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Microarray Studies Reveal Pomc and IL-6 as Biomarker Genes Associated with Hyperprolactinemia-Induced Male Infertility

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A B S T R A C T

This research attempted to establish biomarker genes and pathways involved in impairing the spermatogenesis process of patients with hyperprolactinemia. The gene expression dataset of GSE36314 was acquired using the GPL8300 platform from Gene Expression Omnibus (GEO) database, NCBI. BRB-Array Tools were used to analyze the microarray data for the identification of Differentially Expressed Genes (DEGs) between patients with hyperprolactinemia and controls. There were 138 upregulated genes and 14 downregulated genes detected in total. Analysis of Gene Ontology demonstrated enrichment in terms of modifications to the molecular functioning of hormonal activities, biological functioning of cell-cell signaling, and peptide hormone processing in the hyperprolactinemia group. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis indicated that the DEGs were significantly enriched in 17 pathways. Three important pathways namely, Hippo Signaling pathway, the Neuroactive ligand-receptor interaction pathway, and the GnRH signaling pathway were identified to be directly involved in the hyperprolactinemia group which results in the alteration of the spermatogenesis process. Proopiomelanocortin (POMC) and Interleukin-6 (IL6) genes were ascertained as the hub genes by statistical analysis which were found to be significantly modified and associated with spermatogenesis dysregulation in the hyperprolactinemia group. Conjointly, these data facilitate the identification of biomarkers for determining drug targets and also provide insights into the etiology underlying hyperprolactinemia-induced male infertility.

***Key words*:** Biomarkers, GnRH signaling, Hippo signaling, Hormonal activities, Interleukin-6, Proopiomelanocortin, