Chapter

Prolactin Proteoform Pattern Changed in Human Pituitary Adenoma Relative to Control Pituitary Tissues

Xianquan Zhan and Shehua Qian

Abstract

PRL gene-encoded prolactin is synthesized in the ribosome in the pituitary and then secretes into blood circulation to reach its target organ and exerts its biological roles, for example, involving in production, growth, development, immunoregulation, and metabolism. Multiple post-translational modifications and other unknown factors might be involved in this process to cause different prolactin proteoforms with differential isoelectric point (p*I*) and relative mass (M_r). Pituitary adenomas are the common disease occurring in pituitary organ to affect the endocrine system. Two-dimensional gel electrophoresis (2DGE) was used to separate prolactin proteoforms according to their p*I* and M_r, followed by identification with Western blot and mass spectrometry (MS) analyses. Six prolactin proteoforms were identified in control pituitary tissues, and this prolactin proteoform pattern was significantly changed in different hormone subtypes of nonfunctional pituitary adenomas (NF^-, LH^+, FSH^+, and LH^+/FSH^+) and prolactinomas (PRL^+). Further, bioinformatics analysis revealed that different prolactin proteoforms might bind to different short- or long-PRL receptor-mediated signaling pathways. These findings clearly demonstrated that prolactin proteoform pattern existed in human pituitary and changed in different subtypes of pituitary adenomas. It is the scientific data to in-depth study prolactin functions, and to discover the prolactin proteoform biomarkers for PRL-related adenomas.

Keywords: prolactin proteoforms, pituitary, pituitary adenomas, nonfunctional pituitary adenomas, prolactinomas, biomarker

1. Introduction

Prolactin (PRL) is a multifunctional hormone which is synthesized and secreted by pituitary [1]. Human PRL gene is located on chromosome 6 [2]. The secretory mode of PRL is autocrine and paracrine [3], and the secretion of PRL is pulsating and circadian rhythm [4]. The concentration of PRL in human serum has a certain reference range, and when its concentration is too high or too low, it will have a certain impact on the body. Dopamine can inhibit the secretion of

PRL, and there are cases where dopamine is used to treat hyperprolactinemia [1]. PRL's biological functions include production, growth, development, immunoregulation, and metabolism [5, 6]. PRL can exert its biological functions only when it binds to its receptor and activates some signaling pathways [7]. According to the concept of proteoforms, a protein is defined as a set of proteoforms, due to different splicing, post-translational modifications (PTMs), and even unknown factors. Each proteoform has its own specific isoelectric point (p*I*) and molecular weight (M_r). For human PRL in the UniProt protein database, its p*I* is 6.5 and M_r is 25.88 kDa. However, Ben-Jonathan et al. found that human serum contained PRLs with M_r > 100, 40–60 and 16 kDa, besides the PRL with 25.88 kDa [8]. Qian et al. found six PRLs with different p*I* and M_r in human pituitary tissues by two-dimensional gel electrophoresis (2DGE) and mass spectrometry (MS) [9]. Similarly, Zhan et al. found 24 growth hormone (GH) with different p*I* and M_r in human pituitary tissues by 2-DGE and MS [10]. A possible reason of this difference of p*I* and M_r in human PRL and GH is that they undergo PTMs or splicing [11]. A proteoform is a specific form that protein exerts its final functions, which is derived from a gene undergoing splicing, transcription, translation, PTMs, translocation/re-distribution, and interaction with other molecules, etc. [12].

Recently 2DGE and MS have been recognized as high throughput and useful tool to study proteoforms [13–15]. 2DGE is able to separate each proteoform in the first dimension—isoelectric focusing (IEF) based on proteoform charge difference, and in the second dimension—sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) based on proteoform relative mass difference [16]. Therefore, 2DGE achieves proteoform separation based on the difference in p*I* and M_r of proteoforms. And then the protein (exactly proteoforms) on the 2D gel is transferred to a polyvinylidene fluoride membrane for detection with a specific antibody. The immunoreactive positive 2D gel spots represent the proteoforms of a protein. The proteoform in each immunoreactive positive spot was subjected to in-gel digestion with trypsin, and identification with MS [17, 18]. MS is a key technique to identify organic molecules and analyze the extreme structure of certain substances [19]. Especially, top-down MS can quickly and extremely accurately determine the molecular weight of biomacromolecules, which enables proteomics research from protein general identification to advanced structural studies and protein-protein interaction studies. Moreover, with the development of MS technology, the accuracy and sensitivity of mass spectrometers have been greatly improved. MS has its absolute advantages in the use of less sample, faster analysis, and simultaneous separation and identification. Therefore, 2DGE in combination with MS is presenting as a super-high approach in separation and identification of large-scale human proteoforms [14]. If the stable isotope labeling is introduced to prepare the protein sample prior to 2DGE-MS, then 2DGE-MS can also quantify the abundance of a proteoform between two given conditions such as tumors vs. controls [20].

Pituitary adenomas are the common disease occurred in pituitary organ to severely impact on the human endocrine system. PRL is an important pituitary hormone. It has important scientific merit in clarification of PRL proteoform pattern changed in different subtypes of pituitary adenomas compared to control pituitaries. This book chapter focuses on the PRL proteoforms in human pituitary and investigates the PRL proteoform pattern alterations in pituitary adenomas relative to controls, with 2DGE and MS. These findings provide the scientific data to in-depth study the PRL functions and to discover PRL proteoform biomarker for PRL-related adenomas.

2. Methods

2.1 Pituitary tissue samples and preparation of protein samples

Eight human post-mortem control pituitary tissues, five PRL-positive prolactinoma tissues, three non-hormone expressed nonfunctional pituitary adenoma (NF-NFPA) tissues, three luteinizing hormone (LH)-positive NFPA tissues, three follicle-stimulating hormone (FSH)-positive NFPA tissues, and three LH/FSH-both positive NFPA tissues were used to extract proteins, with the previously described procedure [21, 22]. The extracted protein of each tissue sample was used for 2DGE and MS analysis.

2.2 2DGE

A amount (70 μg) of proteins was diluted into 350 μL of protein sample buffer (7 mol/L urea, 2 mol/L thiourea, 40 g/L CHAPS, 100 mmol/L dithiothreitol (DTT), 5 mol/L immobilized pH gradient (IPG) buffer pH 3–10 NL, and a trace of bromophenol blue, followed by rehydration (18 h, 20°C) of precast IPG strips pH 3–10 NL (180 x 3 × 0.5 mm) in 18-cm IPG strip holder on an IPGphor instrument, and IEF (25°C) with parameters (Gradient 250 V and 1 h for 125 Vh, gradient at 1000 V and 1 h for 500 Vh, gradient at 8000 V and 1 h for 4000 Vh, step-and-hold at 8000 and 4 h for 32,000 Vh, step-and-hold at 500 V and 0.5 h for 250 Vh to achieve a total of 36,875 Vh). After IEF, the proteins on IPG strip were reduced (15 min) with DTT, and alkylated with iodoacetamide, followed by separation with 12% SDS-PAGE (250 × 215 × 1.0 mm) in an Ettan DALT II system (GE Healthcare, up to 12 gels at a time) with a constant voltage (250 V, 360 min). All 2DGE-arrayed proteins were stained with silver-staining [23], and then digitized and analyzed with Discovery Series PDQuest 2D Gel Analysis software [24, 25]. Each sample was performed for 3–5 times.

2.3 2DGE-based Western blot

The proteins in the 2D gel were partially transferred to a polyvinylidene fluoride (PVDF) membrane (0.8 mA/cm^2 for 80 min) using Amersham Pharmacia Biotech Nova Blot semi-dry transfer instrument, followed by blocking (1 h, room temperature) with bovine serum albumin (BSA), incubation (1 h, room temperature) with rabbit anti-hPRL antibodies, incubation (1 h, room temperature) with goat anti-rabbit alkaline phosphatase conjugated IgG, and visualization with 5-bromo-4-chloro-3-indolyl phosphate. The detailed procedure was described previously [9].

2.4 In-gel digestion with trypsin and MS identification of PRL

The proteins in each Western blot-positive spot was performed in-gel digestion with trypsin, purification of tryptic peptides with ZipTipC18, followed by analysis with three types of MS instruments, including MALDI-TOF MS [24], LC-ESI-Q-IT MS [24], and MALDI-TOF-TOF MS [9]. The detailed procedure was described previously [9, 24]. The obtained peptide mass fingerprint (PMF) and tandem mass spectrometry (MS/MS) data were used to search Swiss-Prot human database for protein determination and PTM analysis.

2.5 Bioinformatics and statistical analysis

The phosphorylation sites, O-glycosylation sites, and N-glycosylation sites in the PRL amino acid sequence were predicted with NetPhos 3.1 Server

(http://www.cbs.dtu.dk/services/NetPhos) [26, 27], NetOGlyc 4.0 Server (http://www.cbs.dtu.dk/services/NetOGlyc) [28], and NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc) [29]. The PRL proteoform pattern changes were tested with the Chi-square test among different subtypes of pituitary adenomas ($p < 0.05$).

3. Results and discussion

3.1 The amino acid sequences of human PRL prohormone and mature PRL

In human pituitary, the PRL prohormone is synthesized in the ribosome, with 227 amino acids (position 1–227; 25.9 kDa), containing a signal peptide (position 1–28) (**Table 1**), which was assigned with Swiss-Prot accession No. P01236. However, the mature human PRL only contains 199 amino acids (position 29–227; 22.9 kDa), which removed the signal peptide (position 1–28), and secreted into the circulation system to bind to its target organ for exerting PRL function.

3.2 PRL proteoform pattern in human pituitaries

The PRL proteoform pattern was found in human pituitaries. Qian et al. [9] found six PRL proteoforms with 2DGE in human pituitaries and then verified four of six PRL proteoforms with 2DGE-based Western blot in human pituitaries (**Figures 1** and **2**). The p*I* and M_r of these PRL proteoforms are slightly different. Each PRL proteoform was digested with trypsin, and followed by MS and MS/MS analysis (**Figures 3** and **4**). The characteristic tryptic peptide are calculated to determine whether the signal peptide (position 1–28) in each PRL proteoform (**Table 2**), which was compared to the observed ions of each PRL proteoform. It found all PRL proteoforms all contained the tryptic peptide sequence MNIKGSPWK (position 1–9), which clearly demonstrated that six PRL proteoforms are all PRL prohormone, but not mature PRL.

3.3 PRL proteoform changes in human pituitary adenomas compared to controls

The PRL proteoform pattern changed in different subtypes of pituitary adenomas compared to control pituitaries (**Table 3**). The ratio of each subtype of pituitary

10	20	30	40	50
MNIKGSPWKG	**SLLLLLVSNL**	**LLCQSVAP**LP	ICPGGAARCQ	VTLRDLFDRA
60	70	80	90	100
VVLSHYIHNL	SSEMFSEFDK	RYTHGRGFIT	KAINSCHTSS	LATPEDKEQA
110	120	130	140	150
QQMNQKDFLS	LIVSILRSWN	EPLYHLVTEV	RGMQEAPEAI	LSKAVEIEEQ
160	170	180	190	200
TKRLLEGMEL	IVSQVHPETK	ENEIYPVWSG	LPSLQMADEE	SRLSAYYNLL
210	220			
HCLRRDSHKI	DNYLKLLKCR	IIHNNNC		

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Table 1.
Human PRL prohormone amino acid sequence (position 1–227) and mature PRL (position 29–227). The signal peptide is position 1-28 in the bold letters.

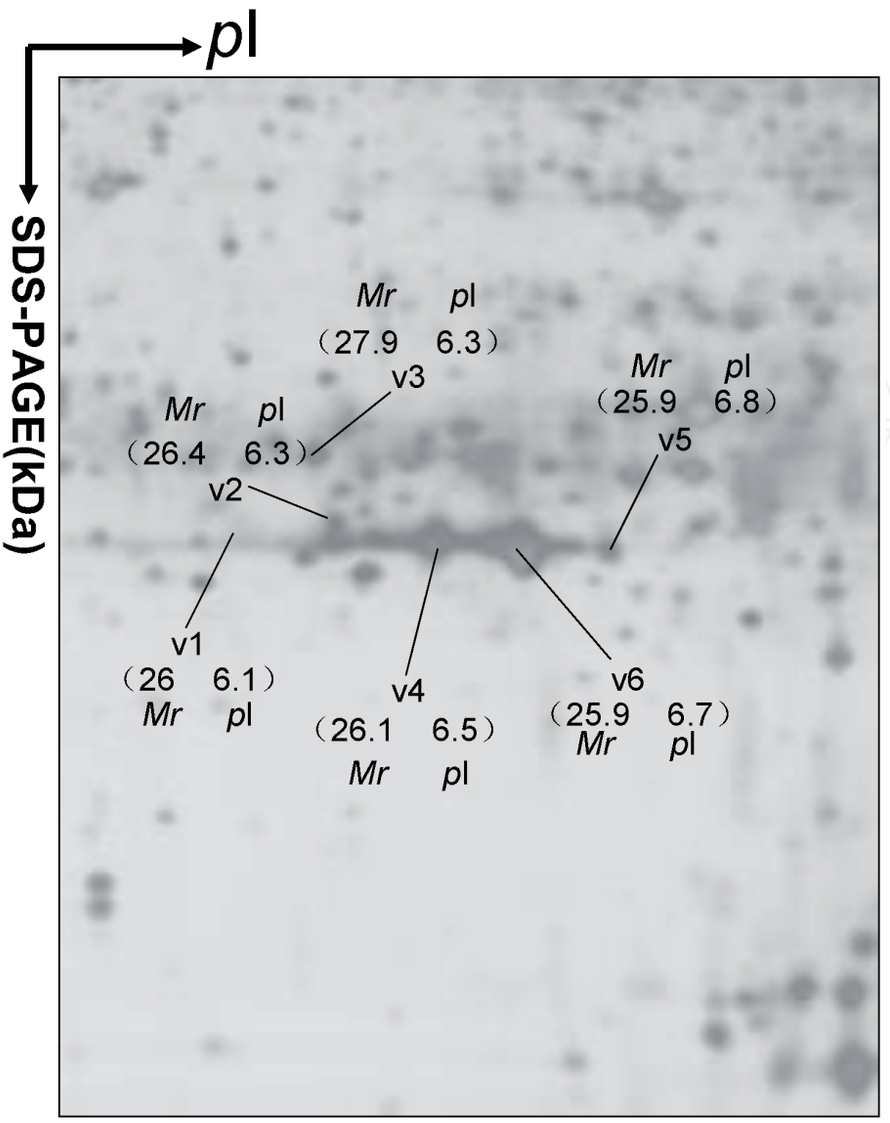

Figure 1.
PRL proteoform pattern in human pituitaries with a 2DGE gel image. Reproduced from Qian et al. [9], with copyright permission from Frontiers in open access article, copyright 2018.

adenoma relative to control pituitaries was decreased or unchanged. The proportional ratio of six PRL proteoforms among five subtypes of pituitary adenomas was changed (**Table 4** and **Figure 5**). In FSH^+/LH^+ and PRL^+ pituitary adenomas, the proportion of PRL proteoform v1 is the largest. In FSH^+ pituitary adenoma, the proportion of PRL proteoform v5 is the largest. The PRL proteoform changes suggest their scientific merit for clinical application.

