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ORIGINAL ARTICLE

MICROARRAY TECHNOLOGY REVEALS POTENTIALLY NOVEL GENES AND PATHWAYS INVOLVED IN NON-FUNCTIONING PITUITARY ADENOMAS

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ABSTRACT

Microarray data of non-functioning pituitary adenomas (NFPAs) were analyzed to disclose novel genes and pathways involved in NFPA tumorigenesis. Raw microarray data were downloaded from Gene Expression Omnibus. Data pre-treatment and differential analysis were conducted using packages in *R*. Functional and pathway enrichment analyses were performed using package GOstats. A protein-protein interaction (PPI) network was constructed using server STRING and Cytoscape. Known genes involved in pituitary adenomas (PAs), were obtained from the Comparative Toxicogenomics Database. A total of 604 differentially expressed genes (DEGs) were identified between NFPAs and controls, including 177 up- and 427 down-regulated genes. Jak-STAT and p53 signaling pathways were significantly enriched by DEGs. The PPI network of DEGs was constructed, containing 99 up- and 288 down-regulated known disease genes (*e.g. EGFR* and *ESR1*) as well as 16 up- and 17 down-regulated potential novel NFPAs-related genes (*e.g. COL4A5*, *LHX3*, *MSN*, and *GHSR*). Genes like *COL4A5*, *LHX3*, *MSN*, and *GHSR* and pathways such as p53 signaling and Jak-STAT signaling, might participate in NFPA development. Although further validations are required, these findings might provide guidance for future basic and therapy researches.

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Keywords: Differentially expressed genes (DEGs); Functional enrichment analysis; Microarray; Non-functioning pituitary adenomas (NFPAs); Protein-protein interaction (PPI).

INTRODUCTION

As a kind of benign adenomas in the pituitary gland, clinically non-functioning pituitary adenomas (NFPAs) are the most common type of pituitary macroadenomas in adults. The NFPAs account for about 34.0% [1] of all pituitary adenomas (PAs) that occur at a prevalence rate of 75-94 per 100,000 [1,2]. Patients with NFPAs generally suffer from headaches, hypopituitarism, hypogonadism and visual field defects. Late diagnosis due to inconspicuous signs and symptoms, extension to the cavernous sinus and sellar floor, resistance to pharmacological therapy and high recurrence rate, make their treatment disappointing and challenging [3]. Approximately 80.0% of NFPAs originate from gonadotroph cells (gonadotroph pituitary adenoma, GnPA) [4], and other NFPAs are mainly associated with null cells (null cell pituitary adenoma, ncPA). The identification of novel therapeutic targets for human NFPAs depend on a good understanding of the molecular mechanism of NFPAs [5].

Progression in understanding the mechanism of PAs, especially NFPAs, has been achieved over the last several years. According to the reports, germline mutations in *AIP* or *MEN1* genes are associated with young age-onset PAs [6,7]. The *HGF* and *c-MET* genes are frequently expressed in PAs, and their expressions are correlated with phos-phorylated Akt expression [8]. Durán-Prado *et al.* [9] identified that sst5TMD4, a truncated variant of so-

matostatin receptor 5, appeared in 85.0% PAs rather than normal pituitary, and it may play an inhibitory role in PAs that possess poor response to somatostatin analogs. Raf/ MEK/ ERK and PI3K/Akt/mTOR signaling pathways are perturbed in NFPAs [10]. As a target of the *SF1* gene in gonadotroph cells, *CYP11A1* is up-regulated in human GnPA, and *Cyp11a1* promotes survival and proliferation of primary cells and cell lines of rat PAs [5]. Rotondi *et al.* [11] suggested that the gonadotroph phenotype was strongly associated with *AIP* expression in NFPAs. The *AIP* level is higher in GnPA than that in ncPA, and both *AIP* and cyclinD1 levels are high in most NFPAs. The *AIP* level correlates with follicle-stimulating hormone β (FSHβ) and cyclinD1 levels in GnPA. However, *AIP* is not involved in the aggressiveness of NFPAs [11]. Recently, *CCNB1* was found to mediate the proliferation-inhibiting role of miR-410, a small non-coding RNA, in GnPA [12]. Additionally, Chesnokova *et al.* [13] have identified that human pituitary tumors originated from gonadotroph cells express abundant *FOXL2*, and both *FOXL2* and *PTTG* promote cluster- ing expression and secretion from gonadotroph cells, thus restraining the proliferation of pituitary cells.

Along with the development of microarray, transcriptome analysis has been widely utilized in understanding tumor mechanism. Based on the gene expression microarray dataset GSE26966, Michaelis *et al.* [14] identified that GADD45β, a downstream effector of p53, is a tumor suppressor in gonadotroph tumor. Its overexpression in mouse gonadotroph cells blocks cell proliferation and promotes apoptosis [14]. Based on the same dataset, Cai *et al.* [15] identified the coexpressed and altered genes involved in gonadotroph tumors and suggest that *ITGA4*, *MPP2*, *DLK1*, *CDKN2A* and *ASAP2* might be biomarkers. However, pathways or functions of the altered genes were not studied by Michaelis *et al.* [14], and the protein-protein interactions (PPIs) between genes were not investigated in the two aforementioned studies [14,15]. In particular, Zhao *et al.* [16] performed an integrated analysis of five available microarray datasets of various PAs, to detect 3994 differentially expressed genes (DEGs) (including 2043 up- and 1951 down-regulated genes), and conducted a PPI network analysis. However, PPIs of more DEGs are needed to be analyzed, and more potential novel PAs-related genes are still unknown. Moreover, molecular mechanisms underlying the pathogenesis of PAs, particular NFPAs, remain unclear, and it is still essential to comprehensively investigate and annotate the alterations in gene expression profiles. In the present study, NFPAs-related microarray data uploaded by Michaelis *et al.* [14] were analyzed to identify significant DEGs, study NFPAs-related functions and pathways, construct interaction network, and identify potential novel NFPAs-related genes.

