Invasiveness-Related Proteomic Variations and Molecular Network Changes in Human Nonfunctional Pituitary Adenomas

Xianquan Zhan, Xiaohan Zhan and Xiaowei Wang

Abstract

The invasive characteristic of nonfunctional pituitary adenoma (NFPA) is an important clinical problem without a clear molecular mechanism, which severely challenges its treatment strategy. Clarification of the proteomic alterations between invasive and non-invasive NFPA is the key step for in-depth understanding of its mechanisms and discovering reliably invasive biomarkers. Two-dimensional gel electrophoresis (2DGE)-based comparative proteomics was carried out between four invasive and four non-invasive NFPA. A total of 64 upregulated protein-spots and 39 downregulated protein-spots were identified among 24 (invasive n = 12; non-invasive n = 12) 2DGE maps (ca. 1200 spots/gel). Mass spectrometry identified 30 upregulated proteins and 27 downregulated proteins between invasive and non-invasive NFPA. Those 57 differentially expressed proteins are involved in multiple biological functions, including oxidative stress, mitochondrial dysfunction, MAPK signaling alteration, proteolysis abnormality, CDK-C signaling, amyloid processing, and TR/RXR activation. These findings provide important clues to insights into molecular mechanisms of invasive NFPA and to discovery of effective biomarkers for effective treatment of invasive NFPA patients.

Keywords: invasive nonfunctional pituitary adenoma, two-dimensional gel electrophoresis, mass spectrometry, proteome, comparative proteomics, invasive biomarker

1. Introduction

Invasive pituitary adenoma is a type of pituitary adenoma that locally invades contiguous anatomy structures surrounding pituitary gland [1–6]. In fact, the rate of local invasion is about 40% of pituitary adenoma patients with macroscopic observation, and even up to 80% of pituitary adenoma patients with microscopic observation [1, 7, 8] although most pituitary adenomas are align. Magnetic resonance imaging (MRI) is commonly used method to measure the size of pituitary adenomas, and can classify pituitary adenomas into giant adenomas (>40 mm), macro-plus adenomas (20–30 mm), macroadenomas (10–20 mm), and microadenomas (<10 mm) [5, 7]. Furthermore, based on preoperative MRI and perioperative observation, pituitary adenomas are classified into grade I (enclosed
microadenoma, <10 mm), grade II (enclosed macroadenoma, >10 mm), grade III (localized perforation of the sellar floor), and grade IV (diffuse destruction of the sellar floor) [9]. Grades III and IV are commonly looked as invasive pituitary adenomas. Invasiveness is very challenging clinical problem in pituitary adenoma patient, which reasons are that (1) invasiveness suppresses and/or damage surrounding structures because of the limited intracranial cavity and around important structure tissues, and (2) invasiveness causes incomplete removal of pituitary adenoma in neurosurgery to increase risks of complications including recurrence and poor outcome and need adjuvant therapy (radiotherapy or medications) [1]. However, the molecular mechanisms of pituitary adenoma invasiveness remain unclear, although some studies [10] found more vascular evidence in invasive pituitary adenomas compared to non-invasive tumors to indicate the role of angiogenesis [10], and some molecular and genetic changes in invasive pituitary adenomas including downregulation and methylation of CDH13 (H-cadherin) and CDH1 (E-cadherin) [11], loss of death-associated protein kinase and CpG island methylation [12], and loss of heterozygosity at 11q13 (MEN1 locus) and 13q (retinoblastoma gene RB locus) without mutation and overexpression of p53 and without homozygous deletions of p15 or p16 [13]. Multiomics analysis is an effective approach to investigate systematically molecular mechanisms of invasiveness of pituitary adenomas [14–19]. Quantitative transcriptomics analysis [9, 20] identified differentially expressed gene (DEG) profiling (346 DEGs, including 233 upregulated and

**Figure 1.**

Experimental flow-chart to comparatively study the proteomes between invasive and non-invasive NFPAs. Reproduced from Zhan et al. [5], with copyright permission from Wiley-VCH, copyright year 2014.
113 downregulated) between invasive and non-invasive NFPAs. However, protein and its proteoforms are the functional performer of each gene, proteome is much more complex than transcriptome, and the coefficient of correlation is very low (about 0.4) in consistence analysis between proteome and transcriptome for the same tissue sample [21, 22]. Therefore, it is necessary to use proteomics for pituitary adenoma invasiveness [23, 24]. A comparative proteomics experiment revealed 30 differentially expressed proteins (DEPs) profiling between invasive and non-invasive pituitary adenoma tissues [25], however, this study did not distinguish the functional and non-functional pituitary adenomas (FPAs, and NFPAs). This chapter focused on the proteomic variations and molecular network changes in invasive relative to noninvasive NFPAs, investigated with two-dimensional gel electrophoresis (2DGE) coupled with mass spectrometry (MS) and pathway network analysis. The findings offer the scientific data to discover protein biomarkers for effective treatment of invasive NFPAs. An experimental flow-chart is shown to study proteomes between invasive and noninvasive NFPAs (Figure 1).

2. Materials and methods

2.1 2DGE analysis of pituitary adenoma specimen

The invasive (n = 4) and non-invasive (n = 4) NFPA tissues with pathological diagnosis were used in this study. Each tissue sample was used to individually extract proteins, and the protein content was quantified. Each tissue sample was analyzed with 2DGE for 3–4 times [5, 22]. For each 2DGE analysis, 150 μg proteins were used for isoelectric focusing (IEF) with IPG strips pH 3–10 NL (180 × 3 × 0.5 mm). After IEF, the proteins were reduced and alkalinized, and then were separated with the 12% PAGE resolving gel (250 × 215 × 1.0 mm), followed by visualization with modified silver-staining [26]. The PDQuest 2D gel analysis software (version 7.1.0; Bio-Rad) was used to digitize and compare 2DGE gel images between invasive and non-invasive NFPAs. A total of 12 gel images for invasive NFPAs and 12 gel images for non-invasive NFPAs were used in this analysis to determine each DEPs with a 3-fold cutoff values and p < 0.05. In addition, four standard proteins, including myoglobin (17 kDa; p. 7.6), carbonic anhydrase (29 kDa; p. 7.0), ovalbumin (45 kDa; p. 5.1), and amyloglucosidase (89/70 kDa; p. 3.8), were applied to measure the observed pI and Mr on the 2D gel.

2.2 Mass spectrometry analysis of 2DGE-separated proteins

The protein that contains in gel spot was digested in-gel with trypsin, followed by ZipTipC18 purification [5, 26]. For LC-ESI-MS/MS analysis, the purified tryptic peptides were eluted in 6 μl of 85% acetonitrile plus 0.1% TFA, air-dried, and then resuspended in 6 μl of 85% acetonitrile plus 0.1% formic acid. The prepared peptide samples were analyzed by LC-ESI-qTOF mass spectrometer to obtain MS/MS spectrum. For MALDI-TOF-MS analysis, the ZipTipC18 peptides were directly eluted on MALDI plate with 2 μl of a-cyano-4-hydroxycinnamic acid solution (seven cycles), and dried, and then were analyzed with Voyager DE STR MALDI-TOF mass spectrometer to obtain peptide mass fingerprint (PMF). The MS/MS data and PMF data were used to search SwissProt database with Mascot software for protein identification.

2.3 Bioinformatics

The software NIHDAVID (version 6.7, http://david.abcc.ncifcrf.gov/summary.jsp) was used to carry out gene-ontology (GO) analysis, including cellular
components (CC), molecular functions (MF), and biological processes (BP), and furtherly were categorized into different functional clusters. Ingenuity pathway analysis (IPA) (www.ingenuity.com) [27] was applied to obtain statistically significant signaling pathways with identified DEP data between invasive and non-invasive NFPAs.

3. Results and discussion

3.1 2DE pattern and DEP profile between invasive and noninvasive NFPA proteomes

Each NFPA tissue sample (four invasive NFPAs and four non-invasive NFPAs) was analyzed by 2DGE for 3–5 times to guarantee at least three high-quality gel images. Thus, 24 high-quality 2DGE images (12 gel-images for invasive NFPAs; 12 gel images for non-invasive NFPAs) were obtained. About 1200 spots (an average of 1172 spots for invasive NFPAs and 1213 spots for non-invasive NFPAs) were present in each gel image (Figure 2), and most of spots were distributed within pH 4–9 and Mr 15–150 kDa [21]. The average between-gel matched percentage was 64% (61–67%) among invasive NFPA gels, and 67% (61–69%) among non-invasive NFPA gels. The positional deviation of the matched-spots was 2.05 ± 0.89 mm in the IEF direction and 1.41 ± 0.65 mm in the SDS-PAGE direction. For each sample, the average correlation coefficient (r) of the normalized volumes for between-gel
matched-spots was 0.74 (range, 0.59–0.83), with a best-fit line of:

\[ y = 0.8685x + 0.0804 \quad (r = 0.87; n = 811) \]

The normalized spot volumes between 12 invasive NFPA gels and 12 non-invasive NFPA gels were compared to determine a differential protein spot with at least 3-fold change and \( p < 0.05 \). For example, Spot-2010 was identified as differential protein spots downregulated in invasive NFPA s compared to non-invasive NFPA s (Figure 3). With the same approach, 103 differential spots were identified, including 64 upregulated and 39 downregulated protein spots in invasive NFPA s relative to non-invasive NFPA s (Table 1 and Figure 1). It clearly demonstrated that the proteome was significantly different between invasive and non-invasive NFPA s.