3.4 Bioinformatics prediction of potential factors to form PRL proteoforms and pathway networks

PRL is a hormone which is secreted by pituitary gland. PRL has a variety of biological functions. Only when it reaches a specific target organ and binds to its receptor can it play its biological function (**Figure 6**). PRL can bind to short PRL receptor or long PRL receptor and then plays its biological functions. The long or short PRL receptors definitely bind to different PRL proteoforms. PRL proteoforms are definitely derived from a PRL gene undergoing splicing, transcription, translation, PTMs, translocation/re-distribution, and interaction with other molecules, etc. Therefore, phosphorylation sites in hPRL (position 1–227) were predicted

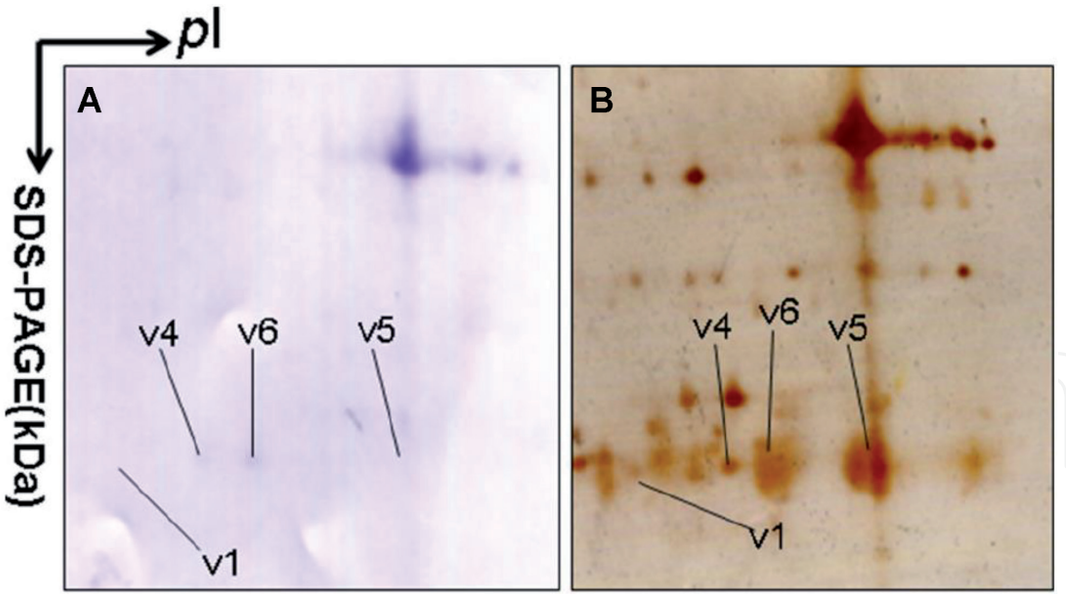

Figure 2.
Verification of PRL proteoforms with 2DGE-based Western blot in human pituitaries. (A) Western blot image. (B) Silver-stained image. Reproduced from Qian et al. [9], with copyright permission from Frontiers in open access article, copyright 2018.

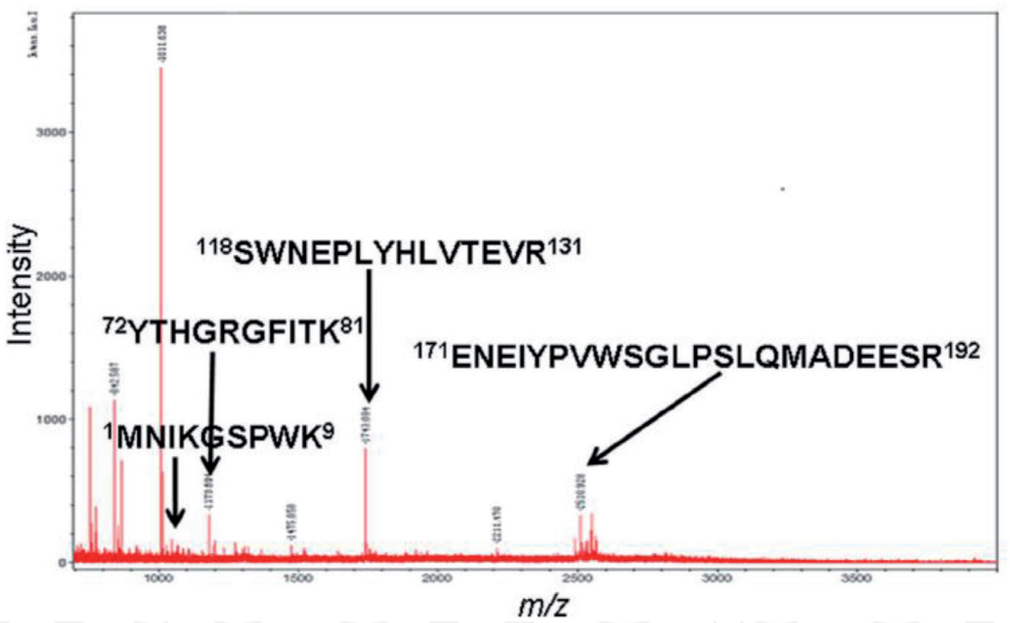

Figure 3.
PMF analysis of hPRL that was contained in spot v6. Reproduced from Qian et al. [9], with copyright permission from Frontiers in open access article, copyright 2018.

with NetPhos 3.1 Server with a score more than 0.5. It obtained 22 statistically significantly phosphorylation sites in hPRL (position 1–227). N-glycosylation sites in hPRL (position 1–227) were predicted with NetNGlyc 1.0 Server with score more than 0.5. It obtained 10 statistically significant N-glycosylation sites in hPRL (position 1–227). O-glycosylation sites in hPRL (position 1–227) were predicted with NetOGlyc 4.0 Server with score more than 0.5. It obtained six statistically significant O-glycosylation sites in hPRL (position 1–227). These data suggest that PTMs such as phosphorylation and glycosylation might be the important reason to cause the PRL proteoforms.