MATERIALS AND METHODS

Microarray Data. Microarray dataset of gene expression, GSE26966 [14], was downloaded from the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE26966). In this dataset, nine normal human pituitary samples were collected from individuals without an endocrine dysfunction at autopsy 2-18 hours post death, and 14 NFPAs samples were obtained from patients at the time of transsphenoidal surgery after obtaining the patient's or their families' permission [14]. Moreover, the 14 NFPA samples contained 10 human GnPA samples [histological analysis: >5.0% staining for α-subunit (ASU), follicle-stimulating hormone (FSH) or lutein-izing hormone (LH)] and four ncPA samples (histological analysis: <5.0% staining for ASU, FSH or LH) [14]. Clinical characteristics of tumor samples were: male/female = 8/6, mean age (years) = 61.4, invasive/non-invasive = 7/7, and recurrent/non-recurrent = 5/9. Clinical characteristics of normal controls were: male/female = 4/5 and mean age (years) = 55.9 years that had no significant difference in comparison with tumor samples (p value = 0.39) [14]. Raw microarray data were collected using Affymetrix Human Genome U133 Plus 2.0 Array (http://www.ncbi.nlm.nih. gov/geo/query/acc.cgi?acc=GPL570) in the previous study [14].

Pre-Treatment and Differential Analyses. Robust multi-array average algorithm in the affy package (from http://www.bioconductor/org/package/release/bioc/ html/affy.html) [17] in *R* was chosen for background correction, data normalization, and calculation of expression values. T-test in package simpleaffy [18] was performed, and fold change (FC) values were determined. Then, p values were corrected using the Bonferroni method, and corrected p value <0.05 and [$\log_2$ FC] >2 were set as the cut-off to identify DEGs. Thereafter, package Pheatmap (https://cran.r-project/org/web/packages/pheatmap/index. html) [19] in *R* was utilized to cluster genes and samples based on the expression values of DEGs.

Functional and Pathway Enrichment Analyses. Gene Ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were conducted using package GOstats (http://www.biocon ductor.org/packages/release/bioc/GOstats.html) [20]. The p value <0.05 was set as the threshold. User data mapping

module in the KEGG database (http://www.kegg/jp) was utilized to visualize the significantly enriched pathways.

Construction of Protein-Protein Interaction Network. For all of the identified DEGs, a PPI network was constructed with information from a well-known online server, Search Tool for the Retrieval of Interacting Genes/Proteins version 10 (STRING v10) (http://string-db.org) [21]. Only the PPIs with a confidence score of >0.4 were defined as significant PPIs, which were then utilized to construct the PPI network. The network was visualized using software Cytoscape version 2.8 (http://www.cytoscape.org) [22], and node degrees were determined.

Potential Novel Non-Functioning Pituitary Adenoma-Related Genes and Sub-Network. In order to find potential novel disease genes, known genes implicated in pituitary tumorigenesis were obtained from the Comparative Toxicogenomics Database (CTD) (the most recently released version was up-dated on February 9 2016, http://ctdbase.org/) [23]. Afterwards, the appearance of these known genes were checked in the PPI network to see whether the known genes were DEGs. Common genes, namely, the overlapped genes, were marked in the PPI network. Other DEGs were defined as potential novel NFPAs-related genes, as they were significantly altered in NFPA specimens and interacted with known disease genes. Furthermore, the top 10 significant DEGs, and DEGs directly interacting with the top DEGs, were extracted to construct a sub-network.

RESULTS

Differentially Expressed Genes and Clusters. A total of 604 DEGs were acquired between NFPAs and controls, involving 177 up- and 427 down-regulated genes. The top 10 up-regulated genes and top 10 down-regulated genes are shown in Table 1. The 604 DEGs and 23 samples were clustered, and DEGs could well differentiate the disease samples from the healthy controls (Figure 1).

Functions and Pathways. The GO enrichment analysis and KEGG pathway analysis were performed to reveal the key biological functions altered in NFPAs. As shown in Table 2, 12 pathways were significantly enriched, which

Table 1. The top 10 up-regulated genes and top 10 down-regulated genes.

Genes	Log_2 FC	Corrected *p* Value	Gene Title
Up-regulated			
SSBP2	2.04	1.43E-10	single-stranded DNA binding protein 2
CDH10	2.68	1.43E-10	cadherin 10, type 2 (T2-cadedrin)
FAM171A1	2.18	2.45E-10	family with sequency similarity 171, member A1
EFNB3	2.05	8.76E-10	ephrin-B3
PCYT1B	2.16	9.13E-10	phosphate cytidylytransferase 1, choline, β
RNF157	2.26	1.16E-09	ring finger protein 157
CDK18	2.46	1.57E-09	cyclin-dependent kinase 18
LRFN5	3.63	2.01E-09	leucine rich repeat and fibronectin type III domain containing 5
CACNA2D4	4.11	2.92E-09	calcium channel, voltage-dependent, a2/δ subunit 4
PPARGC1B	2.97	5.94E-09	peroxisome proliferator-activated receptor γ, coactivator 1 β
Down-regulated			
GH1	–9.74	1.49E-21	growth hormone 1
CSH1	–8.67	3.69E-15	chorionic somatomammotropin hormone 1 (placental lactogen)
DLK1	–9.33	4.16E-15	δ-like 1 homolog (Drosophila)
CSH2	–9.17	3.14E-13	chorionic somatomammotropin hormone 2
HIP1R	–2.16	5.43E-12	huntingtin interacting protein 1 related
CDKN2A	–2.33	4.74E-11	cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)
MGP	–2.86	8.06E-11	matrix Gla protein
KCNJ6	–3.79	9.50E-11	potassium inwardly-rectifying channel, subfamily J, member 6
SPRY4	–2.32	1.03E-10	sprouty homolog 4 (Drosophila)
MEG3	–5.72	1.60E-10	maternally expressed 3 (non-protein coding)

Corrected *p* value <0.05 and [log_2 FC (fold change)] >2 were set as the cut-off to identify differentially expressed genes.

Table 2. Significantly enriched terms.

Category	Term ID	Corrected *p* Value	Number of DEGs	Number of Genes	Term
KEGG	K04610	3.18E-04	10	69	complement and coagulation cascades
	K04512	3.97E-04	11	84	extracellular matrix-receptor interaction
	K04010	4.55E-03	20	271	MAPK signaling pathway
	K04115	5.32E-02	8	69	p53 signaling pathway
	K00350	6.79E-03	9	87	transforming growth factor β signaling pathway
	K04630	7.62E-03	13	155	Jak-STAT signaling pathway
	K04080	1.14E-02	18	256	neuroactive ligand-receptor interaction
	K04510	1.27E-02	15	202	focal adhesion
	K05218	2.08E-02	7	71	Melanoma
	K05412	2.90E-02	7	76	arrhythmogenic right ventricular cardiomopathy
	K05210	4.63E-02	7	84	colorectal cancer
	K04920	4.70E-02	6	67	adipocytokine signaling pathway
GO BP	GO:0032501	2.26E-24	266	4974	multicellular organismal process
(top 10)	GO:0010243	6.92E-13	57	596	response to organic nitrogen
	GO:0007275	4.19E-12	112	2080	multicellular organismal development
	GO:0048583	2.00E-10	99	1624	regulation of response to stimulus
	GO:0023051	4.23E-10	110	1866	regulation of signaling
	GO:0010646	5.12E-10	110	1872	regulation of cell communication
	GO:0048812	1.38E-09	49	571	neuron projection morphogenesis
	GO:0048667	3.03E-09	48	566	cell morphogenesis involved in neuron differentiation
	GO:0007243	3.09E-09	64	879	intracellular protein kinase cascade
	GO:0022008	6.39E-09	63	900	neurogenesis
GO CC	GO:0005576	1.31E-14	132	2164	extracellular region
(top 10)	GO:0005615	1.19-E-09	61	848	extracellular space
	GO:0005587	1.37E-05	4	6	collagen type IV
	GO:0005581	1.90E-05	12	88	collagen
	GO:0043005	5.12E-05	39	634	neuron projection
	GO:0005578	5.72E-05	18	204	proteinaceous extracellular matrix
	GO:0031012	8.74E-05	9	62	extracellular matrix
	GO:0016323	1.39E-04	15	158	basolateral plasma membrane
	GO:0005887	5.55E-04	59	1216	integral to plasma membrane
	GO:0005584	9.84E-04	2	2	collagen type I
GO MF	GO:0005201	2.40E-10	17	78	extracellular matrix structural constituent
(top 10)	GO:0008201	1.20E-07	18	129	heparin binding
	GO:0097367	1.60E-07	22	191	carbohydrate derivative binding
	GO:0042803	5.71E-06	38	553	protein homodimerization binding
	GO:0005179	9.11E-11	14	110	hormone activity
	GO:0000981	1.75E-05	22	253	sequence specific DNA binding RNA polymerase II transcription factor activity
	GO:0001077	4.36E-05	10	67	RNA polymerase II core promoter proximal region sequence-specific DNA binding transcription factor activity involved in positive regulation of transcription
	GO:0019199	5.05E-05	11	82	transmembrane receptor protein kinase activity
	GO:0005102	1.92E-04	55	1093	receptor binding
	GO:0048407	2.70E-04	4	11	platelet-derived growth factor binding

ID: identifier; DEGs: differentially expressed genes; KEGG: Kyoto Encyclopedia of Genes and Genomes; GO: gene ontology; BP: biological process; CC: cellular component; MF: molecular functions.

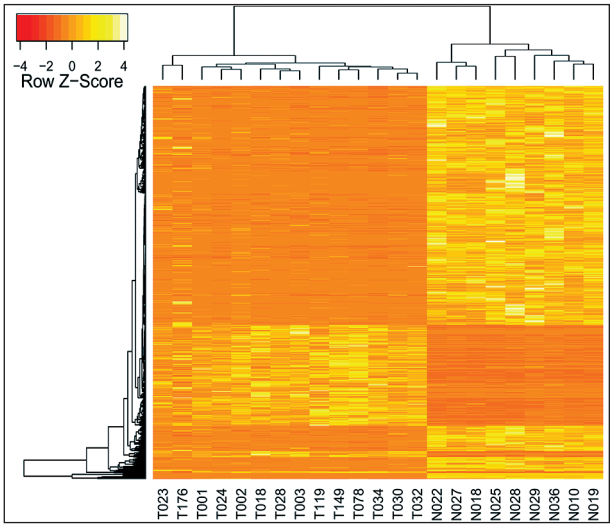

Figure 1. Cluster analysis of DEGs. DEGs: differentially expressed genes; T: tumor samples; N: healthy normal samples. Cluster analysis was performed both at gene level (vertical) and sample level (horizontal).

were mainly associated with signaling pathway and receptor interaction. In GO enrichment analysis, DEGs were significantly enriched in 1037 biological process terms mainly about cell communication and signaling, 65 cellular component terms mainly related with an extracellular matrix (ECM), plasma membrane, and collagen, as well as 186 molecular function terms mainly associated with transcription factor activity and receptor binding (Table 2). In order to better understand the positions of DEGs in pathways and their roles in the development of NFPAs, we visualized four significant pathways that had been reported to participate in the pathogenesis of NFPAs or PAs, including MAPK signaling pathway [10] (Figure 2), p53 signaling pathway [24] (Figure 3), transforming growth factor β (TGFβ), signaling pathway [25] (Figure 4), and Jak-STAT signaling pathway [8] (Figure 5).

