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Expression pattern of lncRNAs in pituitary adenomas

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ABSTRACT

Non-functioning pituitary adenomas (NFPAs) are a group of pituitary tumors lacking manifestations linked to high hormone production, such as acromegaly and Cushing's syndrome. NFPA carcinogenesis depends on several molecular players. Long non-coding RNAs (lncRNAs) are a class of molecular players whose role in tumorigenesis has just recently been recognized. In the current study, we appraised expressions of 5 lncRNAs, namely FGD5-AS1, ATP6V0E2-AS1, ARHGAP5-AS1, WWC2-AS2 and EPB41L4A-AS1 in NFPAs versus their corresponding non-tumoral samples. Expressions of ATP6V0E2-AS1, EPB41L4A-AS1, FGD5-AS1 and WWC2-AS2 were significantly increased in NFPA samples compared with adjacent non-tumoral samples (P values = 0.037, 0.007, 0.008 and 0.03, respectively). However, expression of ARHGAP5-AS1 was not different between NFPA samples and controls (P value = 0.62). EPB41L4A-AS1 and FGD5-AS1 could discriminate between NFPA samples and adjacent non-tumoral samples (P values = 0.03 and 0.04, respectively). However, the AUC values were not appropriate. There was a significant positive association between age of NFPA patients and invasiveness of NFPA ($\chi^2 = 4.24$, P value = 0.039). Moreover, there was a significant positive association between diseases duration and CSF leak ($\chi^2 = 11.4$, p value = 0.023). Finally, there was a significant positive association between tumor size and Knosp classification ($\chi^2 = 11.5$, p value = 0.02) and invasiveness of NFPA ($\chi^2 = 6.12$, p value = 0.04). The current study provides information about dysregulation of lncRNAs in NFPAs and warrants additional studies in this field.

1. Introduction

A broad range of lesions of the anterior pituitary are termed as pituitary adenomas (PA). Statistics show that the incidence of clinically significant pituitary neoplasms is 80–100 for every 100,000 people [21]. These are categorized as functional (FPAs) and non-functioning pituitary adenomas (NFPAs) depending on the level of hormone release. NFPAs make up around 30 % of all PAs, being the second most common PA after prolactinomas, which make up 50 % of all PAs [1].

NFPAs lack manifestations linked to high hormone production, such as acromegaly and Cushing's syndrome [6]. NFPAs are often identified based on persistent compressive symptoms (such as headaches or visual field defects) or hypopopituarism [4,15]. The primary reason for persistent tumor and postoperative recurrence is that the tumor may have invaded the internal carotid artery and entered the cavernous sinus when the mass effect first appeared [17]. The median age of diagnosis of NFPAs is 51.5 years (range: 19–79 years) [7]. Additionally, even totally resected neoplasms have a reappearance risk of 10–20 % after 5–10

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Table 1
Log2 fold change and adjusted p values obtained from bioinformatics analyses.

Selected lncRNA	Log2 fold change	Adjusted p.value
ARHGAP5-AS1	1.240983	0.04245685
ATP6V0E2-AS1	2.856039	0.01387096
EPB41L4A-AS1	2.672387	0.00749758
FGD5-AS1	2.541793	0.02155033
WWC2-AS2	2.278828	0.02880773

years, while residual tumors have a recurrence risk of 40 % and 50 % at 5 and 10 years, respectively [3,5,22].

NFPA carcinogenesis depends on several molecular players. Long non-coding RNAs (lncRNAs) are a class of molecular players whose role in tumorigenesis has just recently been recognized [9,10,24]. Recent research indicates that lncRNAs control gene expression at the transcriptional and posttranscriptional stages and that lncRNA malfunction speeds up the development of several malignancies, including PA [2,13,19,23]. In the current study, we appraised expression of five lncRNAs in PAs versus adjacent non-cancerous tissues.

2. Materials and methods

2.1. Selection of lncRNAs to Perform qRT-PCR

According to our recent study on NFPA datasets [11], by analyzing GSE62960 and GSE63357 datasets, a number of 44 lncRNAs were obtained, of which 5 lncRNAs including FGD5-AS1, ATP6V0E2-AS1, ARHGAP5-AS1, WWC2-AS2 and EPB41L4A-AS1 were selected to perform qRT-PCR. These 5 lncRNAs had 2.541793, 2.856039, 1.240983, 2.278828 and 2.672387 $\log_2$ FC, respectively. Table 1 shows parameters related to these lncRNAs.

2.2. Patients

Expression assays were conducted on pituitary adenoma samples and paired normal samples. Tissue samples were excised during surgery from 47 patients admitted to hospital affiliated to Shahid Beheshti University of Medical Sciences during 2021–2022. None of them had received any chemo/radiotherapy before tumor excision. The study protocol was approved by the ethical committee of Shahid Beheshti University of Medical Sciences. Informed consent form was obtained from all participants.

2.3. Expression assays

Total RNA was extracted using RNJia extraction kit (RN983006, Roje Technologies Company, Iran). Subsequently, cDNA was produced from these samples using AddScript cDNA Synthesis Kit (Cat. No. 22701, ADDBIO Company, South Korea). RT-qPCR was conducted using RealQ Plus 2x Master Mix Green with high ROX purchased (AMPLIQON, Denmark), and primers provided by the METABION Company (Germany). Table 2 shows detailed features of primers.

2.4. Statistical methods

Analyses were performed using SPSS v.22.0 (SPSS Inc., Chicago, IL). Graphics were created using GraphPad Prism v.9.0 (GraphPad Software, La Jolla California USA). Expression levels of five lncRNAs, namely FGD5-AS1, ATP6V0E2-AS1, ARHGAP5-AS1, WWC2-AS2, EPB41L4A-AS1 were compared between PA tissues and adjacent normal tissues. Expression levels in each sample were calculated using the Efficiency adjusted Ct of normalizer gene (B2M) - Efficiency adjusted Ct of target gene method (comparative –delta Ct method). The normal/gaussian distribution of the values was assessed by the Shapiro-wilk test. Wilcoxon matched-pairs signed rank test or paired t test was used to identify differentially expressed genes between the adenoma tissues and adjacent normal tissues.

The correlation of expression of studied genes was measured using Spearman correlation coefficient. Mann-Whitney test and Kruskal–Wallis one-way ANOVA were used for comparing gene expression levels between different groups of patients. Chi-square test was used to find out the association between expression of lncRNAs and clinicopathological factors.

The receiver operating characteristic (ROC) curve was illustrated by the GraphPad Prism v.9 software. The p value < 0.05 was considered as significant.

3. Results

3.1. Information about selected lncRNAs

Table 3 shows the characteristics of lncRNAs included in this study.

Table 2
Information about primers and the corresponding amplified regions.

Name	Type	Sequence	Primer Length	PCR Product Length
FGD5-AS1-F	lncRNA	GCCTTGTCCTTCCCTGTTTCA	21	161
FGD5-AS1-R		CTGGGCACTTGATGCTTTCATT	22	
ATP6V0E2-AS1-F	lncRNA	TGGAATGAGAGCAGGAGGAGTG	22	162
ATP6V0E2-AS1-R		AGCGAGATGACGGATAAGGCAG	22	
ARHGAP5-AS1-F	lncRNA	GTTCACGCCACTACCAGCCTAA	22	142
ARHGAP5-AS1-R		AGAAACACACCCAAAGCCCAGT	22	
WWC2-AS2-F	lncRNA	GAAGAGGAAAGGGGTCGTGT	20	172
WWC2-AS2-R		CTAAATGCGGTCAAAGCGGG	20	
EPB41L4A-AS1-F	lncRNA	ACTGGCACTTCTCCCTCCG	19	180
EPB41L4A-AS1-R		CAGAGCAAAAACCACAGCAAGC	22	
B2M-F	mRNA	AGATGAGTATGCCTGCCGTG	20	105
B2M-R		GCGGCATCTTCAAACCTCCA	20	

Table 3
Characteristic features of genes studied in this article.

