 15 times, and the differences were also statistically significant(P<0.01). Fig.1&2.

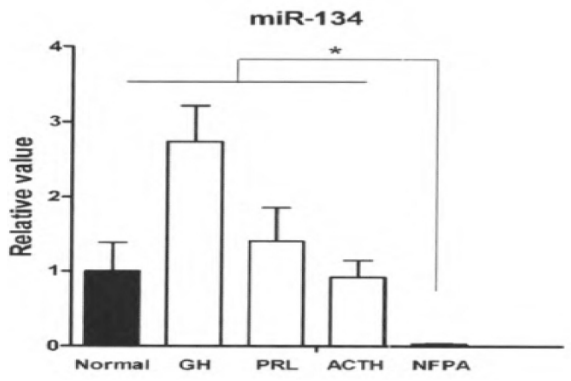

Fig. 1 miR 134 expression level(P<0.01).

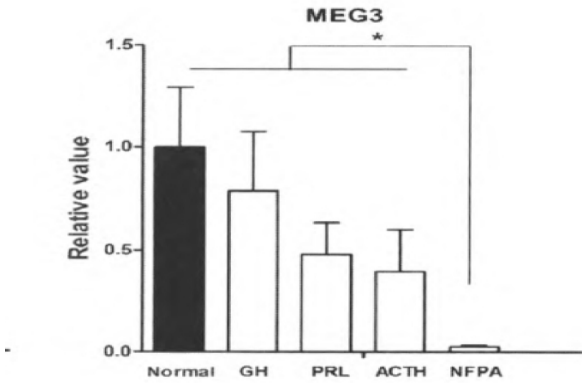

Fig.2 Comparison of the expression levels of MEG3(P<0.01).

2.2 Correlation between the miR−134 and MEG3 expressions of NFPA

Take the expression values of miR-134 and MEG3 of 66 cases in NFPA organization, and conduct the correlation analysis between the two indexes. The analysis results show that the correlation between the two indicators does not reach statistical level (r = 0.187, P = 0.296).

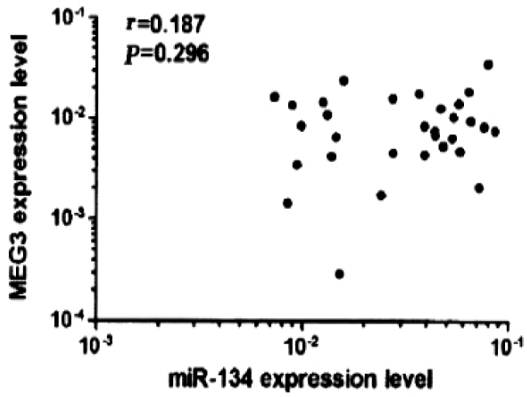

Fig.3 Correlation analysis between the miR-134 and MEG3 expressions of NFPA

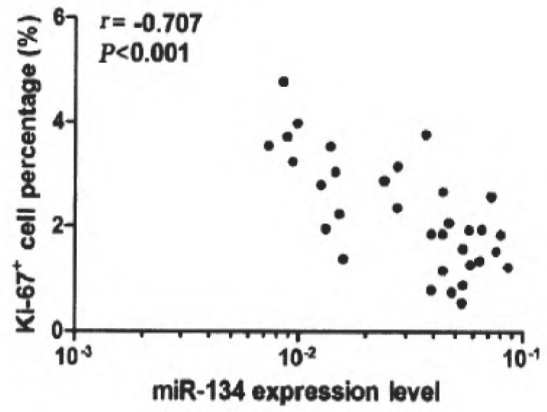

Fig. 4 Analysis of the positive cell ratio of NFPA miR-134 and the correlation with Ki67

2.3 Expression of miR −134 and correlation with cell proliferation

MiR-134 expression and NFPA cell proliferation ability correlation analysis was shown in Figure 4. Take miR-134 expression value (3.48± 0.21) and immunohistochemical Ki-67 positive cells (0.0311 ± 0.269) ratio value in 33 cases. The correlation between the two indexes was analyzed , and the analysis result suggested a negative correlation between the 2 indicators (r = 0.707, P <0.01).