Protein-Protein Interaction Network of Differentially Expressed Genes. For the 604 DEGs, the PPI network was constructed using information from STRING v10 (Figure 6). The whole network consisted of 115 up-regulated DEGs, 305 down-regulated DEGs and 1379 PPIs (Figure 6).

Potential Novel Non-Functioning Pituitary Adenoma-Related Genes and Sub-Network. Known disease genes were obtained from the CTD database (http://ctd

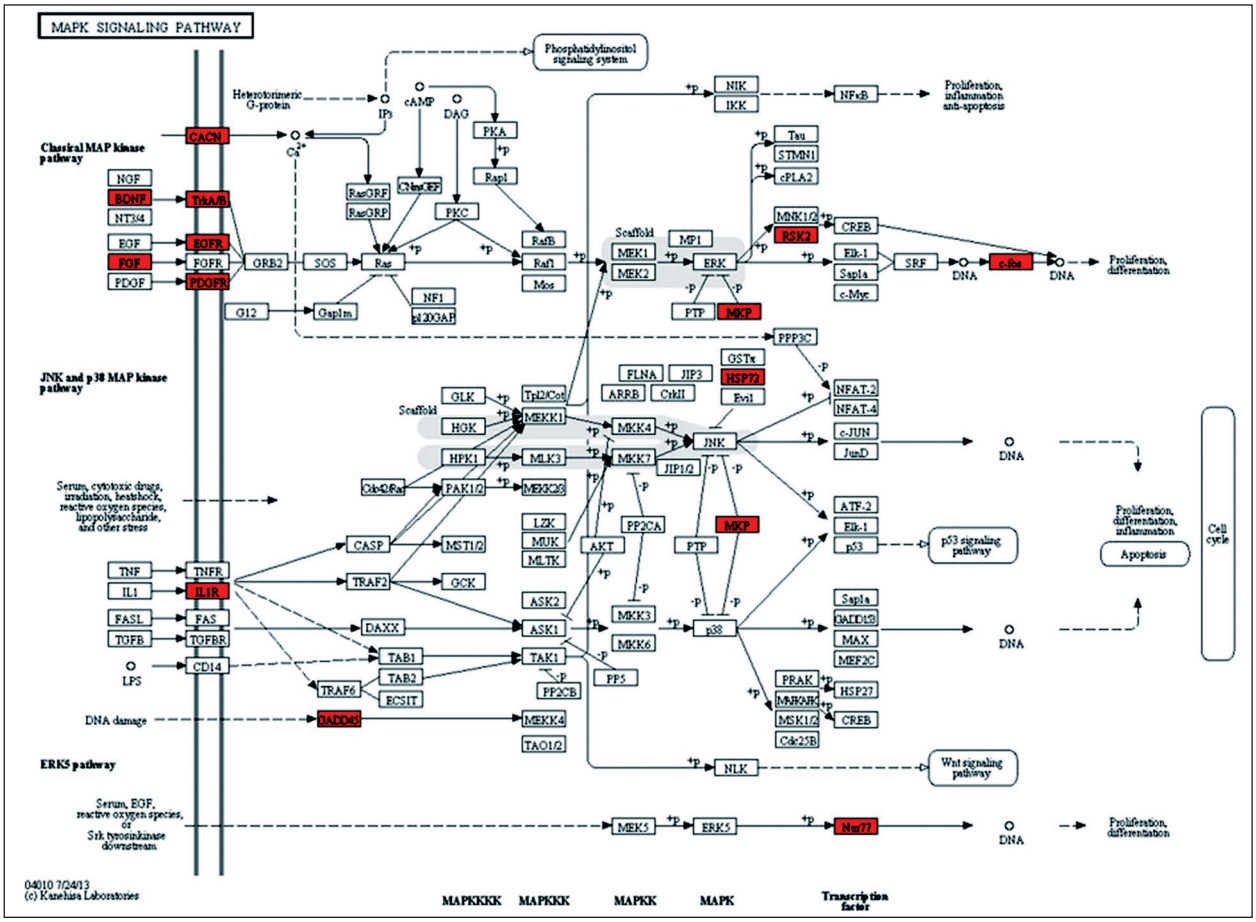

Figure 2. The MAPK signaling pathway. Genes down-regulated in NFPAs are shown in green, while up-regulated genes are in red.

