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Molecular pathogenesis of human prolactinomas identified by gene expression profiling, RT-qPCR, and proteomic analyses

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Abstract The molecular pathogenesis of prolactinomas has resisted elucidation; with the exception of a RAS mutation in a single aggressive prolactinoma, no mutational changes have been identified. In prolactinomas, a further obstacle has been the paucity of surgical specimens suitable for molecular analysis since prolactionomas are infrequently removed due to the availability and effectiveness of medical therapy. In the absence of mutational events, gene expression changes have been sought and detected. Using high-throughput analysis from a large bank of human pituitary adenomas, we examined these tumors according to their molecular profiles rather than traditional immunohistochemistry. We examined six prolactinomas and eight normal pituitary glands using oligonucleotide GeneChip microarrays, reverse transcription-real time quantitative polymerase chain reaction using 10 prolactinomas, and proteomic analysis to examine protein expression in four prolactinomas. Microarray analyses identified 726 unique genes that were statistically significantly different between prolactinomas and normal glands, whereas proteomic analysis identified four differently up-regulated and 19 down-regulated proteins. Several components of the Notch pathway were altered in prolactinomas, and there was an increased expression of the Pit-1 transcription factor, and the survival factor BAG1 but decreased E-cadherin and N-cadherin expression. Taken together, expression profiling and proteomic analyses have identified molecular features unique to prolactinomas that may contribute to their pathogenesis. In the current era of molecular medicine, these findings greatly enhance our understanding and supercede immunohistochemical diagnosis.

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Introduction

Prolactinomas typically result in impaired reproduction, decreased libido, amenorrhea and galactorrhea. Prolactin (PRL) is a polypeptide hormone encoded by a single gene in humans, and is evolutionarily related to growth hormone [1]. The prolactin gene resides on chromosome 6 and is comprised of five coding exons, with 40% homology to the pituitary growth hormone gene on chromosome 17 [2]. Prolactin messenger RNA is expressed at a high level in lactotropes, which comprise approximately 40% of the total pituitary endocrine cells. The prolactin gene is also expressed in a number of extrapituitary sites, notably the uterus and T-lymphocytes, although low-level expression has been found in the brain, skin, breast, and other tissues [3]. In the pituitary, prolactin gene expression is dependent on the pituitary-specific transcription factor Pit-1, whereas in extrapituitary tissues that do not express Pit-1, prolactin gene expression is driven by an alternative upstream promoter that imparts different patterns of hormonal regulation compared with the pituitary [4–6]. The clinical syndrome of hyperprolactinemia is due mainly to the effects of prolactin on the breast and on gonadal function.

Prolactinomas express a high level of dopamine (D-2) receptors and a large majority of patients with prolactinomas, both micro- and macroprolactinomas, can be successfully treated with dopaminergic drugs as first-line treatment, with normalization of prolactin secretion and gonadal function, and significant tumor shrinkage. Surgical resection of prolactinomas is an option for patients who do not respond to or tolerate medical therapy [7].

Molecular genetic studies have demonstrated that these pituitary tumors are monoclonal in origin [8]. The genetic causes of common pituitary tumors remain for the most part unknown [9]. A minority is part of an autosomal dominant syndrome, multiple endocrine neoplasia type 1 (MEN1), which is associated with mutations in the multiple endocrine neoplasia type 1 tumor suppressor gene. Others are associated with loss of heterozygosity on 11q13 chromosome [10–14]. Interestingly, FRα maps to 11q13, and amplification of this region in some carcinomas is associated with over-expression of the FR [15]. We found that FRα was over-expressed in (NF) adenomas [16–18]. Pituitary oncogenes have been intensively studied and three of them gsp, ccnd1, and PTTG are abundant in significant numbers of cases [19]. PTTG-1 plays a pivotal role in cell transformation and is over-expressed in numerous cancer cell lines and tissues [20–23]. A dominant mutation occurs in the Gαs gene in $\sim$30% of somatotrophinomas, but this mutation is rare in other pituitary tumors [24–26].

Currently, pathological identification of prolactinomas is based solely on histology and anterior pituitary hormone immunohistochemistry. A comprehensive molecular profile of these tumors has not been hitherto described. Such molecular profiling is essential for understanding pituitary biology and events leading to tumorigenesis. An initial report by Evans et al. in our laboratory based on a single

Table 1 Clinical and pathological characteristics of prolactinomas used in this study. Sex, age of patient and a brief description of tumor type are given

Patient	Sex, age	Serum PRL level, tumor size,	Immunohistochemistry
T217[a]	M, 39	Prolactinoma, 1,070 ng/ml (postoperative level, preop level not available) 3.2 × 3.6 × 3.2 cm	PRL 3+
T240[a]	M, 39	Prolactinoma, 605 ng/ml 4.5 cm	PRL 3+
T273[a, b]	M, 36	Prolactinoma, 1,918 ng/ml 2.0 × 2.1 × 2.5 cm	PRL 3+
T192[a, b]	M, 41	Prolactinoma, 1,176 ng/ml, 3 × 2.5 × 2.0 cm	PRL 3+
T131[a, b]	F, 52	Prolactinoma, 359 ng/ml, 2.5 × 3.5 × 2.8 cm	PRL 3+
T87[a, b]	M, 48	Prolactinoma with calcification,	PRL+
T277	M, 55	Prolactinoma, 1,520 ng/ml, 3.0 × 3.0 cm	PRL 3+
T279	M, 20	Prolactinoma, 174 ng/ml, 3.5 × 2.5 cm	PRL 3+, GH 2+
T320	M, 50	Prolactinoma, 3,035 ng/ml, 3.0 cm	PRL 3+
T326	F, 31	Prolactinoma, 4,643 ng/ml, 5.0 cm	PRL 3+

Adenomas were graded blindly by a neuropathologist from 0 to 4 for intensity of staining for each peptide hormone. Immunohistochemical characterization of Hormornes in Prolactinomas were described [17]

[a] Samples were analyzed by microarray

[b] Samples were analyzed by proteomics

All samples were analyzed by RT-qPCR

cDNA prolactinoma array [16] assured the feasibility and promise of this approach that has now provided the basis for robust data of gene expression and proteomics in 10 prolactinomas compared to normal pituitary glands.

In this study, microarray analysis identified 726 unique genes that were statistically significantly different between prolactinomas and normal tissues, with 201 genes up-regulated and 525 down-regulated; whereas proteomic analysis identified four up-regulated and 19 down-regulated proteins. Several components of the Notch pathway were altered in prolactinomas. Prolactinomas also showed an increased expression of the Pit-1 transcription factor, the non-classical MHC antigens HLA-G and HLA-F and increased expression of the survival factor BAG1. Conversely there was decreased E-cadherin and N-cadherin expression. These findings greatly enhance our understanding and supercede immunohistochemical diagnosis.

Materials and methods

Patients and tumor characterization

Prolactinomas were obtained during transsphenoidal surgery from patients at Emory University Hospital (Table 1) as part of an ongoing accession of human pituitary tumors. Informed consent for inclusion in this study was obtained. Because pituitary adenomas are anatomically and pathologically distinct from the normal anterior lobe, they are easy to dissect under the surgical microscope. All tumors were microdissected and removed using the surgical microscope, rinsed in sterile saline, snap-frozen in liquid nitrogen, and stored (−80°C) until analysis. Each tumor fragment was then confirmed independently by a neuropathologist as being homogenous and unadulterated by histology and immunohistochemistry prior to molecular analysis.