Name/Gene ID	Accession number	Location	Official Full Name	Gene type
ATP6V0E2-AS1	NR_027040.1	7q36.1	ATP6V0E2 antisense RNA 1	ncRNA
EPB41L4A-AS1	NR_015370.2	5q22.1	EPB41L4A antisense RNA 1	ncRNA
FGD5-AS1	NR_046251.1, NR_046252.1, NR_046253.1, NR_046254.1, NR_046255.1	3p25.1	FGD5 antisense RNA 1	ncRNA
WWC2-AS2	NR_024008.1	4q35.1	WWC2 antisense RNA 2	ncRNA
ARHGAP5-AS1	NR_027263.1	14q12	ARHGAP5 antisense RNA 1	ncRNA

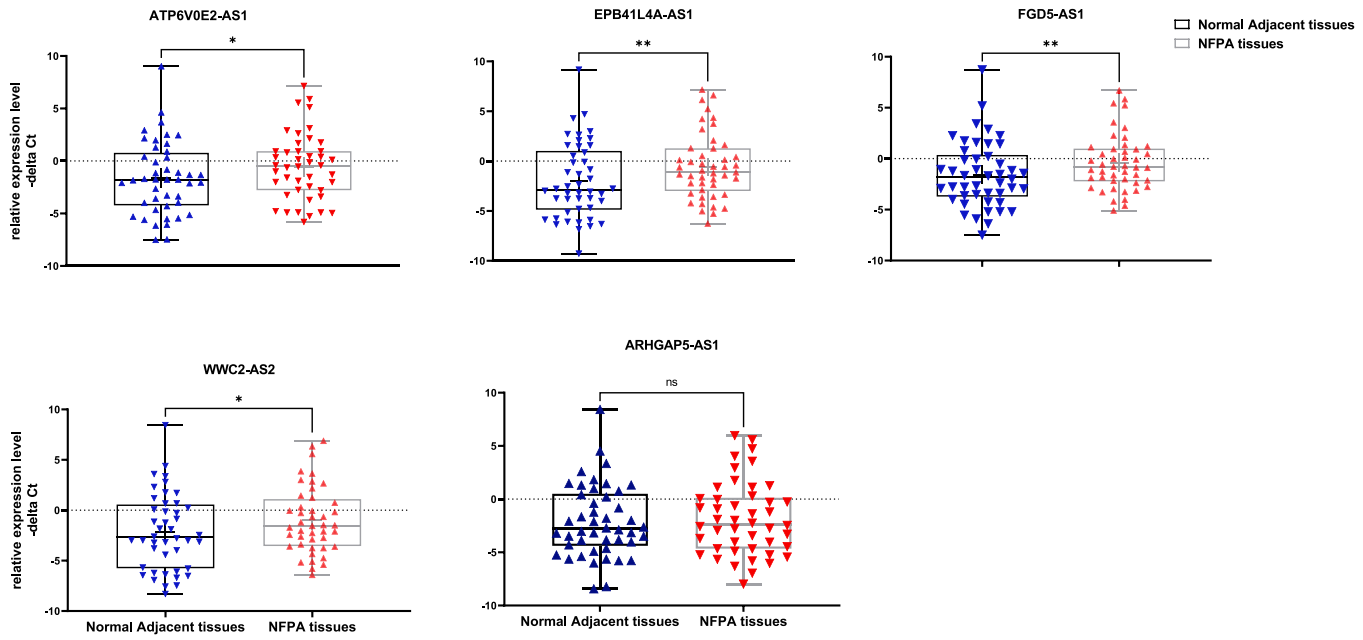

Fig. 1. Relative expression levels of five lncRNAs in non-functional pituitary adenoma (NFPA) tissues relative to the adjacent normal tissues as described by –delta Ct values (Ct Housekeeping gene- Ct Target gene). Data was analyzed using the Wilcoxon rank-sum test or paired t test, and $P < 0.05$ was considered statistically significant. Asterisks indicate significant difference between two mentioned groups (*P value < 0.05, **P value < 0.01 ns; non-significant).

3.2. General information

Table S1 shows general information about 47 NFPA samples included in this study.

3.3. Expression assays

We detected significant differences in the expression of FGD5-AS1, ATP6V0E2-AS1, WWC2-AS2 and EPB41L4A-AS1 between NFPA samples and corresponding non-tumoral samples (Fig. 1).