Furthermore, each DEP in the differential spot was identified with MS [26]. For MALDI-TOF-MS PMF analysis, all interfering masses derived from contaminants including keratins, trypsin, matrix CHCA, and other unknown ones, were removed from MS spectrum of analyzed sample to obtain a corrected mass list for PMF data (Figure 4). Those nine masses labeled in Figure 4B were used with MASCOT PMF search tool to search Swiss-Prot database, and matched to the corresponding tryptic peptides from 78 kDa glucose-regulated protein (GRP78_HUMAN; P11021) (Figure 5), which was the DEP identified in the differential Spot-1809. With the same method, 43 DEPs was identified with PMF analysis (Figure 1 and Table 1). For LC-ESI-MS/MS analysis, the tryptic peptides were separated by LC and then sequenced by MS/MS on the qTOF MS instrument, followed by MASCOT MS/MS data search in the human Swiss-Prot database. For example, six tryptic peptides from Spot-7604 were sequenced and matched to ATP synthase subunit alpha (ATPA_HUMAN; P25705) (Figure 6). With the same method, 11 DEPs were identified with MS/MS data (Figure 1 and Table 1). A total of 57 DEPs, including 30 upregulated and 27 downregulated, were identified in invasive compared to non-invasive NFPA s (Table 1).

### 3.2 Functional characteristics of DEPs identified in invasive relative to noninvasive NFPA s

A total of 54 DEPs out of 57 DEPs were eligible for GO analysis to identify the significant BPs, CCs, and MFs, which are further grouped with hierarchical cluster into to functional clusters (Table 2). It clearly demonstrated those DEPs participated in multiple biological functions to associate with NFPA invasiveness, including peptidase and proteolysis, nucleotide metabolism, mitochondrial functions and oxidative stress, and protein kinase and cell signaling.

A total of 54 DEPs out of 57 DEPs were accepted for IPA pathway-network analysis to identify significant molecular networks and signaling pathways and...
| SSP | Swiss Prot No. | Protein name | Mr (kDa) | pI  | Fold |
|-----|----------------|--------------|----------|-----|------|
| 0011 | Q00535 | Cyclin-dependent kinase 5 | 17.56 | 33.74 | 4.04 | 7.57 | 13.6 |
| 0045 | P04434 | Ig kappa chain V-III region VH (fragment) | 14.35 | 12.86 | 4.04 | 5.63 | 10.2 |
| 0029 | P00742/Q8N4Z0 | Chain 1: factor X light chain/putative Ras-related protein Rab-42 | 16.72 | 54.73/11.59 | 4.90 | 5.68/5.84 | 3.0 |
| 0101 | P23297 | Protein S100-A1 | 21.07 | 10.54 | 4.12 | 4.39 | 18.6 |
| 0411 | P04264 | Keratin, type II cytoskeletal 1 | 37.57 | 66.17 | 5.05 | 8.15 | 5.4 |
| 0221 | Q5JXM2 | Methyltransferase-like protein 24 | 25.65 | 41.87 | 4.71 | 9.41 | 4.6 |
| 0416 | P01040 | Cystatin-A | 39.04 | 11.00 | 5.04 | 5.38 | 4.6 |
| 0402 | Q14314 | Fibroleukin | 40.94 | 50.82 | 4.24 | 7.08 | 7.7 |
| 0511 | P08779/P4040 | Cytokeratin 16/catalase | 45.70 | 51.27/59.95 | 4.89 | 4.98/6.90 | 16.0 |
| 1712 | P56817 | Beta-secretase 1 | 61.24 | 56.36 | 5.25 | 5.24 | 8.5 |
| 2608 | P78536 | Disintegrin and metalloproteinase domain-containing protein 17 | 52.29 | 94.56 | 5.48 | 5.5 | 3.5 |
| 2133 | Q9BYM8 | RanBP-type and C3HC4-type zinc finger-containing protein 1 | 24.67 | 59.35 | 5.52 | 5.47 | 6.5 |
| 2730 | Q8N3R9 | MAGUK p55 subfamily member 5 | 68.52 | 77.53 | 5.62 | 5.77 | 3.2 |
| 2707 | Q9Y3B9 | RRP15-like protein | 66.35 | 31.64 | 5.53 | 5.39 | 3.6 |
| 3308 | P29466 | Caspase-1 | 35.79 | 45.81 | 5.86 | 5.63 | 4.0 |
| 3013 | P07108 | Acyl-CoA-binding protein | 13.27 | 10.04 | 5.98 | 6.12 | 3.5 |
| 3512 | A2VDF0 | Fucos mutarotase | 42.54 | 16.93 | 5.98 | 5.49 | 14.4 |
| 4407 | Q9UIY3 | RWD domain-containing protein 2A | 37.06 | 34.21 | 6.13 | 6.01 | 24.3 |
| 4701 | Q99797 | Mitochondrial intermediate peptidase | 65.37 | 81.38 | 6.04 | 6.6 | 12.4 |
| 4615 | Q96BJ3 | Axin interactor, dorsalization-associated protein | 56.24 | 35.17 | 6.25 | 6.13 | 3.4 |
| 4807 | Q16891 | Mitochondrial inner membrane protein | 80.13 | 84.03 | 6.09 | 6.08 | 10.6 |
| 7014 | P18988 | Hemoglobin beta-2 chain (PANLE) | 16.98 | 15.93 | 7.33 | 7.25 | 3.4 |
| 7021 | P06576 | ATP synthase subunit beta | 12.29 | 56.56 | 6.98 | 5.26 | 4.2 |
| 6313 | Q8NA31 | Coiled-coil domain-containing protein 79 | 33.06 | 84.55 | 6.92 | 7.29 | 3.2 |
| 8512 | Q8N823 | Zinc finger protein 611 | 41.66 | 81.39 | –1.00 | 9.16 | 37.4 |
| 8513 | Q9Y6N3 | Calcium-activated chloride channel regulator family member 3 | 42.45 | 30.29 | –1.00 | 8.42 | 3.2 |
| 8212 | Q9P267/P01834 | Methyl-CpG-binding domain protein 5/15 Ig kappa chain C region | 28.38 | 159.90/11.61 | –1.00 | 9.17/5.58 | 7.8 |
| 7616 | Q9P267 | Methyl-CpG-binding domain protein 5 | 55.37 | 16.12 | 7.14 | 9.17 | 6.1 |
| 1602 | A4FU49 | SH3 domain-containing protein 21 | 53.77 | 70.52 | 5.13 | 5.6 | –7.4 |
| 2010 | P60983 | Gli maturation factor beta | 15.82 | 16.87 | 5.45 | 5.19 | –5.0 |
| 2106 | P01241/P02792 | Chain 1: somatotropin/ferritin light chain | 21.87 | 24.85/20.06 | 5.44 | 5.29/5.51 | –5.9 |
| 1809 | P11021 | 78 kDa glucose-regulated protein | 78.19 | 72.4 | 5.24 | 5.07 | –4.6 |
| 2101 | P01241 | Chain 1: somatotropin | 23.95 | 24.85 | 5.38 | 5.29 | –11.1 |
molecular networks. Three molecular networks were identified (Figure 7). The hub molecules among those three molecular networks included ATPase, MAPK, ERK, ERK1/2, p38, Jnk, NFkB, AKT, PKA, PKC, EGFR, K-RAS, insulin, UBC, CCND1, IFNG, ESR1, CDK5, calmodulin, and S100A1, which are obviously associated with cancer biological systems. About 19 statistically significant canonical pathways were mined from DEPs data (Figure 8), including superoxide radical degradation, mitochondrial dysfunction, eNOS signaling, inhibition of matrix metalloprotease, CDK5 signaling, endoplasmic reticulum stress pathway, ketolysis, ketogenesis, TR/RXR activation, amyloid processing, endothelin-1 signaling, and more.

Table 1. Differentially expressed proteins between invasive and non-invasive NFPAs identified with 2DGE and mass spectrometry (fold > 3-fold or < −3-fold).