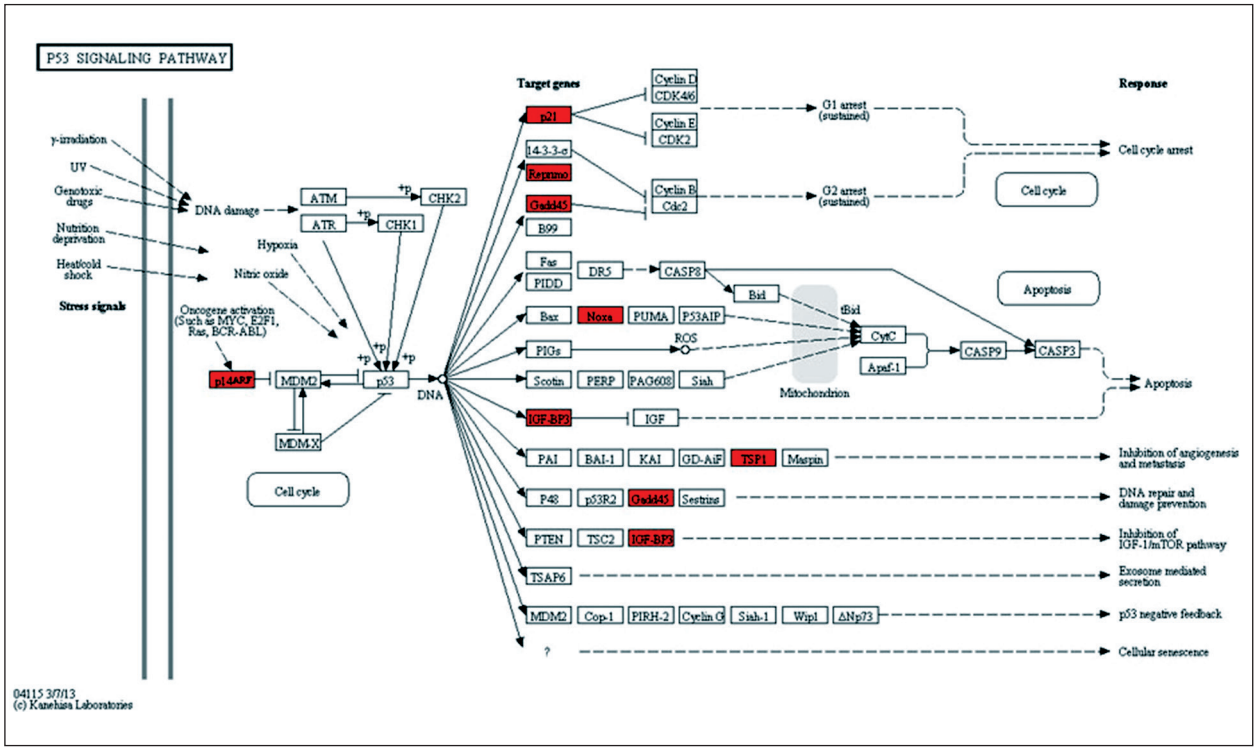

Figure 3. The p53 signaling pathway. Genes down-regulated in NFPAs are shown in green, while up-regulated genes are in red.

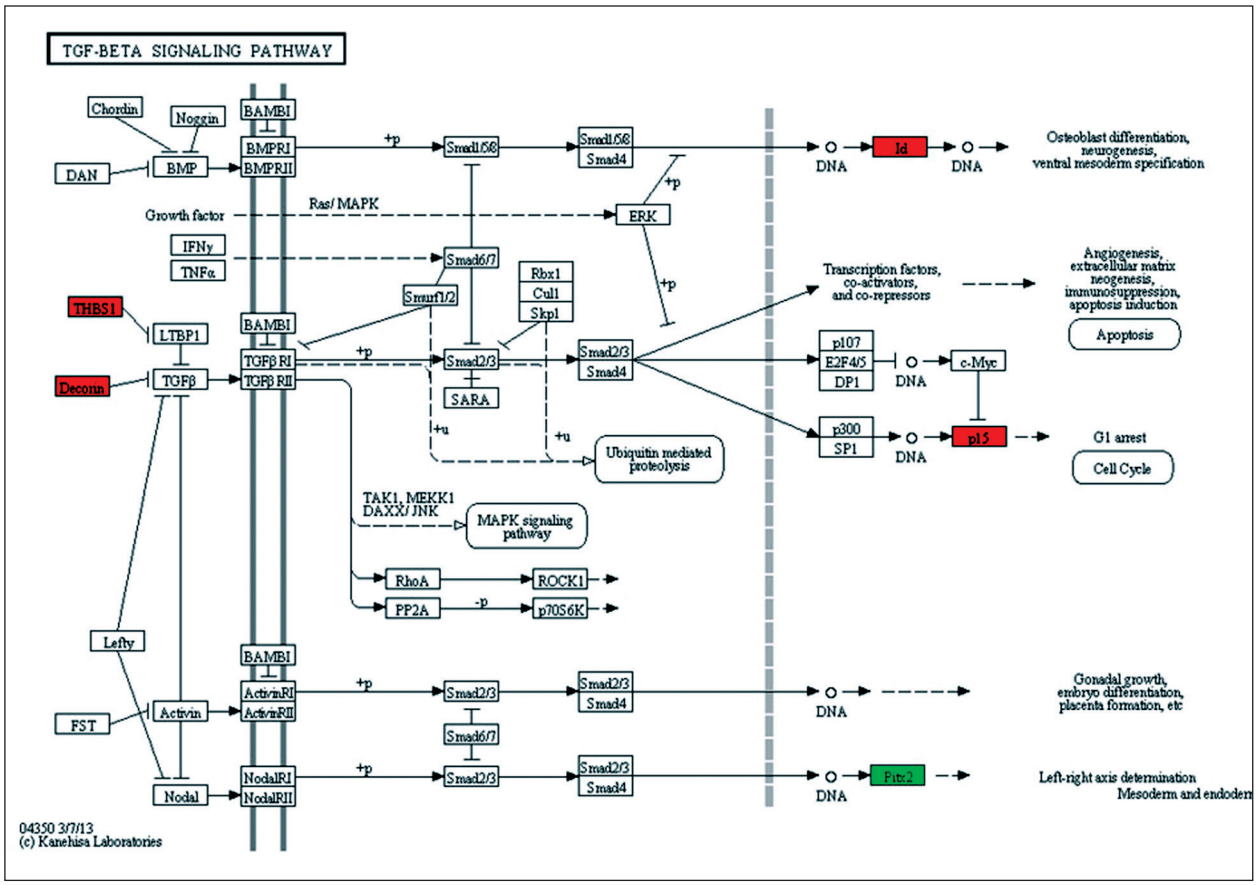

Figure 4. The TGFβ signaling pathway. Genes down-regulated in NFPAs are shown in green, while up-regulated genes are in red.