Expressions of ATP6V0E2-AS1, EPB41L4A-AS1, FGD5-AS1 and WWC2-AS2 were significantly increased in NFPA samples compared with adjacent non-tumoral samples (P values = 0.037, 0.007, 0.008 and 0.03, respectively). However, expression of ARHGAP5-AS1 was not different between NFPA samples and controls (P value = 0.62). Table 4 summarizes this information.

EPB41L4A-AS1 and FGD5-AS1 could discriminate between NFPA samples and adjacent non-tumoral samples (P values = 0.03 and 0.04, respectively) (Fig. 2). However, the AUC values were not appropriate (Table 5).

We also evaluated the correlation between expression levels of ATP6V0E2-AS1, EPB41L4A-AS1, FGD5-AS1, WWC2-AS2 and ARHGAP5-AS1 in NFPA tissues as well as adjacent non-tumoral tissues (Table 6). All pairs of lncRNAs reached the significance level of $P < 0.001$.

There was a significant positive association between age of NFPA patients and invasiveness of NFPA ($\chi^2 = 4.24$, P value = 0.039). Moreover, there was a significant positive association between diseases duration and CSF leak ($\chi^2 = 11.4$, p value = 0.023). Finally, there was a significant positive association between tumor size and Knosp classification ($\chi^2 = 11.5$, p value = 0.02) and invasiveness of NFPA ($\chi^2 = 6.12$, p value = 0.04). Table 7 shows this information.

Table 4
The results of expression study of five lncRNA genes in non-functional pituitary adenoma (NFPA) tissues compared with the adjacent normal tissues. The expression ratio of each gene is shown as mean and 95 % Confidence interval and SEM.

Studied genes	Expression ratio (95 % CI)	SEM	P Value
ATP6V0E2-AS1	2.1 (1.04–4.19)	0.049	**0.037**
EPB41L4A-AS1	2.77 (1.33–5.76)	0.52	**0.007**
FGD5-AS1	2.26 (1.24–4.11)	0.42	**0.008**
WWC2-AS2	2.25 (1.08–4.68)	0.52	**0.03**
ARHGAP5-AS1	1.19 (0.56–2.55)	0.54	0.62

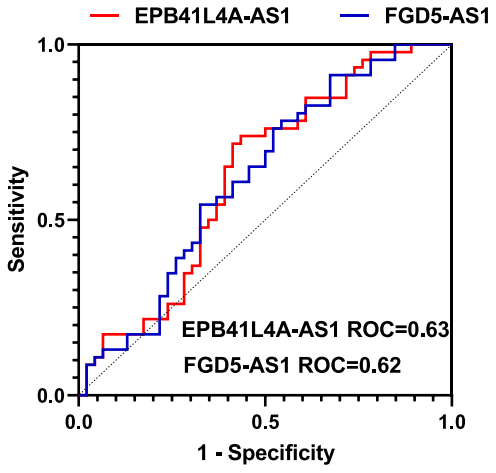

Fig. 2. The receiver operating characteristic (ROC) curve of EPB41L4A-AS1 and FGD5-AS1 lncRNAs for discrimination of NFPA tumors from adjacent normal tissues. AUC indicates area under the ROC curve.

Table 5
The results of ROC curve analysis for five lncRNA genes for discrimination of NFPA tumors genes from adjacent normal tissues.

ATP6V0E2-AS1				EPB41L4A-AS1				FGD5-AS1				WWC2-AS2				ARHGAP5-AS1			
AUC ± SD	Sensitivity	Specificity	P Value	AUC±SD	Sensitivity	Specificity	P Value	AUC±SD	Sensitivity	Specificity	P Value	AUC±SD	Sensitivity	Specificity	P Value	AUC±SD	Sensitivity	Specificity	P Value
0.6 ± 0.06	0.57	0.66	0.1	0.62 ± 0.05	0.57	0.58	0.03	0.62 ± 0.05	0.78	0.48	0.04	0.6 ± 0.06	0.85	0.27	0.1	0.52 ± 0.06	0.6	0.48	0.76

Table 6
Spearman's correlations between five LncRNA genes expression levels among the non-functional pituitary adenoma tumor tissues ($N = 46$) and adjacent normal tissues ($N = 46$).