| SSP | Swiss-Prot No. | Protein name | Mr (kDa) | pI | Fold |
|-----|---------------|--------------|----------|----|------|
|     |               |              | Exp.     | Theor. | Exp.     | Theor. |
| 2625 | P07332       | Tyrosine-protein kinase Fes/Fps | 52.98  | 94.12 | 5.54  | 6.27  | −7.6 |
| 4517 | Q8TB05       | UBA-like domain-containing protein 1 | 47.39  | 19.06 | 6.26  | 6.14  | −5.6 |
| 3612 | Q96DQ5       | DEP domain-containing protein 7 | 50.55  | 58.62 | 5.88  | 7.62  | −4.6 |
| 5711 | P38405       | Guanine nucleotide-binding protein G (olf) subunit alpha | 73.36  | 44.79 | 6.49  | 6.23  | −4.1 |
| 5415 | A6NHL2       | Tubulin alpha chain-like 3 | 36.72  | 50.68 | 6.56  | 5.68  | −10.7 |
| 6207 | P32785       | Kinesin light chain 1 | 27.69  | 63.74 | 6.78  | 5.73  | −11.6 |
| 5702 | P42704       | Leucine-rich motif-containing protein, mitochondrial | 73.4  | 159   | 6.35  | 5.81  | −5.8  |
| 6414 | Q9UL42       | Paraneoplastic antigen Ma2 | 38.19  | 41.71 | 6.8   | 4.84  | −10.3 |
| 6513 | Q9UPQ3       | Arf-GAP with GTPase, ANK repeat and PH domain-containing protein 1 | 44.44  | 95.38 | 6.91  | 8.18  | −17.9 |
| 6608 | Q7Z377 /
Q9Y6G9 | Zinc finger protein 572/cytoplasmic dynein 1 light intermediate china 1 | 48.91  | 63.12/  | 6.75  | 8.32/  | −7.3 |
| 6603 | Q9HD45       | Transmembrane 9 superfamily member 3 | 53.47  | 68.58 | 6.68  | 6.83  | −22.5 |
| 6616 | P01859       | Ig gamma-2 chain C region | 52.36  | 35.9  | 6.87  | 7.66  | −33.0 |
| 7022 | P02080       | Hemoglobin beta-C | 14.09  | 15.68 | 7.27  | 11.58 | −13.8 |
| 7604 | P25705       | ATP synthase subunit alpha, mitochondrial | 53.63  | 59.83 | 6.99  | 9.16  | −94.3 |
| 7302 | Q96CN7       | Isochorismatase domain-containing protein 1 | 32.25  | 32.5  | 6.99  | 6.96  | −3.9  |
| 7519 | Q99542       | Chain 1: matrix metalloproteinase-19 | 43.84  | 57.36 | 7.45  | 7.22  | −7.0  |
| 7802 | Q96KP1       | Exocyst complex component 2 | 80.76  | 105.1 | 6.98  | 6.46  | −16.3 |
| 7708 | Q02338       | D-beta-hydroxybutyrate dehydrogenase, mitochondrial | 72.25  | 38.53 | 7.11  | 9.11  | −4.9  |
| 8503 | Q9NP18       | Fanconi anemia group F protein | 44.22  | 42.46 | 7.53  | 9.11  | −8.0  |
| 8405 | P17066       | Heat shock 70 kDa protein 6 | 38.49  | 71.44 | 7.55  | 5.81  | −25.6 |
| 8409 | P25101       | Endothelin-1 receptor | 41.13  | 49.89 | −1.00 | 8.73  | −7.6  |

*It was identified with LC-ESI-MS/MS, and the others with MALDI-TOF-PMF. Fold (+) means that it is upregulated in invasive relative to noninvasive NFPAs. Fold (−) means that it is downregulated in invasive relative to noninvasive NFPAs. Exp. pI = 1.00 means that it was out of the pI range of standard markers. Reproduced from Zhan et al. [5], with copyright permission from Wiley-VCH, copyright year 2014.
Figure 4.
All interfering masses from contaminants derived from the margin blank gel on a silver-stained 2D gel map (A) were removed from MALDI-TOF-MS spectrum derived from the proteins in Spot-1809 (B) to obtain a corrected mass list for PMF data that were labeled as the symbol *. Reproduced from Zhan et al. [5], with copyright permission from Wiley-VCH, copyright year 2014.

A. Summary of Mascot search result

Figure 5.
Mascot search results from PMF data (Spot-1809). Modified from Zhan et al. [5], with copyright permission from Wiley-VCH, copyright year 2014.
Invasiveness-Related Proteomic Variations and Molecular Network Changes in Human...
DOI: http://dx.doi.org/10.5772/intechopen.85546

A. MATRIX SCIENCE MASCOT Search Results (Spot-7604)
MS data file: C:\Zhan\Paper\QTOF\20130719-2iq-7604.pk
Dataset: SwissProt 2013_08 (540,732 sequences; 192,091,492 residues)
Taxonomy: Homo sapiens (human) (20,267 sequences)
Timestamp: 29 Aug 2013 at 09:54:19 GMT

MASCOT Score distribution

![MASCOT Score distribution graph]