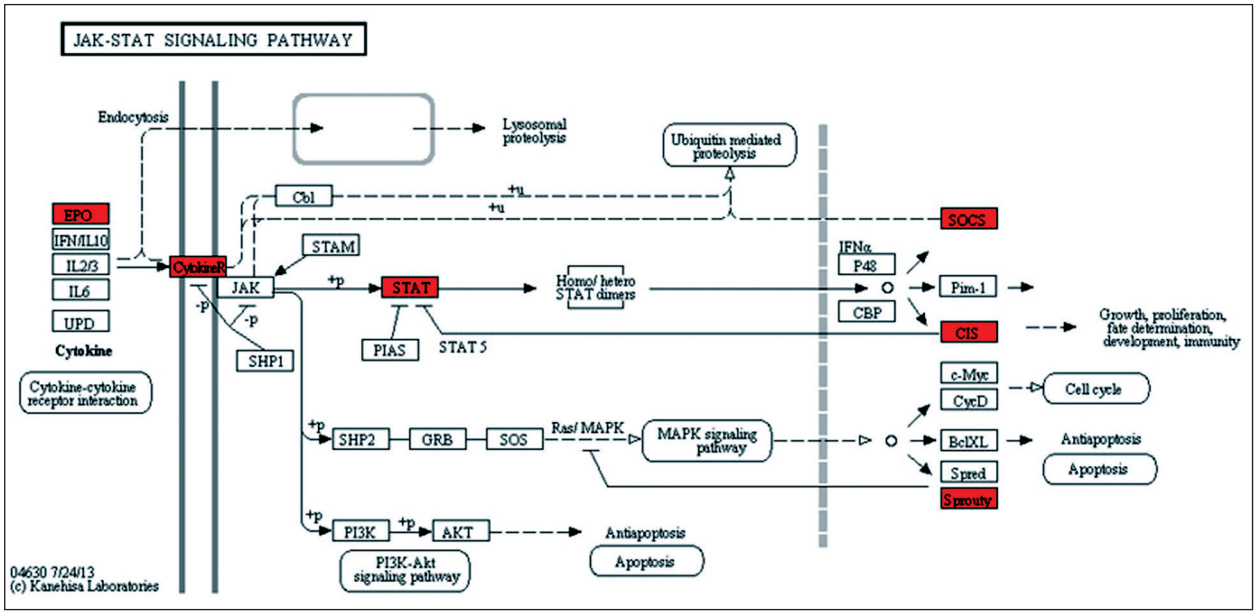

Figure 5. Jak-STAT signaling pathway. Genes down-regulated in NFPAs are shown in green, while up-regulated genes are in red.

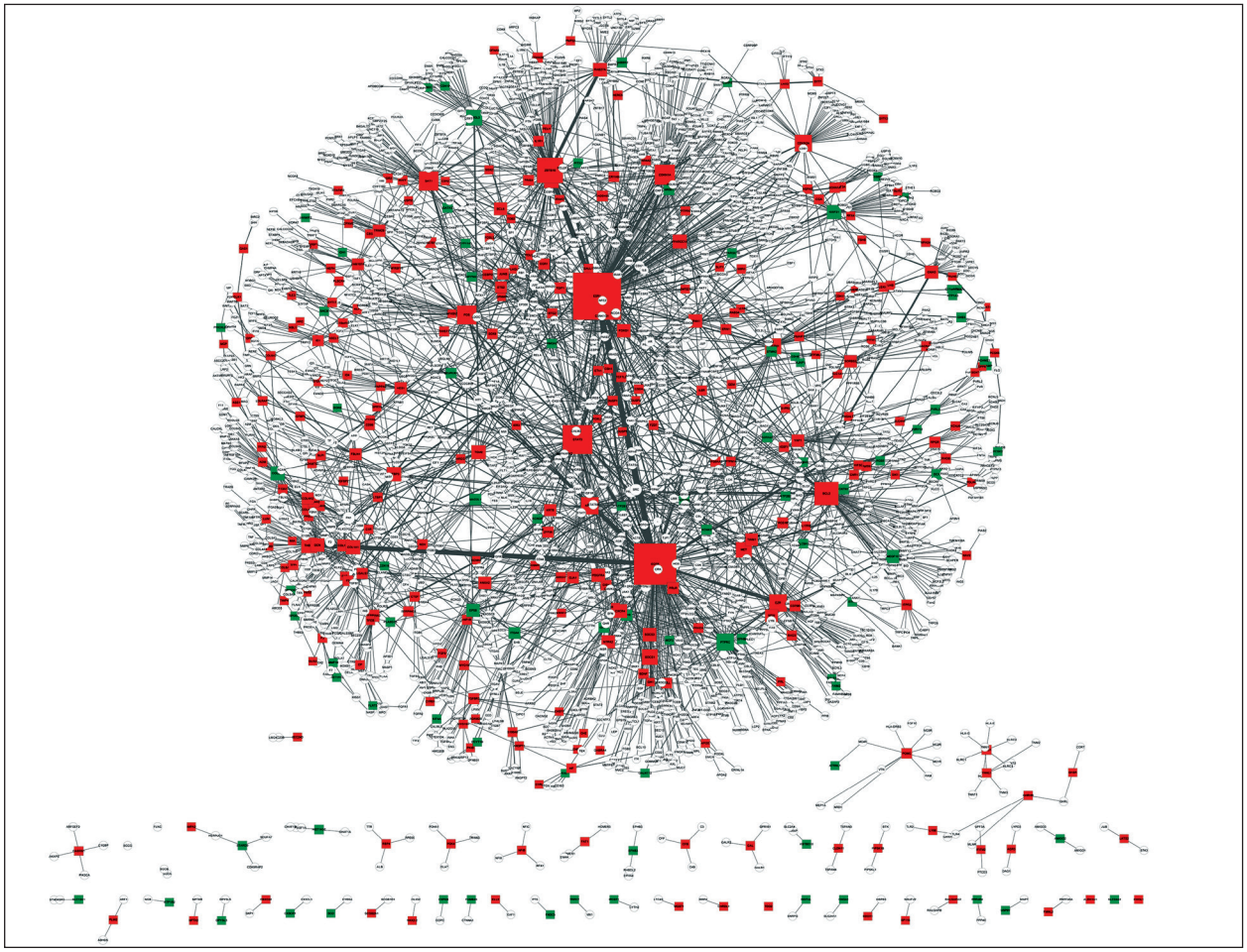

Figure 6. The whole PPI network of DEGs. Red nodes represent the genes up-regulated in NFPAs, and green nodes represent the genes down-regulated in NFPAs. Circle nodes stand for known disease genes, whereas triangle nodes stand for potential novel disease genes. Node size positively correlates with node degree, namely, the number of neighbors. PPI: protein-protein interaction; DEGs: differentially expressed genes; NFPAs: non-functioning pituitary adenomas.