	EPB41L4A-AS1		FGD5-AS1		WWC2-AS2		ARHGAP5-AS1	
	adjacent	Tumor	adjacent	Tumor	adjacent	Tumor	adjacent	Tumor
ATP6V0E2-AS1	0.94**	0.87**	0.95**	0.79**	0.89**	0.88**	0.92**	0.81**
EPB41L4A-AS1			0.93**	0.92**	0.9**	0.97**	0.92**	0.89**
FGD5-AS1					0.92**	0.95**	0.93**	0.81**
WWC2-AS2							0.89**	0.88**

** significance level of $p < 0.001$.

Table 7
Comparison of expression levels of five lncRNA genes in NFPA patients with different clinicopathologic factors. Mann-Whitney test and Kruskal–Wallis one-way ANOVA were used for comparing gene expression levels between different groups.

Parameters	Subclasses	Number of patients (%)	Relative expression level of ATP6V0E2-AS1 (mean±SD)	P-value	Relative expression level of EPB41L4A-AS1(mean±SD)	P-value	Relative expression level of FGD5-AS1 (mean±SD)	P-value	Relative expression level of WWC2-AS2 (mean±SD)	P-value	Relative expression level of ARHGAP5-AS1 (mean±SD)	P-value
Tumor subtypes	NFPA	33	-0.78±0.52	0.69	-0.58±0.58	0.9	-0.5±0.47	0.83	-1.01±0.56	0.81	-1.84±0.6	0.79
	NFPA +CD+AP	11	-0.28±1.13		-0.66±1.13		-0.32±1.05		-0.78±1.05		-1.86±1.1	
age	22-48	22	-0.62±0.62	0.67	-0.21±0.66	0.33	-0.3±0.58	0.48	-0.59±0.58	0.29	-1.6±0.61	0.51
	49-77	22	-0.7±0.73		-0.98±0.79		0.62±0.66		-1.3±0.79		-2.01±0.84	
gender	Female	11	-1.65±0.92	0.28	-1.97±0.94	0.13	-1.51±0.71	0.16	-2.49±0.86	0.085	-2.87±1.1	0.27
	Male	33	-0.32±0.55		-0.14±0.59		-0.11±0.52		-0.44±0.56		-1.5±0.58	
Disease's duration	<1 y	22	-0.22±0.77	0.69	-0.34±0.85	0.96	-0.13±0.71	0.86	-0.78±0.81	0.91	-1.32±0.86	0.81
	1 y	10	-0.97±1.06		-0.98±1		-0.9±0.93		-1.3±0.94		-2.13±0.86	
	>=2 y	12	-1.19±0.55		-0.76±0.69		-0.7±0.59		-0.97±0.71		-2.56±0.81	
Tumor Size (cm)	<500 mm^2	14	-0.73±1	0.87	-0.19±1.06	0.96	-0.23±0.99	0.73	-0.78±1.05	0.96	-1.24±1.08	0.87
	500-800mm^2	15	-0.47±0.9		-0.65±1		-0.17±0.71		-0.82±0.95		-1.89±0.94	
	>800 mm^2	15	-0.77±0.59		-0.93±0.6		-0.95±0.58		-1.24±0.54		-2.37±0.68	
csf leak	No	20	-0.69±0.77	0.64	-0.4±0.76	0.86	-0.3±0.65	0.57	-0.85±0.76	0.52	-1.75±0.75	0.5
	Low flow	10	-1.24±1.04		-1.2±1.15		-1.08±1.06		-1.7±1.15		-2.67±1.36	
	High flow	14	-0.2±0.73		-0.45±0.91		-0.25±0.72		-0.55±0.77		-1.39±0.79	
knosp classification	1	10	-0.41±0.61	0.73	-0.49±0.75	0.67	-0.38±0.79	0.67	-0.66±0.7	0.61	-1.1±0.83	0.51
	2	16	-0.76±0.91		-1.01±0.95		-0.84±0.79		-1.48±0.92		-2.08±0.96	
	3	18	-0.7±0.79		-0.29±0.85		-0.17±0.7		-0.64±0.81		-2.05±0.84	
invasiveness	invasive	7	-0.57±0.53	0.86	-0.44±0.57	0.49	-0.36±0.49	0.64	-0.8±0.55	0.64	-1.74±0.58	0.59
	non invasive	37	-1.12±1.11		-1.42±1.05		-0.95±0.85		-1.7±1.05		-2.42±1.15	
drug history	Yes	9	-1±0.48	0.33	-0.98±0.54	0.34	-0.78±0.48	0.16	-1.2±0.51	0.51	-2.23±0.55	0.21
	No	35	0.67±1.32		0.87±1.32		0.78±0.96		0.07±1.33		-0.32±1.25	