**Protein family summary**

| Code | Swiss-Prot No | Score | Protein |
|------|---------------|-------|---------|
| 1    | ATPA_HUMAN    | 89    | ATP synthase subunit alpha, mitochondrial |
| 2    | K2CI_HUMAN    | 76    | Keratin, type II cytoskeletal 1 OS=Homo sapiens |
| 3    | FIBB_HUMAN    | 50    | Fibrinogen beta chain OS=Homo sapiens |
| 4    | K2ZE_HUMAN    | 50    | Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens |

B. Protein View for ATPA_HUMAN

ATPA_HUMAN/ATP synthase subunit alpha, mitochondrial  OS=Homo sapiens  GN=ATPAH  PE=1  SV=1  Database: SwissProt/Score: 55/100/Score coverage: 16%

**Sequence:**

MS/MS fragmentation of TQAI(DVPVGEEGLGR (Query 307)

**MS/MS Fragmentation of**

TQAI(DVPVGEEGLGR (Query 307)

**Found in** ATPA_HUMAN in SwissProt, ATP synthase subunit alpha, mitochondrial OS=Homo sapiens GN=ATPAH PE=1 SV=1

**Match to Query 307: 1624.559848 from(813.287200;2+) intensity(1415.4703) index(240)**

**Data file:** C:\Zhan\Paper\QTOF\20130719-2iq-7604.pk

Figure 6.
Mascot search results from a representative LC-ESI-MS/MS data from proteins in Spot-7604. Modified from Zhan et al. [5], with copyright permission from Wiley-VCH, copyright year 2014.
| Category Term Count | P-value | Proteins (DEPs) |
|---------------------|---------|-----------------|
| **Annotation Cluster 1** |
| **GOTERM_BP_FAT** Regulation of protein kinase cascade | 5 | 5.56E - 03 | P29466, Q96Bf3, P00742, P01241, P04040 |
| **GOTERM_BP_FAT** Positive regulation of signal transduction | 5 | 1.00E - 02 | P29466, P00742, P01241, P04040, P78536 |
| **GOTERM_BP_FAT** Positive regulation of protein kinase cascade | 4 | 1.22E - 02 | P29466, P00742, P01241, P04040 |
| **GOTERM_BP_FAT** Positive regulation of cell communication | 5 | 1.45E-02 | P29466, P00742, P01241, P04040, P78536 |
| **Annotation Cluster 2** |
| **GOTERM_MF_FAT** Endopeptidase activity | 6 | 3.99E - 03 | P29466, P00742, Q99542, Q99797, P56817, P78536 |
| **GOTERM_MF_FAT** Peptidase activity, acting on L-amino acid peptides | 6 | 1.89E - 02 | P29466, P00742, Q99542, Q99797, P56817, P78536 |
| **GOTERM_MF_FAT** Peptidase activity | 6 | 2.25E - 02 | P29466, P00742, Q99542, Q99797, P56817, P78536 |
| **GOTERM_BP_FAT** Proteolysis | 8 | 2.92E - 02 | P29466, P00742, Q99542, Q9BYM8, Q99797, P04264, P56817, P78536 |
| **GOTERM_MF_FAT** Metalloendopeptidase activity | 3 | 3.53E - 02 | Q99542, Q99797, P78536 |
| **Annotation Cluster 3** |
| **GOTERM_CC_FAT** Mitochondrial lumen | 5 | 3.29E - 03 | Q02338, P06576, P42704, P25705, Q99797 |
| **GOTERM_CC_FAT** Mitochondrial matrix | 5 | 3.29E - 03 | Q02338, P06576, P42704, P25705, Q99797 |
| **GOTERM_CC_FAT** Mitochondrial part | 7 | 5.08E - 03 | Q02338, P06576, P42704, P25705, Q99797, P04040, Q16891 |
| **GOTERM_CC_FAT** Organelle envelope | 7 | 6.20E - 03 | Q02338, P06576, P42704, P25705, P04040, P25101, Q16891 |
| **GOTERM_CC_FAT** Envelope | 7 | 6.29E - 03 | Q02338, P06576, P42704, P25705, P04040, P25101, Q16891 |
| **GOTERM_CC_FAT** Organelle inner membrane | 5 | 1.20E - 02 | Q02338, P06576, P42704, P25705, Q16891 |
| **GOTERM_CC_FAT** Mitochondrial envelope | 5 | 2.67E - 02 | Q02338, P06576, P25705, P04040, Q16891 |
| **GOTERM_CC_FAT** Organelle membrane | 8 | 2.68E - 02 | Q02338, P06576, P42704, P25705, P11021, P04040, P25101, Q16891 |
| **GOTERM_CC_FAT** Mitochondrial membrane part | 3 | 4.58E - 02 | P06576, P25705, Q16891 |
| **GOTERM_CC_FAT** Mitochondrial inner membrane | 4 | 5.06E - 02 | Q02338, P06576, P25705, Q16891 |
| **Annotation Cluster 4** |
| **GOTERM_BP_FAT** Response to organic substance | 7 | 1.63E - 02 | P29466, Q00535, P01241, P38405, P25101, P17066, P78536 |
| **Annotation Cluster 5** |
### Category Term Count P-value Proteins (DEPs)

| Category           | Term                                                | Count | P-value         | Proteins (DEPs)                                                                 |
|--------------------|-----------------------------------------------------|-------|-----------------|-------------------------------------------------------------------------------|
| GOTERM_BP_FAT      | Proteolysis                                         | 8     | 2.92E - 02      | P29466, P00742, Q99542, Q9BYM8, Q99797, P04264, P56817, P78536               |
| GOTERM_BP_FAT      | Protein processing                                  | 3     | 4.13E - 02      | P29466, Q99797, P04264                                                        |
| GOTERM_BP_FAT      | Protein maturation                                  | 3     | 4.81E - 02      | P29466, Q99797, P04264                                                        |
| Annotation Cluster 6 |                                      |       |                 |                                                                               |
| GOTERM_BP_FAT      | Response to alkaloid                                | 3     | 1.05E - 02      | Q00535, P38405, P25101                                                        |
| GOTERM_BP_FAT      | Response to organic substance                       | 7     | 1.63E - 02      | P29466, Q00535, P01241, P38405, P25101, P17066, P78536                      |
| GOTERM_BP_FAT      | Positive regulation of molecular function           | 6     | 2.56E - 02      | Q00535, P01241, P38405, P04040, P25101, P78536                             |
| GOTERM_BP_FAT      | Positive regulation of protein kinase activity      | 4     | 2.61E - 02      | Q00535, P01241, P25101, P78536                                             |
| Annotation Cluster 7 |                                      |       |                 |                                                                               |
| GOTERM_BP_FAT      | Purine ribonucleotide binding                       | 11    | 2.91E - 02      | Q9Y6G9, P06576, P07332, Q8N4Z0, Q00535, P25705, Q9UPQ3, P11021, P38405, P17066, A6NHL2 |
| GOTERM_BP_FAT      | Ribonucleotide binding                              | 11    | 2.91E - 02      | Q9Y6G9, P06576, P07332, Q8N4Z0, Q00535, P25705, Q9UPQ3, P11021, P38405, P17066, A6NHL2 |
| GOTERM_BP_FAT      | Purine nucleotide binding                           | 11    | 3.80E - 02      | Q9Y6G9, P06576, P07332, Q8N4Z0, Q00535, P25705, Q9UPQ3, P11021, P38405, P17066, A6NHL2 |
| GOTERM_BP_FAT      | Nucleotide binding                                  | 12    | 4.37E - 02      | Q9Y6G9, P06576, P07332, Q8N4Z0, Q00535, P25705, Q9UPQ3, P11021, P38405, P17066, A6NHL2 |

### Annotation Cluster 8
semaphoring signaling in neurons, axonal guidance signaling, neuregulin signaling, and primary immunodeficiency signaling [5]. Also, 10 significant toxicological events were identified with those DEP data, including mitochondrial dysfunction, decreased permeability transition/transmembrane potential/depolarization of mitochondria and mitochondrial membrane, anti-oxidative response panel, and TR/RXR activation. Our previous studies also revealed that MAPK-signaling

| Category                | Term                | Count | P-value   | Proteins (DEPs)                                                                 |
|-------------------------|---------------------|-------|-----------|--------------------------------------------------------------------------------|
| GOTERM_BP_FAT           | Proteolysis         | 8     | 2.92E−02  | P29466, P00742, Q99542, Q9BYM8, Q99797, P04264, P56817, P78536                  |
| Annotation Cluster 9    |                     |       |           |                                                                                |
| GOTERM_MF_FAT           | Calcium ion binding | 7     | 4.37E−02  | P06576, P00742, QY6N3, Q99542, P23297, P11021, Q99797                         |
| Annotation Cluster 10   |                     |       |           |                                                                                |
| GOTERM_CC_FAT           | Cell surface        | 5     | 1.45E−02  | P06576, P00742, P11021, P56817, P78536                                       |

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Table 2. The functional categories of 54 DEPs identified by GO analysis.