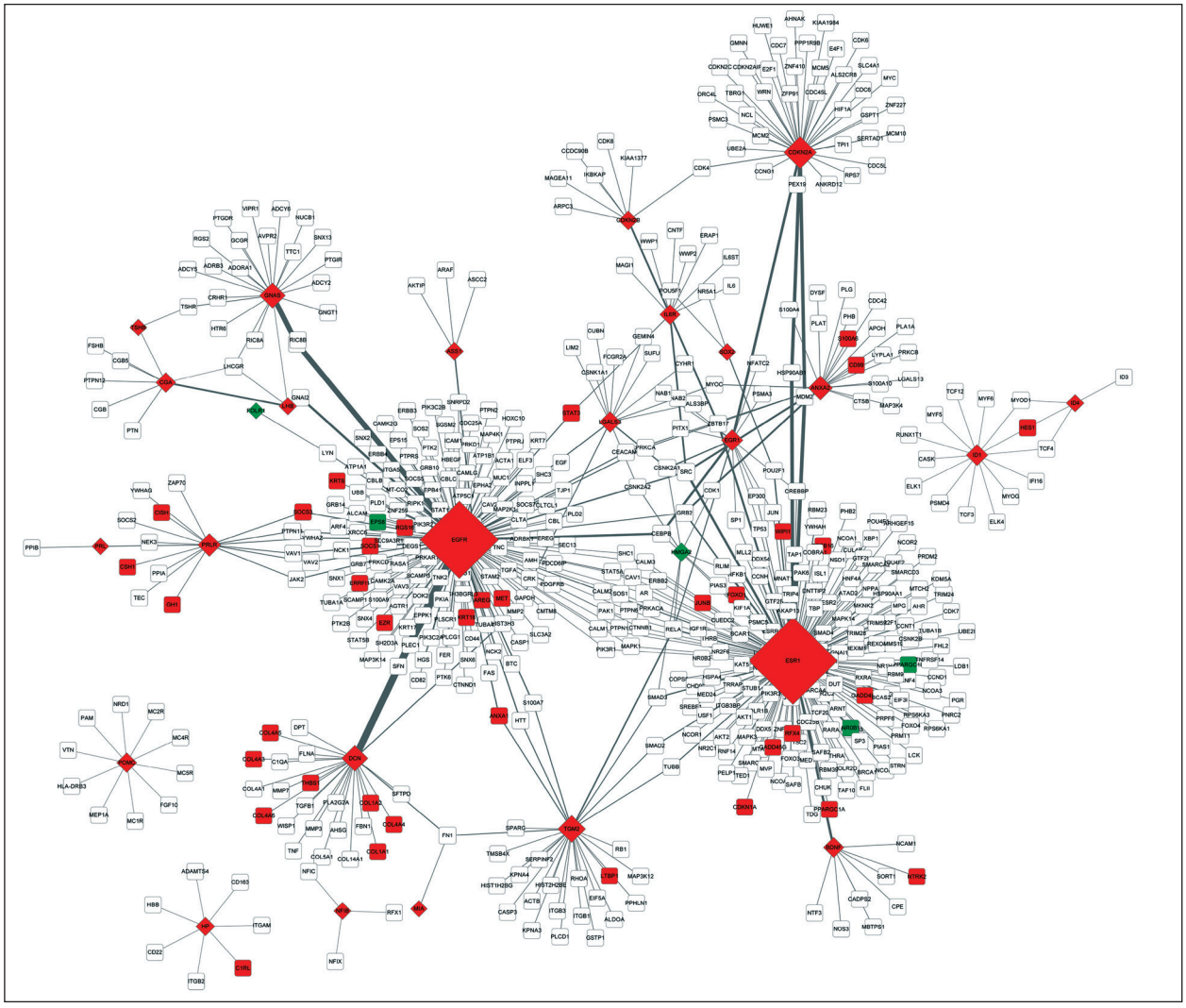

Figure 7. The PPI sub-network containing the top 10 DEGs. Red nodes represent the genes up-regulated in NFPAs, and green nodes represent the genes down-regulated in NFPAs. Circle nodes stand for known disease genes, whereas triangle nodes stand for potential novel disease genes. Node size positively correlates with node degree, namely, the number of neighbors.
PPI: protein-protein interaction; DEGs: differentially expressed genes; NFPAs: non-functioning pituitary adenomas.

base.org/) and compared with the DEGs in the PPI network. Consequently, 99 up- and 288 down-regulated DEGs were known disease genes, *e.g. EGFR* (epidermal growth factor receptor, degree = 63) [10,26-28] and *ESR1* (estrogen receptor 1, degree = 48) [29] (Figure 6). In contrast, 16 up- and 17 down-regulated DEGs were potential novel NFPA-related genes, *e.g. COL4A5* (collagen type IV α5, degree = 17), *LHX3* (LIM homeobox protein 3, degree = 11), *MSN* (moesin, degree = 11) and *GHSR* (growth hormone secretagogue receptor, degree = 10) (Figure 6). Moreover, *COL4A5* interacted with known NFPA-related genes such as *EGFR*, *LHX3* interacted with known NFPAs-related genes like *PRL* (Prolactin), and *MSN* interacted with known NFPA-related genes such as *EGFR*. Among the top 10 up-regulated genes and top 10 down-regulated genes, only 12 DEGs interacted with other DEGs [*e.g. CDKN2A* (cyclin-dependent kinase inhibitor 2A)-*IDH1* (isocitrate dehydrogenase 1)], and all 12 DEGs were known disease genes [*e.g. DLK1* (δ-like 1 homologue)] (Figure 7). In addition, potential NFPA-related gene *GHSR* interacted with the top DEG *GH1* (growth hormone 1).