Eight normal pituitary glands obtained from the National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases (Bethesda, MD, $n = 3$) and from National Disease Research Interchange (Philadephia, PA, $n = 5$) were used as controls for microarray and RT-qPCR analyses. Eight normal pituitary glands obtained from the Memphis Regional Medical Center ($n = 7$), and the National Disease Research Interchange (Philadelphia, PA, $n = 1$) were used as controls in proteomics.

Synthesis of biotin-labeled cRNA and microarray hybridization

Each pituitary tumor was extracted separately for total RNA and analyzed separately for the microarray. Isolation of total RNA was previously described [16, 17]. Briefly, total RNA (100 μg) was purified, using the RNeasy Mini Kit (Cat.No.74104, QIAGEN Inc., Valencia, CA) with minor modifications. Total RNA was eluted twice with 50 μl of 65°C DEPC-treated water. Synthesis (cDNA), labeling, and microarray hybridization were performed as previously described [18].

For microarray analysis, three normal pituitary glands and six prolactinomas were analyzed using HG-U95Av2 GeneChips (Affymetrix, Santa Clara, CA) at the Emory/VA Medical Center in Atlanta, GA. These samples were analyzed in duplicate, starting from the extraction of total RNA, GeneChip hybridization, washing, scanning, and data analysis. Five additional normal pituitary glands were analyzed once using the same chips, HG-U95Av2 GeneChips at the Moffit Comprehensive Cancer Center, University of South Florida, Tampa, FL.

Reverse transcriptase-real time quantitative polymerase chain reaction

To validate the findings of gene transcript measurements by the microarray approach, we performed Reverse Transcriptase-real time quantitative Polymerase Chain Reaction (RT-qPCR) of selected gene transcripts. We measured the expression levels of five genes in eight normal pituitary glands and 10 prolactinomas in a blinded fashion. The eight normal pituitary glands were the same as used in the microarray, and six of the 10 prolactinomas were also used in the microarray. RT-qPCR was performed as described [16–18] except the RT reaction products was diluted in water 5-fold for the candidate genes and 1,000-fold for 18S RNA. The five genes analyzed were transducin-like enhancer of split 4 (TLE4, GenBank M99439), angiopoietin-1 (ANGPT1, GenBank D13628), DnaJ (Hsp40) homolog, subfamily B, member 5 (DNAJB5, GenBank AF088982), Notch homolog 3 (NOTCH3, GenBank U97669), and transforming growth factor, beta receptor III (TGFBR3, GenBank L07594). TLE4, ANGPT1, DNAJB5, and NOTCH3 were increased 5.2-, 5.4-, 3.8-, and 5.3-fold, respectively, whereas TGFBR3 was decreased 10-fold in all prolactinomas compared to normal pituitary controls by microarray analysis. The primers of these genes were selected with Primer Express software version 2.0 (PE Applied Biosystems), BLASTed against all *H. sapiens* gene sequences in GenBank for selectivity, and synthesized by the Microchemical Facility at Emory University. The primers of these genes were as the following:

Ribosomal RNA (18S rRNA from PE Applied Biosystem) was used as an internal control. Briefly, after purifying with an RNeasy Mini Kit, total RNA (3 μg) of

	Forward primer	Reverse primer
TLE4	GGAGCCAGCTGTATTGACATTTC	ACCTGACCGTGTTGTCCAAAC
ANGPT1	CAGAAAACAGTGGGAGAAGATATAACC	AGTTGCCATCGTGTTCTGGAA
DNAJB5	CTCCAACCCCTTCGATATCTTCT	CCATGTCATCTGGGTCAAAGC
NOTCH3	TCTCAGACTGGTCCGAATCCAC	CCAAGATCTAAGAACTGACGAGCG
TGFBR3	GATAATGGATTTCCGGGAGATATG	TGCAATTAAACACCACGATTTCA

each sample was DNAse I digested (Cat. No. 18068-015, Invitrogen, http://www.invitrogen.com) as recommended by the manufacturer, and reverse transcribed in 20 µl, using 150 ng of random prime hexamers, 0.5 mM of deoxynucleotide triphosphate, and 50 units of Superscript^{TM} reverse transcriptase as recommended by the manufacturer (Cat. No 11904-018, Invitrogen, http://www.invitrogen.com). All PCR reactions were performed at least in duplicate, and were cycled in the GeneAmp 5700 Sequence Detection System at 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. The specificity of the PCR reactions was determined from the dissociation curve analysis. The standard curve of each gene, and 18S rRNA were performed each time the genes were analyzed. All PCR products were in the geometric range of the PCR amplification. The quantity of the specific genes that was obtained from the standard curves was normalized to that of the 18S rRNA of the same sample. The fold-change of each gene was calculated as the ratio of the mean of the normalized mRNA of the prolactinomas compared to that of the normal pituitary controls.

Microarray-data analysis

Gene expression data from 12,625 probe sets on the HG-U95Av2 GeneChips were processed into. CEL files using Affymetrix MAS 5.0, and were uploaded to GeneTraffic Software (Iobion, La Jolla, CA) where they were normalized using the GCRMA method [27]. After data normalization, genes with uniformly low expression (maximum signal in any sample <32) were removed from consideration, leaving 7,241 probe sets for analysis using Significance Analysis of Microarrays (SAM) software [28]. Relevant parameters for the SAM analysis were—Imputation engine: 10-Nearest Neighbor, Number of Permutations: 500, RNG Seed: 1234567, Delta: 1.063, Fold-Change: 2.0. Normalized expression data from the 297 significant probe sets were analyzed by a two-dimensional hierarchical clustering, using Spotfire DecisionSite 8.1 software (http://spotfire.tibco.com/products/decisionsite_microarray_analysis.cfm). Data was clustered using unweighted averages and ordered using average Euclidian distance.

For K-nearest neighbor (KNN) prediction, the normalized RT-qPCR data was analyzed with GenePattern software (http://www.broad.mit.edu/cancer/software/genepattern/) and both the KNN cross-validation and class prediction modules were used (KNN = 3). For these analyses the five genes (or features) that were included were TLE4, ANGPT1, DNAJB5, NOTCH3, and TGFBR3.