Figure 7. Significant molecular networks changed in invasive NFPAs. (A) Network 1 functioned in inflammatory disease and inflammatory response. (B) Network 2 functioned in tumor morphology, cancer, cell-to-cell signaling, and interaction. (C) Network 3 functioned in tissue morphology, nervous system development and function, and organismal development. A black solid edge means a direct relationship. A black unsolid edge means an indirect relationship. A red node means upregulated proteins. A green node means downregulated proteins. Reproduced from Zhan et al. [5], with copyright permission from Wiley-VCH, copyright year 2014.
abnormality, oxidative stress, mitochondrial dysfunction, and TR/RXR activation are significantly associated with NFPAs and invasive NFPAs [27], and the changed molecule-pattern in each pathway-system was different between NFPA and invasive NFPA, which might contribute to the pathological processes of invasive NFPAs. Furthermore, ketogenesis and ketolysis, proteolysis abnormality, amyloid processing, and CDK5 signaling abnormality were also obviously related to invasive NAPFs. Therefore MAPK-signaling abnormality, oxidative stress, mitochondrial dysfunction, ketogenic and ketolysis, CDK5 signaling abnormality, ketogenesis and ketolysis, and amyloid processing were significantly associated with invasive characteristics of invasive NFPAs, and pathway-network-based molecule patterns benefit to identify reliable biomarkers for invasive NFPAs.

4. Conclusions

Invasiveness is serious clinical problem in human pituitary adenomas. It is necessary to clarify its molecular mechanisms and discover effective biomarkers to guide management of invasive NFPAs. This 2DGE-based comparative proteomics and bioinformatics successfully identified proteomic variation profiling and pathway-network changes in human invasive NFPAs compared to noninvasive NFPAs, found 103 differential protein spots (64 upregulated and 39 downregulated) in invasive versus noninvasive NFPA 2DE maps, and identified 57 DEPs (30 upregulated and 27 downregulated), which are significantly involved in pathogenetic process of invasive NFPAs, with altered pathway networks including MAPK-signaling abnormality, oxidative stress, mitochondrial dysfunction, ketogenesis and ketolysis, CDK5 signaling abnormality, TR/RXR activation, proteolysis abnormality, and amyloid processing. Moreover, some important hub-molecules were identified to associate with cancer biological processes, including ATPase.
MAPK, ERK, ERK1/2, p38, Jnk, NfkB, AKT, PKA, PKC, EGFR, K-RAS, insulin, UBC, CCND1, IFNG, NfYB, ESR1, CDK5, calmodulin, and S100A1. Those DEPs, changed pathway networks, and hub-molecules provided new insights into molecular mechanisms of NFPA invasiveness, and important resource for discovery of effective biomarkers to guide the management of invasive NFPAs.

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Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations.

Author’s contributions

X.Z. conceived the concept, designed the book chapter, and wrote and critically revised the book chapter, coordinated and was responsible for the correspondence work and financial support. X.H.Z and X.W participated in experiments. X.H.Z edited the English language. All authors approved the final manuscript.

Acronyms and abbreviations

| Acronym   | Description                                      |
|-----------|--------------------------------------------------|
| BP        | biological processes                             |
| CC        | cellular components                              |
| DEP       | differentially expressed protein                 |
| ESI       | electrospray ionization                          |
| FPA       | functional pituitary adenomas                    |
| IEF       | isoelectric focusing                             |
| IPA       | ingenuity pathway analysis                       |
| IPG       | immobilized pH gradient                          |
| LC        | liquid chromatography                            |
| MALDI     | matrix-assisted laser desorption/ionization      |
| MF        | molecular functions                              |
| Mr        | relative mass                                    |
| MRI       | magnetic resonance imaging                       |
| MS        | mass spectrometry                                |
| MS/MS     | tandem mass spectrometry                         |
| NFPA      | nonfunctional pituitary adenoma                  |
| pi        | isoelectric point                                |
| PMF       | peptide mass fingerprint                         |
| SDS-PAGE  | sodium dodecyl sulfate-polyacrylamide gel electrophoresis |
| TOF       | time-of-flight                                   |
| 2DGE      | two-dimensional gel electrophoresis              |
Author details

Xianquan Zhan\textsuperscript{1,2*}, Xiaohan Zhan\textsuperscript{1,2} and Xiaowei Wang\textsuperscript{1,2}

1 Key Laboratory of Cancer Proteomics of Chinese Ministry of Health, Xiangya Hospital, Central South University, Changsha, P.R. China

2 State Local Joint Engineering Laboratory for Anticancer Drugs, Xiangya Hospital, Central South University, Changsha, P.R. China

*Address all correspondence to: yjzhan2011@gmail.com

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