DISCUSSION

Non-functioning pituitary adenomas comprise about 34.0% of pituitary tumors, while their molecular mechanism is still incompletely understood [5]. In the current study, we comprehensively analyzed the gene expression

profile of NFPAs and healthy pituitary glands. As a result, 604 DEGs were identified between NFPAs and controls, including 177 up- and 427 down-regulated genes, which were much less than those identified by Michaelis *et al.* [14]. However, in the current study, we analyzed the same microarray data using different software, algorithms, and analysis criteria (corrected p value <0.05 and [$\log_2$ FC] >2) in order to focus on the DEGs that were more significant.

In the current study, mean FC of the up-regulated genes was 6.6, and mean FC of the down-regulated genes was –19.2, which were different from those in the previous study by Michaelis *et al.* [14] (4.5 and –32.2, respectively). The differences of mean FC values might be caused by the different DEG sets in the two studies [14]. The major DEGs found by Michaelis *et al.* [14] had similar expression change patterns in the current study, *e.g.* for the *PLAGL1*, CDKN1A, *RPRM*, *PMAIP1*, *MDM2*, *GADD45A*, *GADD45B* and *GADD45G* genes.

Of the top DEGs, *DLK1*, *GH1*, CDKN2A and *MEG3* were significantly down-regulated in NFPAs in comparison with normal pituitary glands in this study. According to the report, the *MEG3* and *DLK1-MEG3* locus are silenced in human NFPAs of gonadotroph origin, and *DLK1-MEG3* locus plays a tumor suppressor role in NFPAs [30]. Based on proteome data and microarray data or reverse transcription quantitative real-time polymerase chain reaction analysis, Moreno *et al.* [31] found that *DLK1*, *GH1* and *PRL* are down-regulated in NFPAs when compared with normal pituitary glands, whereas *IDH1* is significantly up-regulated. The *CDKN2A* and *DLK1* are considered as biomarkers of gonadotroph tumors by Cai *et al.* [15], and gene silencing mediated by hypermethylation of the CpG island within exon 1 in *CDKN2A* is associated with NFPAs [32]. As clearly shown in Figure 7, the expression change patterns of known disease genes *DLK1*, *GH1*, *PRL*, *CDKN2A* and *IDH1*, were consistent with the aforementioned studies [30-32], demonstrating the high accuracy of our results.

Expressions of *EGFR* in NFPAs varied in different studies [10,26-28]. In the current study, *EGFR* showed low expression in NFPAs (Figure 7), and it interacted with known disease gene *CDKN2A*, indicating that low expression of *EGFR* might be associated with NFPAs. We also found that *CDKN2A* was a top DEG, and it interacted with 22 DEGs in the whole PPI network and most DEGs in the PPI sub-network, suggesting that *CDKN2A* might play a crucial role in the progression of NFPAs.

Furthermore, potential novel genes were identified (Figure 6), especially *COL4A5*, *LHX3*, *MSN* and *GHSR*. The role of these genes in NFPAs has not been investigated by previous studies. According to the report, mRNA level of *GHSR* in NFPAs is lower than that in growth hormone-producing PAs [33]. In the present study, *COL4A5*, *LHX3*, *MSN* and *GHSR* were significantly down-regulated in NFPAs in comparison with normal controls, and they interacted with known NFPA-related genes such as *EGFR*, *PRL*, and *GH1*. These results indicated that *COL4A5*, *LHX3*, *MSN* and *GHSR* might participate in the initiation and progression of NFPAs *via* interaction with *EGFR*, *PRL* and *GH1*, respectively.

We found DEGs were significantly enriched in the p53 (Figure 3) and Jak-STAT signaling pathways (Figure 5), which had been reported to take part in PAs pathogenesis [8,24]. The p53 signaling pathway is involved in biological processes such as cell cycle arrest, apoptosis, senescence, DNA repair and changes in metabolism. Expression level of p53 correlates with the proliferative state of PAs [24]. The Jak-STAT pathway is an important downstream pathway for growth factor receptors and cytokine receptors, and it is involved in the regulation of cell proliferation and survival [34,35]. As all of the DEGs mapped on these pathways were remarkably down-regulated in NFPAs, p53 and Jak-STAT signaling pathways might play roles in the progression of NFPAs.

In addition, DEGs were significantly enriched in GO terms mainly about cell communication, signaling, ECM, plasma membrane, collagen, transcription factor activity and receptor binding (Table 2). The ECM, plasma membrane, and receptor binding are the basis of cell communication and signaling between pituitary cells, which play crucial roles in the development and invasion of PAs [36, 37]. As DEGs mapped on these GO terms were remarkably dysregulated in NFPAs, cell communication and signaling might contribute to the progression of NFPAs.

In conclusion, a number of genes (*e.g. COL4A5*, *LHX3*, *MSN* and *GHSR*) identified in this study, might be potential novel NFPA-related genes. Furthermore, cell communication and signaling pathways (*e.g.* p53 and Jak-STAT) might be implicated in the pathogenesis of NFPAs. Currently, no effective medical therapies are available for NFPAs, due to their unclear mechanism. Although further validation is required, our findings might provide information to guide future researchers and even benefit the development of medical therapy for NFPAs.

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