Two-dimensional gel electrophoresis (2DGE) of pituitary proteins

Two-dimensional (2D) gel electrophoresis, image analysis of digitized 2D gels and mass spectrometry characterization of proteins were also described [18]. The detailed experimental protocols have been published [29, 30]. Each whole control pituitary gland (0.45–0.70 g) was separately homogenized (4°C) in 10 ml of 2 M acetic acid/0.1% mercaptoethanol with a Polytron homogenizer (13,000 rpm, Model P710/35; Brinkmann Insruments, Westbury, NY, USA). The homogenate was sonicated (20 s) with a Fisher sonicator (Model FS30H; Pittsburgh, PA, USA), and an aliquot (1 ml) of the homogenate was lyophilized and stored (−80°C) until analysis. Each tumor tissue (15–75 mg) was separately homogenized in 2 ml of 2 M acetic acid/0.1% mercaptoethanol with the same procedure. Prior to protein redissolution for first dimension IEF, the protein content in the lyophilized pituitary adenoma was measured with a bicinchoninic acid (BCA) protein assay reagent kit (Pierce 23227, Rockford, IL, USA). For an 18 cm IPG strip (pH 3–10, nonlinear (NL), Amersham Biosciences, Piscataway, NJ, USA), the lyophilized pituitary sample that contained 70 µg of pituitary protein was mixed with a volume (250 µl) of the dissolving solution that contained these final concentrations (7 M urea, 2 M thiourea, 4% w/v CHAPS, 100 mM DTT, 2% v/v Pharmalyte, and a trace of bromophenol blue). The mixture was vortexed (5 min) and sonicated (5 min), and remained standing (40 min). A volume (110 µl) of rehydration solution, which contains the final concentrations (7 M urea, 2 M thiourea, 4% w/v CHAPS, 60 mM DTT, 0.5% v/v IPG buffer pH 3–10 NL, and a trace of bromophenol blue) was added into the above mixture. The mixture was sonicated (5 min), remained standing (40 min), was vortexed (5 min), and was centrifuged (20 min, 13,000 × g,

Model OM3590; International Equipment, Needham Heights, MA, USA). The supernatant (360 μl) is the "protein sample solution", which was used for Isoelectric focusing IEF. SDS-PAGE was performed on a PROTEAN plus Dodeca^{TM} Cell (Bio-Rad, Hercules, California, USA) that can analyze up to 12 gels at a time with a 12% PAGE resolving gel (190 × 205 × 1.0 mm) that was cast with a PROTEAN plus Multi-casting Chamber (Bio-Rad). The 2DGE-separated proteins were visualized with a modified silver-staining method.

Image analysis of digitized 2D gels

The silver-stained 2D gels were digitized, and were analyzed qualitatively and quantitatively with the PDQuest 2D Gel Analysis software for a PC computer (version 7.1.0, Bio-Rad, Hercules, CA, USA). The total density in a gel image was used to normalize each spot-volume in the gel image to minimize the effect of any experimental factor on the quantitative analysis [30–32]. Gel images in the match set were grouped into: control and prolactinoma.

Mass spectrometry characterization of proteins

Each differential spot between control gels and prolactinoma gels was excised from the 2D gels, and was subjected to in-gel trypsin digestion [29]. That mixture of tryptic peptides was purified with a ZipTipC18 micro-column (Millipore, ZTC18S096, Bedford, MA, USA) according to the manufacturer's instructions. The purified tryptic-peptide mixture was analyzed with a Perseptive Biosystems MALDI-TOF Voyager DE-RP mass spectrometer (Framingham, MA, USA) and with an LCQ^{Deca} mass spectrometer (LC-ESI-Q-IT) equipped with a standard electrospray source (Thermo-Finnigan, San Jose, CA, USA). For MALDI-TOF MS analysis, the peptide-mass fingerprinting (PMF) data were generated, and for LC-ESI-Q-IT analysis, the amino acid sequence of each LC-separated tryptic peptide was obtained. The MALDI-TOF MS PMF data were used to identify the protein by searching the SWISS-PROT/TrEMBL database with PeptIdent software (http://us.expasy.org/tools/peptident.html). The LC-ESI-Q-IT MS/MS data were used to identify the protein by searching the SWISS-PROT and NCBInr databases with the SEQUEST software that is a part of the LCQ^{Deca} software package.

Bisulfite modification and methylation-specific PCR

DNA from eight normal and nine prolactinomas used in the microarray and RT-qPCR were bisulfite modified as previously described [33]. Briefly, 2 μg genomic DNA was denatured in 0.2 M NaOH at 37°C and subsequently incubated for 16 h at 50°C in the presence of 0.5 mM hydroquinone and 2.5 M sodium bisulfite. Modified DNA was then desalted and conversion was completed by addition of NaOH to a concentration of 0.3 M, followed by ethanol precipitation. Methylation specific PCR was performed with approximately 80 ng of bisulfite-modified DNA as previously described [34]. Primers for GSTP1 have previously been described [35]. Primers for HLA-G were as follows: methylated, 5'-GGTTGCGATTTGGGGTTCGAC-3′ and 5′-CTATAAAACCACTCCACGCACG-3′; and unmethylated, 5′-ATTGGTTGTGATTTGGGGTTT-GAT-3′ and 5′-ATCTATAAAACCACTCCACACACA-3′.

Results and discussion

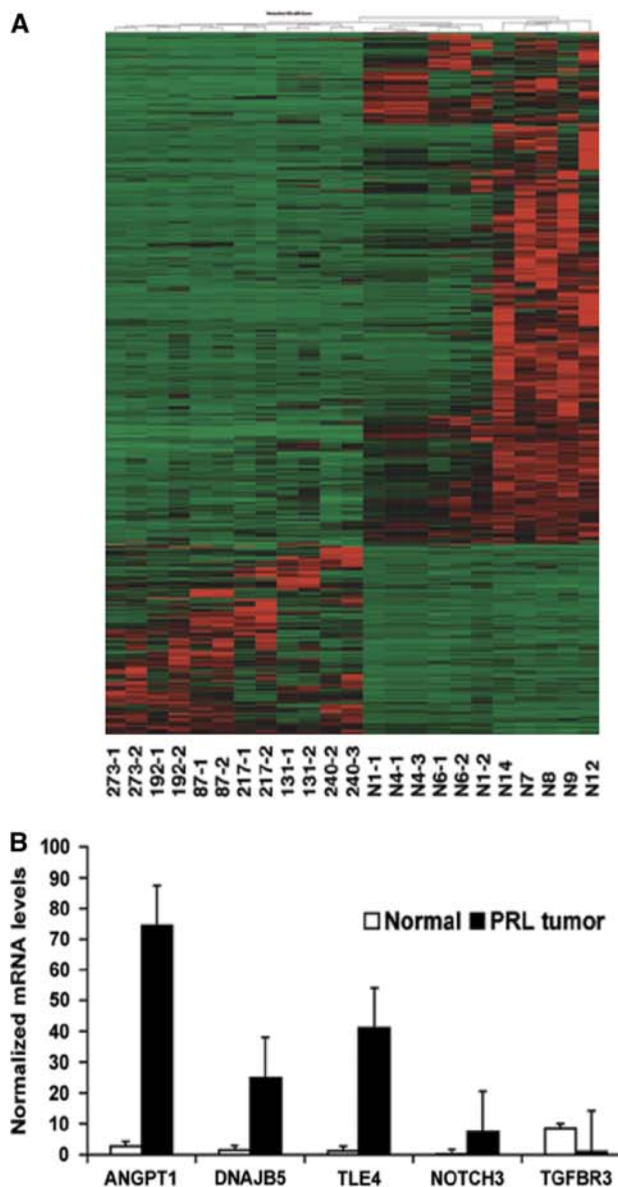
The clinical and pathological characteristics of the 10 prolactinomas are listed in Table 1.

Cluster analysis of gene expression by microarray analysis.

To identify changes in gene expression that correlated with prolactinomas, we performed expression profiling. We prepared total RNA by trizol extraction of serial sections from snap-frozen tissues and analyzed gene expression profiles in duplicate for six prolactinomas and eight normal pituitary glands. To identify genes that were differentially expressed between tumor and normal pituitary samples in a statistically significant manner, we used the SAM software [28] which computes false discovery rates (FDR's) for any user-specified threshold (Δ) for statistical stringency and fold-change. Using a highly conservative threshold ($\Delta = 1.871$, fold-change = 2.0) that provided a median estimate of less than 1% false positive genes, the SAM software identified 818 probe sets corresponding to 726 unique genes that were statistically significantly different in prolactinomas and normal pituitary tissues (Fig. 1A). Normalized expression data from those 818 probe sets was analyzed by two-dimensional hierarchical clustering using unweighted average Euclidian distance metrics.

Figure 1A shows the results from that clustering analysis for the 221 up-regulated and 597 down-regulated probe sets corresponding to 201 significantly up-regulated genes and 525 down-regulated genes. A complete list of those 726 unique genes is given in Supplementary Table S1. Among the 201 significantly up-regulated genes were Ras-induced senescence 1 (RIS1), Pit-1 transcription factor (POU1F1), OCT2 transcription factor (POU2F2), DnaJ (Hsp40) homolog B5 (DNAJB5), angiopoetin 1

Fig. 1 (**A**) Expression pattern of the 818 probe sets that were significantly different between prolactinomas and normal pituitary tissues analyzed by two-dimensional hierarchical clustering. Red, higher expression; green, lower expression; black, non-significant genes. (**B**) Normalized mRNA levels of prolactinomas and normal controls of the expression of TLE4, ANGPT1, DNAJB5, NOTCH3, and TGFB3 by RT-qPCR. The solid rectangular boxes represent the mean mRNA expression of each gene of prolactinoma or control group, and the whisker bars represent the standard error. Changes in the mean mRNAs of those genes were all significant at $P \leq 0.002$ by the Mann–Whitney test. Fold-change was the ratio of the mean of mRNA of the adenoma group to the controls as described in "Methods and Materials". Samples analyzed were: 10 prolactinomas and eight normal pituitary controls. (**C**) KNN classification of prolactinomas and normal pituitaries based on RT-qPCR data



(ANGPT1), engulfment and cell motility 1 (ELMO1), Notch homolog 3 (NOTCH3), transducin-like enhancer of split 4 (TLE4) and Bcl-2 athanogene. Among the 525 significantly down-regulated genes in prolactinomas were transforming growth factor, beta receptor III (TGFBR3), E-cadherin, and the β and γ subunits of the GADD45 complex, zinc finger protein suppression of tumorigenicity 18 (ST18), deleted in lymphocytic leukemia-1 (DLEU1), insulin-like growth factor binding protein 3 (IGFBP3), and frizzled homolog 7 (FZD7).

Next, we compared the expression profiling of genes in prolactinomas versus normal pituitaries and genes in non-functional pituitary adenomas versus normal pituitaries [18]. Table 2 shows genes changed similarly in both prolactinomas and non-functional pituitary adenomas. Table 3 shows genes changed significantly in opposite directions between prolactinomas and non-functional pituitary adenomas. Table 4 shows genes changed more than 2-fold in prolactinomas but not in non-functional pituitary adenomas.

We then compared the expression profile of some important genes in prolactinomas versus normal pituitaries and versus NF. Some of these genes have been responsible for human pituitary tumorigenesis, especially prolactinomas. Others have been implicated in prolactinomas formation by transgenic studies. Table 5 showed that PTTG-1, PRKAR2A, RB1, E2F(s), CD5, CD6, CD8, CD10, CDKN2B (p15), and CDKN1B (p27) had no significant change in prolactinomas versus normal pituitaries or versus NF adenomas. Genes such as GNAS complex locus (Gαs) were over-expressed in prolactinomas compared to normal pituitaries and to NF. Interestingly, prolactin showed no fold change compared to normal pituitaries but it showed change greather than 100-fold compared to NF. MEN1, CDKN1A (p21) and CDKN2C (p18) had no significant change compared to normal pituitaries but over-expressed in prolactinomas, respectively – 2.3-fold, 5.6-fold and 3.1-fold compared to NF. HMGA1 over-expressed, respectively 2.2-fold compared to normal pituitaries but had no significant change compared to NF.

Table 2 Genes changed similarly in both PRL and NF adenomas

Gene ID	Symbol	Fold change (PRL)	Fold change (NF)	Gene ID	Symbol	Fold change (PRL)	Fold change (NF)
34981_at	KCNA5	6.1	5.9	649_s_at	CXCR4	−6.6	−16.4
38750_at	NOTCH3	5.3	4.3	875_g_at	CCL2	−7.8	−18.7
41055_at	KIAA0363	3.3	2.1	40570_at	FOXO1A	−7.9	−5.8
31897_at	DOC1	2.7	2.8	1052_s_at	CEBPD	−8.4	−14.1
36811_at	LOXL1	2.2	3.1	39822_s_at	GADD45B	−9.6	−6.2
33904_at	CLDN3	−2.1	−2.5	1897_at	TGFBR3	−10.1	−6.8
1452_at	LMO4	−2.3	−3.8	37319_at	IGFBP3	−10.8	−3.3
36690_at	NR3C1	−2.6	−2.1	36711_at	MAFF	−12.0	−28.0
38340_at	HIP1R	−2.7	−3.5	32648_at	DLK1	−12.0	−917.4
103_at	THBS4	−2.8	−4.1	32531_at	GJA1	−12.7	−14.7
31874_at	GAS2L1	−2.9	−9.0	1153_f_at	CGB	−14.5	−5.1
33222_at	FZD7	−3.0	−1.9	35663_at	NPTX2	−16.8	−40.1
37819_at	MDM1	−3.4	−2.2	36543_at	F3	−17.7	−7.3
41189_at	TNFRSF25	−3.5	−2.2	38164_at	RPGR	−21.8	−18.6
36550_at	RIN2	−3.6	−3.3	40103_at	VIL2	−23.7	−9.1
41215_s_at	ID2	−3.7	−6.3	41424_at	PON3	−24.0	−12.5
41660_at	CELSR1	−3.9	−3.5	39154_at	GADD45G	−24.4	−30.5
37393_at	HES1	−4.1	−7.7	32243_g_at	CRYAB	−25.8	−9.0
36617_at	ID1	−4.2	−9.7	39051_at	NNAT	−35.3	−5.6
38286_at	AMOT	−4.3	−2.4	40544_g_at	ASCL1	−39.8	−116.8
41536_at	ID4	−4.4	−2.2	36784_at	CSHL1	−54.5	−85.0
1237_at	IER3	−4.8	−5.2	39350_at	GPC3	−70.8	−3.7
39356_at	NEDD4L	−4.9	−3.5	32552_at	RBP4	−252.1	−6.3
39827_at	RTP801	−5.5	−3.1	309_f_at	GH2	−284.3	−578.0
40953_at	CNN3	−5.6	−3.7	35879_at	GAL	−563.7	−487.8
1586_at	IGFBP3	−5.8	−2.6	35378_at	LHB	−1622.2	−13.0
33791_at	DLEU1	−5.8	−2.3	33711_at	POMC	−3148.7	−1666.7
38429_at	FASN	−6.2	−9.5	1332_f_at	GH1	−3787.6	−5000.0