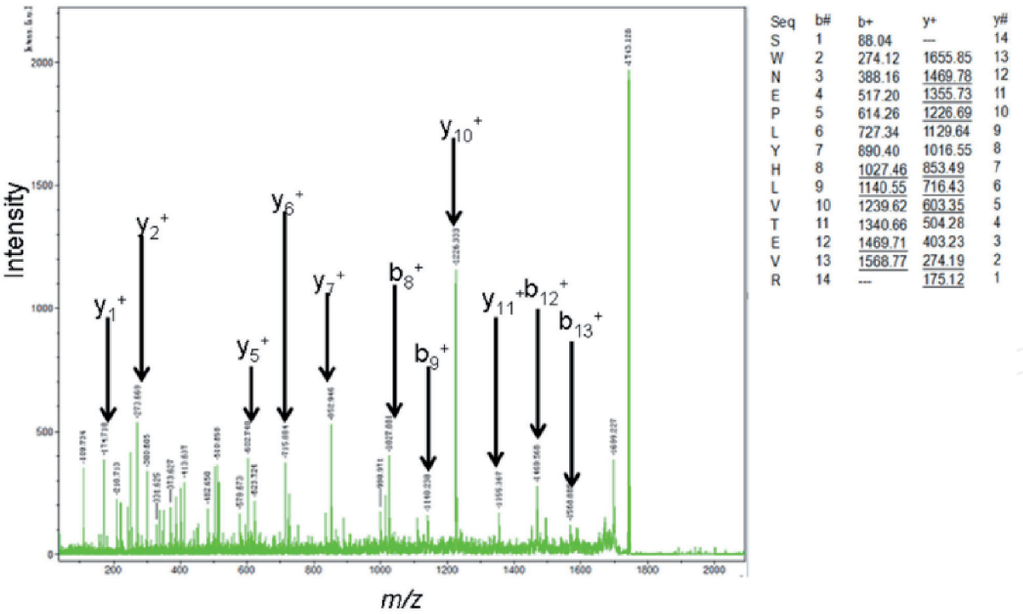

Figure 4.
MS/MS analysis of the tryptic peptide 118SWNEPLYHLVTEVR131 that was derived from PRL in spot v6. Reproduced from Qian et al. [9], with copyright permission from Frontiers in open access article, copyright 2018.

Calc. $[M + H]^+$	Position	Characteristic tryptic peptide sequence	Observed $[M + H]^+$
505.2803	1–4	MNIK	–
1060.5608	1–9	MNIKGSPWK	+
3930.1893	1–38	MNIKGSPWKGSLLLLLVSNLLLCQSVAPLPICPGGAAR	–
574.2983	5–9	GSPWK	–
3443.9268	5–38	GSPWKGSLLLLLVSNLLLCQ SVAPLPICPGGAAR	–
2888.6463	10–38	GSLLLLLVSNLLLCQSVAPL PICPGGAAR	–
3589.0154	10–44	GSLLLLLVSNLLLCQSVAPL PICPGGAARCQVTLR	–
954.5189	29–38	LPICPGGAAR	–
1654.8879	29–44	LPICPGGAARCQVTLR	–
2301.1954	29–49	LPICPGGAARCQVTLRDLFD R	–

+, this peptide ion was observed with mass spectrometry in each MS spectrum.
–, this peptide was not observed with mass spectrometry.
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Table 2.
Characteristic tryptic peptides to determine signal peptide (position1–28) within human PRL proteoforms in human pituitary.

3.5 Potential clinical application of PRL proteoform pattern

Prolactin synthesized in the ribosome in the pituitary secretes into blood circulation to reach its target organ and exert its biological roles, which is closely associated with multiple physiological and pathological processes, including pituitary adenomas. This study found six PRL proteoforms with different with differential isoelectric point (p*I*) and relative mass (M_r) in control pituitary tissues, which were identified with 2DGE coupled with Western blot and MS. This prolactin

PRL proteoform no.	Swiss-Prot no.	*p*I	M_r	Ratio (NF^-: Con)	Ratio (FSH^+/ LH^+: Con)	Ratio (FSH^+: Con)	Ratio (LH^+: Con)	Ratio (PRL: Con)
V1	P01236	6.1	26.0	−8.3	−99.9	−46.2	−12.6	−3.4
V2[a]	P01236	6.3	26.4	−4.9	−3.8	1	−4.1	1
V3	P01236	6.3	27.9	1	−12.3	−14.6	−26.2	1
V4[b]	P01236	6.5	26.1	−100	−19.0	−17.6	−20.1	1
V5	P01236	6.8	25.9	−100	−19.7	−100	−36.7	1
V6	P01236	6.7	25.9	−100	−32.6	−11.3	−33.6	1

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[a]Characterized with LC-ESI MS/MS.
[b]Characterized with LC-ESI-MS/MS and MALDI-TOF PMF.
All other proteins were characterized with MALDI-TOF PMF. Con, control; −, decreased relative to controls; −100, lost relative to controls; 1, no change relative to controls; M_r, kDa.

Table 3.
Prolactin proteoform pattern changed in different subtypes of pituitary adenomas compared to control pituitaries.

PRL proteoform	NF^- (%)	FSH^+/LH^+ (%)	FSH^+ (%)	LH^+ (%)	PRL^+ (%)
V1	2.64	53.34	24.24	9.45	40.48
V2	1.56	2.03	0.52	3.08	11.90
V3	0.31	6.57	7.66	19.65	11.91
V4t	31.83	10.14	9.18	15.08	11.90
V5	31.83	10.52	52.47	27.53	11.91
V6	31.83	17.40	5.93	25.21	11.90
Total	100.00	100.00	100.00	100.00	100.00

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[*]Chi-square test = 360.606, p = 0.000 ($p < 0.01$) among five subtypes of pituitary adenomas.

Table 4.
Proportional ratio changes of PRL proteoforms among five subtypes of pituitary adenomas.

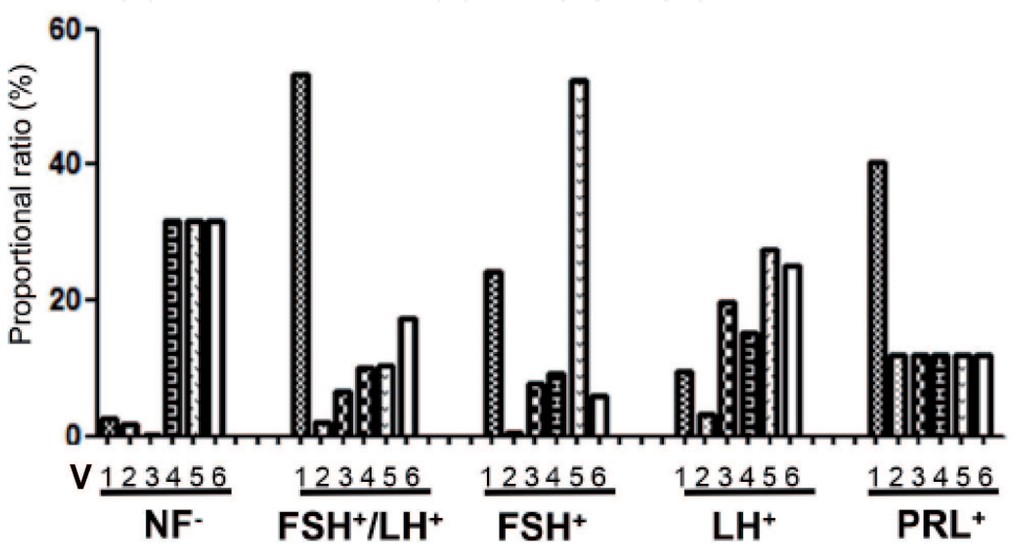

Figure 5.
Proportional ratio changes of PRL proteoforms among five subtypes of pituitary adenomas. Reproduced from Qian et al. [9], with copyright permission from Frontiers in open access article, copyright 2018.

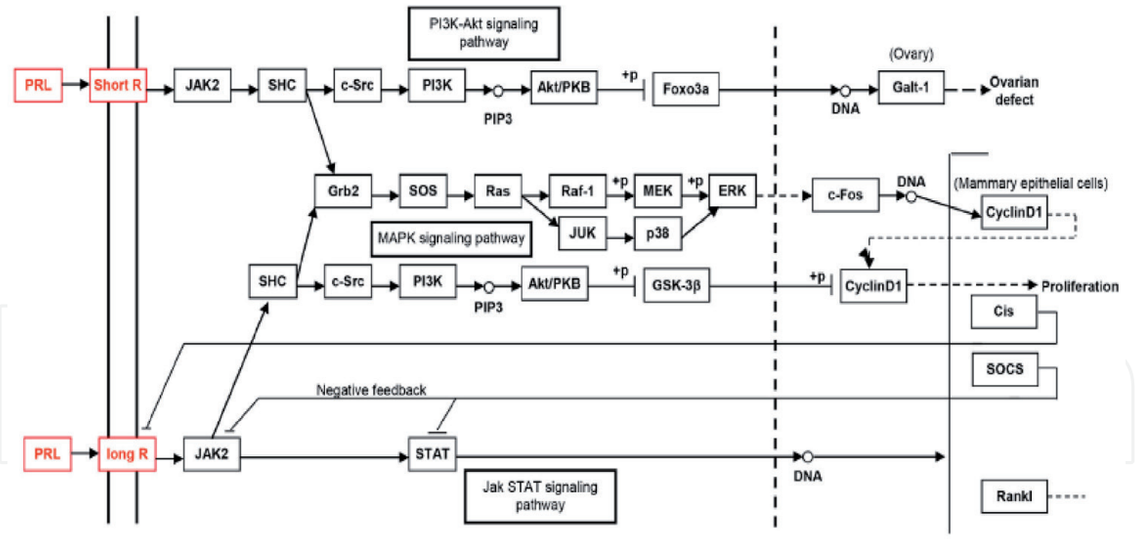

Figure 6.
PRL proteoform-driven signaling pathway via the short or long PRL receptors. Reproduced from Qian et al. [9], with copyright permission from Frontiers in open access article, copyright 2018.