2.4 Correlation of miR−134 expression and NFPA invasiveness

According to the relevant criteria, of the 66 cases of NFPA, there are 38 cases of invasive NFPA and 28 cases of non-invasive NFPA. The result of the comparison between the expression differences of the two groups of miR-134 shows that the expression level of the non-invasive group miR-134 is 5.5 times as high as that of the invasive group, and thus the difference between the two groups is statistically significant (P<0.01); while the expression level of miR-134 of older patients (≥40 years old) is significantly higher than younger patients, and the difference is also statistically significant (P<0.05), but in terms of gender, the difference is not statistically significant(P>0.05).

Table 2 Expression levels of miR-134 in clinical parameters in 66 patients with NFPA ($\bar{x}\pm s$)

Clinical parameters	Cases	miR-134	T	p
Invasiveness			2.832	0.008
Invasive group	28	0.0125± 0.0244		
Non-invasive group	28	0.0692± 0.0597		
Age(year)			2.315	0.027
≥ 40	24	0.0457± 0.0436		
<40	42	0.0196± 0.0182		
Gender			0.515	0.608
Male	26	0.0345± 0.0242		
Female	40	0.0296± 0.493		

3 Discussion

NFPA is the most common pituitary tumor subtype. Epidemiological surveys have showed that about 40% of the NFPA were invasion-like growth[9]. Currently, surgical resection remains the primary method of treatment of invasive NFPA, but neither the trans-sphenoidal surgery through the nose and mouth nor craniotomy is difficult to remove the whole tumor, and after surgery, tumor recurrence recurs among about 20 percent of cases occur[10]; At present, there is no especially effective drugs clinically to treat NFPA, and it is easy for radiotherapy to damage the important tissues in saddle area. Therefore, it is in particular important to find specific molecular markers for early diagnosis of NFPA and targeted therapy.

microRNA is a class of small molecules of 21 to 25 nucleotides which is complementarily binding with 3'UTR through target mRNA, and inhibiting target mRNA translates or causes its degradation, and thus regulates the expression of relative genes. More and more researches have showed that there were abnormal expression of many miRNA in various tumor tissues, indicating that there was correlations between the occurrence of tumors and the miRNA expression[11,12]. Stilling[13] and some other researchers also found 188 kinds of up-regulated miRNA and 160 kinds of down-regulated miRNA in ACTH tumors by using micro-RNA chip technology, and meanwhile they also find the expression of miRNA-122 and miR-493 is significantly more up-graduated than that of ACTH tumor in ACTH pituitary cancer. Besides, Bottoni, Amaral[14,15] and other researchers also found the abnormal expression of miRNAs in ACTH pituitary cancer. However, few researches in miRNA of NFPA are reported at present.

DLKl/MEG3 gene cluster which miR-134 and MEG3 belongs to is made up of many maternal and paternal alleles imprinted genes, the expression of which is confirmed to cause many diseases. This research for the first time confirms that miR-134 is similar to MEG3 in Chinese patients with pituitary tumor, and that its specific expression in NFPA declines. Lages and some other researchers apply qRT-PCR and micro-chip technologies and confirm that in oligodendroglioma cells, the expression of miR-134 and other miRNAs is significantly lower than that of glioblastoma cells and normal cells of the control group, indicating that there also exists subtype differences for the expression of miR-134 expression in gliomas. Therefore, based on the result of this research, it can be inferred that the abnormal expression of miR-134 may be related to the occurrence of certain types of tumors.