Table 3 Genes changed significantly in opposite directions between PRL and NF adenomas

Gene ID	Accession	Symbol	Title	Fold change (PRL)	Fold change (NF)
35692_at	NM_015444	RIS1	Ras-induced senescence 1	7.1	−5.0
39315_at	NM_001146	ANGPT1	Angiopoietin 1	5.4	−5.2
36134_at	NM_006334	OLFM1	Olfactomedin 1	4.0	−4.4
34238_at	NM_001555	IGSF1	Immunoglobulin superfamily, member 1	4.0	−5.2
37005_at	NM_005380	NBL1	Neuroblastoma, suppression of tumorigenicity 1	2.3	−6.1
34013_f_at	NM_000306	POU1F1	Pit-1 transcription factor 1	2.3	−5.3
32076_at	NM_005822	DSCR1L1	Down syndrome critical region gene 1-like 1	−2.1	3.3
225_at	NM_002075	GNB3	G protein beta polypeptide 3	−2.9	3.9
2053_at	NM_001792	CDH2	N-cadherin	−3.7	2.3

CDKN2A (p16) and P8 (p8 protein, a candidate for metastasis 1) were negative (−1.6, −5.8-fold), compared to normal pituitaries but had no significant change compared to NF. CDKN1C (p57) was negative (−1.8-fold) compared to normal pituitaries, but over-expressed (2.2-fold) compared to NF.

Validation of differentially expressed genes by RT-qPCR

To verify the microarray analysis, we measured the expression levels of five genes: TLE4, ANGPT1, DNAJB5, NOTCH3, and TGFBR3 in 10 prolactinomas and eight

Table 4 Genes changed more than 2-fold in PRL but not in NF adenomas

Gene ID	Symbol	Fold change (PRL)	Fold change (NF)	Gene ID	Symbol	Fold change (PRL)	Fold change (NF)
36892_at	ITGA7	8.1	1.3	36118_at	NCOA1	−2.5	−1.0
39250_at	NOV	6.5	−1.4	38146_at	ST18	−2.6	−1.5
39242_at	SYT5	5.1	1.3	36650_at	CCND2	−2.8	1.3
34798_at	BAG1	4.1	−1.5	33396_at	GSTP1	−3.0	1.4
39395_at	THY1	4.0	1.3	39969_at	HIST1H4C	−3.1	−1.3
38632_at	DNAJB5	3.8	−1.4	35277_at	SPON1	−3.6	−1.3
32873_at	KCND2	3.7	1.0	977_s_at	CDH1	−3.9	−1.4
36933_at	NDRG1	3.2	−1.2	41246_at	SERPINE2	−4.1	1.3
37749_at	MEST	3.0	1.1	36627_at	SPARCL1	−4.2	−1.4
37421_f_at	HLA-F	2.9	−1.2	38028_at	DAT1	−4.3	1.2
41237_at	HLA-A	2.9	1.1	39878_at	PCDH9	−5.9	1.1
37383_f_at	HLA-C	2.7	−1.5	36133_at	DSP	−6.8	1.1
40369_f_at	HLA-G	2.3	−1.2	34198_at	PTPN13	−7.5	1.5
32598_at	NELL2	2.2	−1.0	32215_i_at	RHOBTB3	−8.0	−1.4
428_s_at	B2M	2.1	−1.3	39271_at	MIA	−8.2	−1.0
32778_at	ITPR1	−2.3	−1.4	577_at	MDK	−8.5	−1.2
32827_at	RRAS2	−2.3	−1.1	35844_at	SDC4	−18.4	−1.2
39054_at	GSTM1	−2.4	1.5	38650_at	IGFBP5	−20.1	−1.3

normal pituitary glands using RT-qPCR with SYBR Green I dye detection (Applied Biosystems). TLE4, ANGPT1, DNAJB5, and NOTCH3 were increased 5.2-, 5.4-, 3.8-, and 5.3-fold, respectively, whereas TGFBR3 was decreased 10-fold in all prolactinomas compared to controls by microarray analysis. RT-qPCR analysis of those five genes (Fig. 1B) confirmed the changes in mRNA levels that were observed by expression profiling. TLE4 and ANGPT1 mRNA expression were significantly up-regulated 25-fold, DNAJB5 was up-regulated 15-fold, NOTCH3 was up-regulated 34-fold, and TGFB3 was down-regulated 8-fold in prolactinomas compared to normal pituitary controls.

We next used the RT-qPCR data to identify the prolactinomas and normal pituitary glands by their molecular profile using the K-nearest neighbors (KNN) method. With KNN leave-one-out cross-validation the tumor and normal samples were correctly predicted in 94% of the cases using RT-qPCR data for these five genes (Fig. 1C). When the data was separated into independent training sets ($n = 10$) and test sets ($n = 8$), all eight cases were correctly predicted.

Proteomics analysis of prolactinomas versus normal pituitary glands

Four prolactinomas analyzed by microarray were also analyzed by two-dimensional gel electrophoresis (2DGE) and mass spectrometry. The high-resolution and high-reproducibility of 2DGE patterns enabled an accurate comparison between the proteomes from the control pituitary glands versus prolactinomas. Each sample was analyzed ($n = 3$–5), and approximately 1,000 protein spots were detected in each gel. For each sample, the mean between-gel, matched percentage was 85–99% for the controls, and 81–90% for the prolactinomas. For each sample, the correlation coefficient (r) of the normalized volumes for the between-gel matched-spots was >0.73 (range, 0.76–0.92).

The differential spots were determined between control pituitaries ($n = 8$; number of gels = 30) versus prolactinomas ($n = 4$, gels = 12). Fifty-four differential spots (47 decreased spot volumes, 7 increased) were found. The proteins that were contained in the 41 differential protein-spots were MS-characterized, and are listed in Table 6, which contains the corresponding SSP numbers (the coding number generated by the PDQuest software; each SSP number in Table 6 corresponds to the SSP number that was labeled in Fig. 2 in our previous publication) [18], the change-fold (the inimum change-fold = 2.0), and the experimental pI and M_r values of each differential spot.

The differential spots were excised, in-gel trypsin digestion was performed, and MS (MALDI-TOF PMF and/or LC-ESI-Q-IT MS/MS) was used to characterize the protein in each differential spot [29]. Among those 54 differential spots, 41 spots (37 decreased, 4 increased) that represented 23 differentially regulated proteins (19 down-regulated, 4 up-regulated) were characterized (Table 6).