proteoform pattern was significantly changed among different hormone-subtypes of nonfunctional pituitary adenoma (NF^-, LH^+, FSH^+, and LH^+/FSH^+) and prolactinoma (PRL^+) tissues. This result suggests the potentially important clinical value of serum PRL proteoforms. The reason is that pituitary tissues are impossible to obtain for clinical diagnosis, and prolactin must secrete into blood to exert its biological roles, we strongly believe serum PRL proteoforms exist and the serum PRL proteoform pattern changes among different pituitary adenomas. Therefore, we will further analyze serum PRL proteoform pattern changes among different subtypes of pituitary adenomas, and develop the PRL proteoform pattern as biomarker for prediction, diagnosis, or prognostic assessment of pituitary adenoma occurrence, progression, and prognosis.

4. Conclusions

Six PRL proteoforms were identified in human pituitary tissue with 2DGE and MS analyses, and four of six PRL proteoforms were validated with 2DGE-based Western blot, MS, and MS/MS analyses. There were significant differences in PRL proteoform pattern among five different subtypes of pituitary adenomas (LH^+, NF^-, FSH^+, FSH^+/LH^+, and PRL^+) ($P < 0.05$). Moreover, MS analysis revealed that six PRL proteoforms are PRL prohormone. PRL proteoforms might be derived from PTMs such as phosphorylation, deamidation, and glycosylation. Further, different PRL proteoforms might bind to different PRL receptors to produce different physiological functions. These findings provide scientific basis for in-depth understanding of pituitary adenomas, and help develop biomarkers for treatment of pituitary adenoma patients. The serum PRL proteoform pattern has important clinical application value for prediction, diagnosis, and prognostic assessment of pituitary adenomas.

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Conflict of interest

The authors declare that they have no financial and personal relationships with other people or organizations.

Author's contributions

X.Z. conceived the concept, designed the manuscript, wrote and critically revised the manuscript, coordinated, and was responsible for the correspondence work and financial support. Q.S. participated in the literature analysis, data analysis, prepared figures, and wrote partial manuscript.

Abbreviations

DTT	dithiothreitol
FSH	follicle-stimulating hormone
GO	gene ontology
IEF	isoelectric focusing
IPG	immobilized pH gradient
LH	luteinizing hormone
M_r	molecular weight
MS	mass spectrometry
p*I*	isoelectric point
PRL	prolactin
PTM	post-translational modifications
PVDF	polyvinylidene fluoride
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
2DGE	two-dimensional gel electrophoresis

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References

[1] Kobayashi T, Usui H, Tanaka H, Shozu M. Variant prolactin receptor in agalactia and hyperprolactinemia. The New England Journal of Medicine. 2018;**379**(23):2230-2236. DOI: 10.1056/NEJMoa1805171

[2] Owerbach D, Rutter WJ, Cooke NE, Martial JA, Shows TB. The prolactin gene is located on chromosome 6 in humans. Science (New York, N.Y.). 1981;**212**(4496):815-816

[3] Larsen CM, Grattan DR. Prolactin, neurogenesis, and maternal behaviors. Brain, Behavior, and Immunity. 2012;**26**(2):201-209. DOI: 10.1016/j.bbi.2011.07.233

[4] de la Fuente JR, Rosenbaum AH. Prolactin in psychiatry. The American Journal of Psychiatry. 1981;**138**(9):1154-1160. DOI: 10.1176/ajp.138.9.1154

[5] Binart N, Bachelot A, Bouilly J. Impact of prolactin receptor isoforms on reproduction. Trends in Endocrinology and Metabolism: TEM. 2010;**21**(6):362-368. DOI: 10.1016/j.tem.2010.01.008

[6] Xie W, Liu H, Liu Q, Gao Q, Gao F, Han Y, et al. Seasonal expressions of prolactin, prolactin receptor and STAT5 in the scented glands of the male muskrats (*Ondatra zibethicus*). European Journal of Histochemistry: EJH. 2019;**63**(1). DOI: 10.4081/ejh.2019.2991

[7] Bernichtein S, Touraine P, Goffin V. New concepts in prolactin biology. The Journal of Endocrinology. 2010;**206**(1):1-11. DOI: 10.1677/joe-10-0069

[8] Ben-Jonathan N, LaPensee CR, LaPensee EW. What can we learn from rodents about prolactin in humans? Endocrine Reviews. 2008;**29**(1):1-41. DOI: 10.1210/er.2007-0017

[9] Qian S, Yang Y, Li N, Cheng T, Wang X, Liu J, et al. Prolactin variants in human pituitaries and pituitary adenomas identified with two-dimensional gel electrophoresis and mass spectrometry. Frontiers in Endocrinology. 2018;**9**:468. DOI: 10.3389/fendo.2018.00468

[10] Zhan X, Giorgianni F, Desiderio DM. Proteomics analysis of growth hormone isoforms in the human pituitary. Proteomics. 2005;**5**(5):1228-1241. DOI: 10.1002/pmic.200400987

[11] Freeman ME, Kanyicska B, Lerant A, Nagy G. Prolactin: Structure, function, and regulation of secretion. Physiological Reviews. 2000;**80**(4):1523-1631. DOI: 10.1152/physrev.2000.80.4.1523