The ratio of Ki-67 positive cells have been used to determine the proliferation of pituitary adenomas. The result of this study shows that there exists a negative correlation between the expression level of miR-134 and Ki-67 value in NFPA. Niu [8] and some other researchers confirm through external cell lines and animal experiments that miR-134 can block the transcription of downstr-

eam target genes Nanog and thus play the role of anti-cell proliferation, migration and invasion, and it can also promote the apoptosis of tumor cells; In addition, Ye [16] and other researchers find through bio-informatics prediction and experimental discovery that miR-134 may also reduce the expression of target genes encoding proteins VEGFA ; VEGFA is an important tumor-promoting factor, and it is confirmed by some researches that its expression in NFPA increases, and it can contribute to cell proliferation [17,18]. Therefore, miR-134 may affect the proliferation of NFPA cells through the above similar mechanism, which requires further experimental evidence.

In addition, the result of this study shows that among the invasive group and young patients group, the expression level of miR-134 in NFPA significantly decreases; In vitro experiments also confirms that miR-134 can play the role of anti-tumor migration and invasion by inhibiting the expression of FOXMl and blocking epithelial-mesenchymal transition (EMT) in cancer cells[6]. Based on relevant research findings, the author infers that by down-regulation of miR-134 expression, the inhibition effect of some relative genes that contributes proliferation and invasion may be released, and accelerate the growth and infiltration speed of NFPA, and thus make the clinical symptoms in patients appear earlier for diagnosis and medical treatment. Therefore, the down-regulation of miR-134 expression is likely to be one of the important reasons for the occurrence of NFPA tumor and invasion -like growth pattern.

In NFPA, the hyper-methylation status of MEG3 upstream promoter is an important reason for the block of its transcription and its low level of expression [4]. Theoretically, the transcriptional expression of miR-134 and MEG3 that belongs to the same gene clusters are mainly regulated by the same promoter, therefore, this study attempts to analyze the correlation between the expression level of miR-134 and MEG3 in NFPA so as to explore the possible reasons for the down-regulation of miR-134 specific expression. Although the expression level of miR-134 and MEG3 in NFPA both decreases, as the result shows, yet there is no significant correlation between them, indicating the methylation of their shared promoter may not be the only reason for the decrease of their specific expression in NFPA. It has been found that in cancer and other complex diseases, the expression of various genes and protein is regulated by many network-like signals. Braconi [19] and so on have confirmed that miR-29 expression can affect the expression of MEG3 in hepatoma cells; Furthermore, the expression of miR-134 in lung cancer cells is also confirmed to be regulated by TGFJ31 cytokine [6]. Therefore, there could be other mechanisms that affect the expression of both of them in NFPA, such as shear variation, which requires the verification of further clinical high-flux validation research on samples and cell experiments in the post stage.

In conclusion, this study find that at the clinical tissue level, the specific expression of miR-134 in NFPA decreases, and further findings based on clinical data show that there exists correlation between its expression level and patients' age, tumor Ki-67 value and tumor invasiveness and other phenotypes. The result of this research lays an theoretical foundation in advance for the next step of NFPA pathogenesis discovery and molecular targets for the treatment.

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miR-134 在人垂体腺瘤组织中的表达及其意义 *

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摘要 目的: 探讨垂体腺瘤患者 miR-134 的表达及意义，分析其表达水平与无功能垂体腺瘤(non-functioningpituitaryadenomas, NFPA)增殖侵袭能力的相关性。**方法:** 选择 2010 年 6 月至 2013 年 7 月本院收集垂体腺瘤标本 104 例以及 8 例尸检正常腺垂体的临床资料。采用实时荧光定量 PCR、免疫组织化学技术检测 Ki-67、MEG3、miR-134 等在 NFPA 组织中的表达水平，并分析数据。**结果:** miR-134 在 NFPA 组织中表达水平显著低于正常腺垂体和其他类型垂体腺瘤 (P<0.01)；miR-134 的表达水平与 NFPA 患者肿瘤侵袭性、肿瘤细胞 Ki-67 阳性率及发病年龄呈负相关(P<0.01)。**结论:** miR-134 表达下调可能是 NFPA 肿瘤发生及肿瘤呈侵袭样生长的重要因素，miR-134 可作为诊断和评估 NFPA 预后的参考指标。

关键词: 无功能垂体腺瘤；miR-134；增殖侵袭能力

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