Table 5 Fold change vs. Normal and Fold change vs. NF of some important genes

Probe set ID	Gene symbol	Fold change vs. Normal	SAM score vs. Normal	Fold change vs. NF adenomas	SAM score vs. NF adenomas	Title
37448_s_at	GNAS	5.8	1.8	2.3	6.9	GNAS complex locus (G alpha s)
39704_s_at	HMGA1	2.2	3.8	NC	NS	High mobility group AT-hook 1
37741_at	PYCR1	2.2	5.4	5.1	12.8	Pyrroline-5-carboxylate reductase 1
37449_i_at	GNAS	2.1	9.0	2.2	8.0	GNAS complex locus (G alpha s)
37450_r_at	GNAS	2.1	5.3	2.7	6.4	GNAS complex locus (G alpha s)
1713_s_at	CDKN2A	−1.6	−1.5	NC	NS	P16
1787_at	CDKN1C	−1.8	−1.8	2.2	4.0	p57, Kip2
456_at	SMARCD3	−2.1	−3.9	−5.8	−10.5	SWI/SNF related, matrix associated, actin dependent regulator of chromatin D3
37270_at	ATP1B2	−2.1	−1.9	−12.8	−9.4	ATPase, Na+/K+ transporting, beta 2 polypeptide
32230_at	EIF3S2	−2.1	−3.0	2.7	15.6	Eukaryotic translation initiation factor 3, subunit 2 beta, 36kDa
41338_at	AES	−3.3	−7.1	−5.2	−9.5	Amino-terminal enhancer of split
2053_at	CDH2	−3.7	−5.3	−8.6	−8.9	Cadherin 2, type 1, N-cadherin (neuronal)
36423_at	P8	−5.8	−2.6	NC	NS	p8 protein (candidate of metastasis 1)
34198_at	PTPN13	−7.5	−6.2	−11.0	−8.2	Protein tyrosine phosphatase, non-receptor type 13 (APO-1/CD95 (Fas)-associated phosphatase)
35879_at	GAL	−563.7	−6.8	NC	NS	Galanin
878_s_at	PRL	NC	NS	105.2	35.5	Prolactin
2031_s_at	CDKN1A	NC	NS	5.6	8.1	p21, Cip1
897_at	PKD1	NC	NS	5.3	13.5	Polycystic kidney disease 1 (autosomal dominant)
36180_s_at	MAPKAPK2	NC	NS	4.3	14.3	Mitogen-activated protein kinase-activated protein kinase 2
34907_at	AATK	NC	NS	3.9	17.1	Apoptosis-associated tyrosine kinase
31504_at	HDLBP	NC	NS	3.1	14.5	High density lipoprotein binding protein (vigilin)
36053_at	CDKN2C	NC	NS	3.1	15.6	p18
34367_at	PHGDH	NC	NS	2.9	17.5	Phosphoglycerate dehydrogenase
34689_at	TREX1	NC	NS	2.7	15.2	Three prime repair exonuclease 1
33393_at	DDX19	NC	NS	2.5	16.1	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 19 (DBP5 homolog, yeast)
1867_at	CFLAR	NC	NS	2.3	12.8	CASP8 and FADD-like apoptosis regulator
36613_at	IFRD2	NC	NS	2.3	15.8	Interferon-related developmental regulator 2
37531_at	TBC1D5	NC	NS	2.3	14.3	TBC1 domain family, member 5
34395_at	KIAA0354	NC	NS	2.2	13.8	KIAA0354 gene product
926_at	MT1G	NC	NS	2.2	20.5	Metallothionein 1G
1044_s_at	E2F5	NC	NS	2.2	4.1	E2F transcription factor 5, p130-binding
32701_at	ARVCF	NC	NS	2.2	14.8	Armadillo repeat gene deletes in velocardiofacial syndrome
539_at	RYK	NC	NS	−2.0	−8.6	RYK receptor-like tyrosine kinase
37723_at	CCNG2	NC	NS	−2.1	−8.4	Cyclin G2
448_s_at	MEN1	NC	NS	−2.3	−10.0	Multiple endocrine neoplasia I
36940_at	TIAF1	NC	NS	−2.3	−8.0	TGFB1-induced anti-apoptotic factor 1
35520_at	CLDN9	NC	NS	−2.6	−10.7	Claudin 9
38771_at	HDAC1	NC	NS	−3.8	−8.0	Histone deacetylase 1
35536_at	ECE2	NC	NS	−5.2	−10.9	Endothelin converting enzyme 2
32174_at	SLC9A3R1	NC	NS	−5.5	−16.0	Solute carrier family 9 (sodium/hydrogen exchanger), isoform 3 regulatory factor 1
36780_at	CLU	NC	NS	−10.6	−9.9	Clusterin
160024_at	CDK10	NC	NS	NC	NS	Cyclin-dependent kinase (CDC2-like) 10

Table 5 continued

Probe set ID	Gene symbol	Fold change vs. Normal	SAM score vs. Normal	Fold change vs. NF adenomas	SAM score vs. NF adenomas	Title
1206_at	CDK5	NC	NS	NC	NS	Cyclin-dependent kinase 5
1207_at	CDK6	NC	NS	NC	NS	Cyclin-dependent kinase 6
1189_at	CDK8	NC	NS	NC	NS	Cyclin-dependent kinase 8
2034_s_at	CDKN1B	NC	NS	NC	NS	p27, Kip1
1607_at	CDKN2B	NC	NS	NC	NS	P15
1322_at	E2F1	NC	NS	NC	NS	E2F transcription factor 1
2068_s_at	E2F2	NC	NS	NC	NS	E2F transcription factor 2
41632_at	E2F3	NC	NS	NC	NS	E2F transcription factor 3
38706_at	E2F4	NC	NS	NC	NS	E2F transcription factor 4, p107/p130-binding
35984_at	E2F6	NC	NS	NC	NS	E2F transcription factor 6
116_at	PRKAR2A	NC	NS	NC	NS	Protein kinase, cAMP-dependent, regulatory, type II, alpha
40412_at	PTTG1	NC	NS	NC	NS	Pituitary tumor-transforming 1
1570_f_at	RB1	NC	NS	NC	NS	Retinoblastoma 1

NC = No change (<1.5-fold)

NS = Not significant (q-value > 5%)

The 23 MS-characterized differentially expressed proteins were categorized into different functional groups. Those differentially expressed proteins include pituitary hormones, cytokine and cellular signals, cellular-defense and stress-resistance proteins, transport proteins, enzymes, cell growth, cell proliferation, and cell differentiation, those proteins located within the cytoplasmic, mitochondrial, nuclear, lysosomal, peroxisomal, or they are secreted into the plasma. Table 6 indicates the functional categories (second column), and the subcellular location (fifth column). The MS-characterized proteins (Table 6) altered in prolactinomas included: (a) neuroendocrine-related proteins (somatotropin, secretagogin, and mu-crystallin homolog) were down-regulated in the prolactinomas; (b) somatotropin existed in at least 17 isoforms that were down-regulated in the prolactinomas; (c) cell proliferation, differentiation, and apoptosis-related proteins were down-regulated in the prolactinomas; (d) some cell-defense and stress-resistance proteins such as phospholipid hydroperoxide glutathione peroxidase and heat-shock 27 kDa protein were down-regulated in the prolactinomas; (e) some metabolic enzyme-related proteins (for example, isocitrate dehydrogenase [NADP] cytoplasmic, tryptophan 5-hydroxylase 2, matrix metalloproteinase-9, etc.) were up-regulated in the prolactinomas; the presence of the matrix-metalloproteinase (MMP) and its level of expression are related to tumor invasiveness in prolactinomas [36]. The MMP's are peptidases that degrade extracellular matrix and promote angiogenesis and tumor invasion. They are more typical of invasive macroprolactinomas than noninvasive tumors [37]; (f) some cell-signal proteins were down-regulated, and those cell signals are involved in the complex biological roles in the cell growth, proliferation, differentiation, apoptosis, and death cycle.