4. Discussion

LncRNAs are a group of non-coding RNAs that have significant roles in the regulation of gene expression, thus contributing to the pathoetiology of several disorders, particularly cancer [20]. In the current study, we appraised expressions of 5 lncRNAs, namely FGD5-AS1, ATP6V0E2-AS1, ARHGAP5-AS1, WWC2-AS2 and EPB41L4A-AS1 in NFPAs versus their corresponding non-tumoral samples. The basis for selection of these lncRNAs was an *in-silico* method [11]. Thus, there is no explicit data about the role of these lncRNAs in physiological or pathological processes in the pituitary gland. We detected up-regulation of ATP6V0E2-AS1, EPB41L4A-AS1, FGD5-AS1 and WWC2-AS2 in NFPA samples compared with adjacent non-tumoral samples. However, expression of ARHGAP5-AS1 was not different between NFPA samples and controls. A recent study has reported association between single nucleotide polymorphism of ATP6V0E2-AS1 and risk of myeloperoxidase-ANCA associated vasculitis [14]. This lncRNA is an antisense transcript to ATP6V0E2, a gene that encodes an isoform of an essential proton pump component that contribute to the acidification of endosome and lysosome [14]. Over-expression of ATP6V0E2-AS1 has been shown to predict poor prognosis in prostate cancer [12]. LncRNA EPB41L4A-AS1 has been found to regulate glycolysis and glutaminolysis through facilitating nucleolar translocation of HDAC2 [16]. FGD5-AS1 has a role in regulation of cancer cell proliferation and chemoresistance via regulating miR-153–3p/CITED2 axis [8]. This lncRNA also has a role in aggravation of myocardial ischemia-reperfusion injury through regulating expression of miR-129–5p [18]. Future studies are needed to elaborate the mechanism of dysregulation of these lncRNAs in NFPAs and their participation in the pathoetiology of these tumors.

Among lncRNAs with differential expression between these two sets of samples, EPB41L4A-AS1 and FGD5-AS1 could discriminate between NFPA samples and adjacent non-tumoral samples. However, the AUC values were not appropriate. Therefore, we could not suggest these lncRNAs as biomarkers for NFPAs.

There was a significant positive association between age of NFPA patients and invasiveness of NFPA. Moreover, there was a significant positive association between diseases duration and CSF leak. Finally, there was a significant positive association between tumor size and Knosp classification as well as invasiveness of NFPA. However, expression of none of genes was associated with pathological features of NFPAs. Lastly, we detected significant correlations between expressions of FGD5-AS1, ATP6V0E2-AS1, ARHGAP5-AS1, WWC2-AS2 and EPB41L4A-AS1 in both sets of samples which implies their functional relationship. However, this point should be assessed in future studies. In brief, the current study provides information about dysregulation of lncRNAs in NFPAs and warrants additional studies in this field.

Ethics approval and consent to Participant

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent forms were obtained from all study participants. Informed consent forms were obtained from all study participants and from legally authorized representative/next of kin of deceased patients. The study protocol was approved by the ethical committee of Shahid Beheshti University of Medical Sciences. All methods were performed in accordance with the relevant guidelines and regulations.

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CRediT authorship contribution statement

SGF wrote the draft and revised it. MT and NAD designed and supervised the study. SE analyzed the data. BMH, GN, MK, GS and AS performed the experiment and data collection. All the authors read and approved the submitted version.

Declaration of Competing Interest

The authors declare they have no conflict of interest.

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Consent of publication

Not applicable.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.prp.2023.154668.

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