[12] Schaffer LV, Millikin RJ, Miller RM, Anderson LC, Fellers RT, Ge Y, et al. Identification and quantification of proteoforms by mass spectrometry. Proteomics. 2019;**19**(10):e1800361. DOI: 10.1002/pmic.201800361

[13] Oliveira BM, Coorssen JR, Martins-de-Souza D. 2DE: The phoenix of proteomics. Journal of Proteomics. 2014;**104**:140-150. DOI: 10.1016/j.jprot.2014.03.035

[14] Zhan X, Yang H, Peng F, Li J, Mu Y, Long Y, et al. How many proteins can be identified in a 2DE gel spot within an analysis of a complex human cancer tissue proteome? Electrophoresis. 2018;**39**(7):965-980. DOI: 10.1002/elps.201700330

[15] Kurgan N, Noaman N, Pergande MR, Cologna SM, Coorssen JR, Klentrou P. Changes to the human serum proteome in response

to high intensity interval exercise: A sequential top-down proteomic analysis. Frontiers in Physiology. 2019;**10**:362. DOI: 10.3389/fphys.2019.00362

[16] Zhan X, Huang Y, Long Y. Two-dimensional gel electrophoresis coupled with mass spectrometry methods for an analysis of human pituitary adenoma tissue proteome. Journal of Visualized Experiments: JoVE. 2018;**134**:e56739. DOI: 10.3791/56739

[17] Li C, Zhan X, Li M, Wu X, Li F, Li J, et al. Proteomic comparison of two-dimensional gel electrophoresis profiles from human lung squamous carcinoma and normal bronchial epithelial tissues. Genomics, Proteomics & Bioinformatics. 2003;**1**(1):58-67

[18] Wang K, Lin Z, Zhang H, Zhang X, Zheng X, Zhao L, et al. Investigating proteome and transcriptome response of *Cryptococcus podzolicus* Y3 to citrinin and the mechanisms involved in its degradation. Food Chemistry. 2019;**283**:345-352. DOI: 10.1016/j.foodchem.2019.01.052

[19] Kadi AA, Yin W, Rahman A. In-vitro metabolic profiling study of potential topoisomerase inhibitors 'pyrazolines' in RLMs by mass spectrometry. Journal of Chromatography B, Analytical Technologies in the Biomedical and Life Sciences. 2019;**1114-1115**:125-133. DOI: 10.1016/j.jchromb.2019.03.026

[20] Zhan X, Li N, Zhan X, Qian S. Revival of 2DE-LC/MS in proteomics and its potential for large-scale study of human proteoforms. Med One. 2018;**3**(5):e180008. DOI: 10.20900/mo.20180008

[21] Moreno CS, Evans CO, Zhan X, Okor M, Desiderio DM, Oyesiku NM. Novel molecular signaling and classification of human clinically nonfunctional pituitary adenomas identified by gene expression profiling and proteomic analyses. Cancer Research. 2005;**65**:10214-10222. DOI: 10.1158/0008-5472.can-05-0884

[22] Evans CO, Moreno CS, Zhan X, Mccabe MT, Vertino PM, Desiderio DM, et al. Molecular pathogenesis of human prolactinomas identified by gene expression profiling, RT-qPCR, and proteomic analyses. Pituitary. 2008;**11**:231-245. DOI: 10.1007/s11102-007-0082-2

[23] Zhan X, Desiderio DM. Mass spectrometric identification of in vivo nitrotyrosine sites in the human pituitary tumor proteome. Methods in Molecular Biology. 2009;**566**:137-163. DOI: 10.1007/978-1-59745-562-6_10

[24] Zhan X, Desiderio DM. A reference map of a human pituitary adenoma proteome. Proteomics. 2003;**3**:699-713. DOI: 10.1002/pmic.200300408

[25] Peng F, Li J, Guo T, Yang H, Li M, Sang S, et al. Nitroproteins in human astrocytomas discovered by gel electrophoresis and tandem mass spectrometry. Journal of the American Society for Mass Spectrometry. 2015;**26**:2062-2076. DOI: 10.1007/s13361-015-1270-3

[26] Blom N, Sicheritz-Ponten T, Gupta R, Gammeltoft S, Brunak S. Prediction of post-translational glycosylation and phosphorylation of proteins from the amino acid sequence. Proteomics. 2004;**4**:1633-1649. DOI: 10.1002/pmic.200300771

[27] Blom N, Gammeltoft S, Brunak S. Sequence and structure-based prediction of eukaryotic protein phosphorylation sites. Journal of Molecular Biology. 1999;**294**:1351-1362. DOI: 10.1006/jmbi.1999.3310

[28] Steentoft C, Vakhrushev SY, Joshi HJ, Kong Y, Vester-Christensen MB,

Schjoldager KT, et al. Precision mapping of the human O-GalNAc glycoproteome through SimpleCell technology. The EMBO Journal. 2013;**32**:1478-1488. DOI: 10.1038/emboj.2013.79

[29] Gupta R, Brunak S. Prediction of glycosylation across the human proteome and the correlation to protein function. Pacific Symposium on Biocomputing. 2002;**2002**:310-322. DOI: 10.1142/9789812799623_0029