Discussion

In general, due to the limitation we compared the data of prolactinomas to normal pituitaries knowing that normal pituitaries are heterogeneous population. Some of prolactinomas are homogeneous. They in general express PRL, but sometime they express PRL and GH as hormones. The non-functional pituitary adenomas are not homogeneous; cell population includes null cell (17%), oncocytoma (6%), silent corticotroph (8%), silent somatotroph (3%), and gonadotrophs (40–79%). Prolactinomas and non-functional pituitary adenomas derive from normal pituitary glands.

Interestingly, when we compared the data of prolactinomas versus normal pituitaries and versus non-functional pituitary adenomas (Table 5), some genes were over-expressed in one comparison but not over-expressed in other. Some genes were not significantly changed in prolactinomas versus normal pituitaries or versus NF adenomas. Our data (Table 5) showed that PTTG-1, PRKAR2A, RB1, E2F(s), CD5, CD6, CD8, CD10, CDKN2B (p15), and CDKN1B (p27) had no significant change in prolactinomas versus normal pituitaries or versus NF adenomas. Some genes such as GNAS complex locus (Gαs) were over-expressed in prolactinomas for both comparisons. This result agreed with Picard C et al. where Gsα transcripts were exclusively maternal in all 15

Table 6 The MS-characterized differentially expressed proteins in human hyperprolactinomas versus controls, and the corresponding functional category

SSP No.	Protein and functional category	Swiss-prot No.	pI; M_r (exp.)	Subcellular location	Prolactinoma
A. Down-regulated proteins in PRL-secreted pituitary adenomas					
I. Neuro-endocrine and hormones					
1011	Chain 1: somatotropin	P01241	5.33; 19.43	Secreted	133.2(−)
1017	Chain 1: somatotropin	P01241	5.33; 16.47	Secreted	9.7(−)
	Splice isoform 2 of somatotropin precursor	P01241 (isoform)			
1102	Chain 1: somatotropin	P01241	5.14; 25.89	Secreted	(–)
1103[a]	Somatotropin precursor	P01241	5.14; 22.79	Secreted	7.5(−)
	Growth hormone variant precursor	P01242			
1109	Chain 1: somatotropin	P01241 (isoform)	5.26; 21.81	Secreted	(–)
1121	Chain 1: somatotropin	P01241	5.28; 22.95	Secreted	14.8(−)
1122	Chain 1: somatotropin	P01241	5.14; 25.36	Secreted	(–)
1123	Chain 1: somatotropin	P01241	5.14; 21.82	Secreted	(–)
2002	Splice isoform 2 of somatotropin precursor	P01241 (isoform)	5.56; 19.37	Secreted	16.0(−)
2003	Chain 1: somatotropin	P01241	5.57; 18.89	Secreted	(–)
2101	Chain 1: somatotropin	P01241	5.38; 23.95	Secreted	(–)
2106	Chain 1: somatotropin	P01241	5.44; 21.87	Secreted	41.7(−)
2115	Chain 1: somatotropin	P01241	5.62; 19.86	Secreted	(–)
2128[b]	Chain 1: somatotropin	P01241	5.67; 22.79	Secreted	(–)
2129[b]	Chain 1: somatotropin	P01241	5.44; 22.95	Secreted	24.5(−)
3004	Chain 1: somatotropin	P01241	5.88; 19.37	Secreted	161.0(−)
3107	Chain 1: somatotropin	P01241	5.87; 22.65	Secreted	(–)
4106	Chain 1: prolactin	P01236	6.15; 26.02	Secreted	3.4(−)
1311	Secretagogin	O76038	5.25; 30.06	Cytoplasmic	2.2(−)
1402	Mu-crystallin homolog	Q14894	5.14; 37.50	Cytoplasmic	7.6(−)
II. Cytokine and cellular signal-related proteins					
0020	Splice isoform IL15-S21AA of interleukin-15 precursor	P40933-2 (isoform)	4.45; 16.46	Nucleus and cytoplasmic	(–)
1010	14-3-3 protein tau	P27348	5.33; 15.85	Cytoplasmic	43.5(−)
8101	Chain 1: insulin-like growth factor binding	P24592	7.47; 22.07	Secreted	(–)
2303	Serine/threonine protein phosphatase 2A, 55 kDa regulatory subunit B, alpha isoform	Q00007	5.43; 32.01		6.4(−)
0112	Alpha crystallin C chain	Q9UJY1	4.77; 20.11		(–)
III. Cellular defense and stress resistance					
8102	Chain 1: phospholipid hydroperoxide glutathione peroxidase	P36969	7.50; 20.90	Mitochondrial and cytoplasmic	26.1(−)
3210[b]	Heat shock protein 27	P04792	5.87; 27.57	Cytoplasmic or nucleus	2.7(−)
IV. mRNA splicing, transport or translation-related enzym					
6103	N6-adenosine-methyltransferase 70 kDa subunit	Q86U44	6.72; 22.12	Nuclear	(–)
V. DNA-binding proteins					
2311	L-Myc-1 protooncogene protein	P12524	5.58; 34.50	Nuclear (potential)	17.8(−)
VI. Metabolic enzyme					
1806	Tissue transglutaminase	P21980	5.21; 84.58		(–)
2624	Chain 1: dipeptidyl-peptidase II	Q9UHL4	5.40; 52.60	Lysosomal and intracellular vesicles	2.6(−)
VII. Cell proliferation, differentiation, apoptosis-related proteins					
1404	Chain 1: MIMECAN	P20774	5.23; 36.96	Bone	(–)

Table 6 continued

SSP No.	Protein and functional category	Swiss-prot No.	pI; M_r (exp.)	Subcellular location	Prolactinoma
VIII. Transport proteins					
1117	Chain 1: apolipoprotein A-I	P02647	5.33; 25.82	Secreted	3.2(−)
7004[a]	Hemoglobin beta unit variant	gi 1066765	7.06; 17.47	Red blood cell	(–)
7014[a]	Hemoglobin beta-2 chain (PANLE)	P18988	7.33; 16.98	Red blood cell	(–)
	Hemoglobin beta chain (CERAE)	P02028			
	Hemoglobin alpha-3 chain (PANTR)	P01935			
7016[a]	Hemoglobin alpha-2 chain (BOSMU)	P01968	7.41; 12.52	Red blood cell	
	Hemoglobin beta-2 chain (PANLE)	P18988			
IX. Others					
0029	Chain 1: Factor X light chain	P00742	4.90; 16.72	Plasma; synthesized in the liver	83.0(−)
0511	Cytokeratin 16	P08779	4.89; 45.70		(–)
B. Up-regulated proteins in PRL-secreted pituitary adenomas					
I. Metabolic enzyme-related proteins					
6508	Isocitrate dehydrogenase [NADP] cytoplasmic	O75874	6.75; 43.55	Cytoplasmic and peroxisomal	1.9(+)
4616	Tryptophan 5-hydroxylase (Neuronal tryptophan hydroxylase)	Q8IWU9	6.26; 54.24	Brain specific	6.4(+)
7519	Chain 1: matrix metalloproteinase-9	Q99542	7.45; 43.84	Secreted	2.7(+)
II. Cellular defense response					
4012	Cytochrome c oxidase polypeptide VIb	P14854	6.11; 13.52		9.3(+)

[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

(−) = decreased relative to controls; (–) = lost relative to controls; (+) = increased relative to controls. M_r = kDa. Each SSP number in Table 5 corresponds to the SSP number that was labeled in Fig. 2 in our previous publication [18]

heterozygous prolactinomas except 2, a paternal GSα expression was found in 25 of 28 heterozygous somatotroph adenomas [38].

Prolactinomas have the highest level of TLE4 expression compared with other functional adenomas and non-functional tumors [39]. While this initial report by Ruebel et al. was based on a single prolactinoma array [39], we can now confirm high levels of TLE4 expression in 10 prolactinomas (6 analyzed by microarrays only and 10 analyzed by a combination of microarrays and RT-qPCR). TLE4 probably represses transcription by multiple mechanisms. For example, a glycine/proline-rich domain in the central variable region functions to recruit the histone deacetylase Rpd3 to the template. Histone deacetylase then presumably silences transcription by altering local chromatin structure, making the chromatin more compact and transcriptionally inactive. Many aspects of TLE function remain to be explored, including its role in pituitary tumorigenesis [39, 40].

Previous data have implicated VEGF and FGF-2 as angiogenic factors in prolactinomas [37]; here we also postulate a role for Angiopoietin-1 (Ang-1) based on the finding of its up-regulation in prolactinomas evidenced by microarrays and confirmed by RT-qPCR. Angiopoietin-1 is an important regulator of angiogenesis [41] and plays a stabilizing role, maintains cell-cell interactions and inhibits apoptosis. Ang-1 is also involved in recruiting and sustaining peri-endothelial support cells, and in mediating interactions between the endothelial cell and the basement membrane. Ang-1 is also necessary for the maturation of newly formed vessels [41]. In addition, the pro-angiogenic factor angiopoietin (ANGPT1) was strongly up-regulated in prolactinomas.

The dopamine (D-2) was up-regulated by microarray analysis of prolactinomas consistent with the known expression of this receptor on these tumors. This expression is the basis of dopamine agonist therapy of prolactinomas, conversely resistance to dopamine agonist therapy may be related to decreased expression of the receptor. The epithelial adhesion molecule E-cadherin (CDH1) and the proto-cadherin 9 (PCDH9) were decreased 4-fold and 6-fold in prolactinomas, respectively. The neuronal adhesion molecule, N-cadherin (CDH2) was also decreased in prolactinomas. IGFBP3 and IGFBP5 mRNA were down-regulated in prolactinomas. Of significance was the increase in expression of multiple MHC class I antigen-presenting genes (HLA-A, HLA-C, HLA-G, HLA-F, B2M). Two of these genes, HLA-F and HLA-G, are non-

classical MHC class I genes that help prevent maternal-fetal immune rejection, and thus may aid prolactinomas to avoid immune surveillance [42]. Furthermore, these genes can be regulated by DNA methylation, as can glutathione S-transferase pi (GSTP1), an oxidative free-radical scavenger that is lost early in prostate cancer development by methylation of its promoter that is repressed in prolactinomas [43]. However, analysis of the GSTP1 and HLA-G promoters by methylation-specific PCR showed that the GSTP1 and HLA-G promoters are primarily unmethylated in normal pituatiary tissue and remain unmethylated in prolactinomas (Supplementary S2).

Prolactinomas may avoid apoptosis by up-regulating the expression of Bcl-anthanogene (BAG1), an anti-apoptotic factor that is essential for survival of neurons [44]. BAG1 is a co-chaperone for the heat-shock protein Hsp70, interacts with C-Raf, B-Raf, Akt, and Bcl-2, and may be important for expression of IAP proteins. BAG 1 is found at high levels in several human cancers and was described as up-regulated 5.0-fold in a single human prolactinoma by microarray [45]. While this initial report by Morris et al. was based on a single prolactinoma array [45], we can now confirm high (4.0 folds) levels of BAG 1 expression in 10 prolactinomas (6 analyzed by microarrays only and 10 analyzed by a combination of microarrays and RT-qPCR).

NOTCH3, DLK1, HES1, ASCL1, and FDZ7, which play roles in the Delta-Notch and Wnt developmental pathways were altered in prolactinomas, similar to a previous finding we reported in non-functional tumors suggesting a shared molecular genesis [18]. This suggests that targeting of the Notch pathway may be of benefit in prolactinomas as proposed for non-functional pituitary adenomas, particulary in patients for whom dopamine agonist therapy is ineffective or may be inappropriate due to the high dosage required [18, 46]. In addition, the transcriptional repressors ID1, ID2, ID4, and FOXO1A are all decreased in prolactinomas. Genes important for cell-cycle arrest, such as GADD45B, GADD45G, are also down-regulated in prolactinomas as in non-functional pituitary adenomas consistent with a permissive environment for increased proliferation.

TGF-β1 and TGFBR3 were both down-regulated. TGF-β1 is a negative regulator of growth and proliferation, and is produced in and secreted by pituitary lactoropes, the cells that produce prolactin. TGF-β1 is a potent inhibitor of estrogen-induced lactrotropic proliferation and PRL secretion [47, 48]. Thus, down-regulation TGF-β1 and TGFBR3 in these tumors is consistent with a major role in the tumorigenesis and secretory activity of prolactinomas.

Two potential tumor suppressor genes, Ras-induced senescence 1 (RIS1) and neuroblastoma suppression of tumorigenicity 1 (NBL1), were up-regulated in prolactinomas. As expected, the pituitary-specific transcription factor Pit-1 (POU1F1) was increased in prolactinomas. The increased Pit-1 levels may account for the expression of prolactin that defines this subtype of pituitary adenomas.

One notable negative finding in the microarray analysis was that of a disintegrin and metalloproteinases, specifically ADAM 28. The ADAM28 was down-regulated 3.0-fold in prolactinomas. This was somewhat suprising in that high levels of expression of ADAM28s have been discovered in other tumors, for example in non-small cell lung carcinoma. The expression levels of ADAM28 were significantly higher in the carcinomas with lymph node metastasis compared with cavernous without metastasis, suggesting the possibility that ADAM28 plays a role in cell proliferation and progression of human lung carcinomas [49, 50]. Furthermore, a related protein ADAMTS6 is up-regulated in invasive prolactinomas. One possible explanation for its finding in our prolactinomas is that up-regulating ADAM28 protein may be associated with a more aggressive phenotype and is therefore not seen in more benign tumors.

In conclusion, expression profiling and proteomic analyses have identified molecular features unique to prolactinomas that may contribute to their pathogenesis. In the current era of molecular medicine, these findings greatly enhance our understanding and supercede immunohistochemical diagnosis.

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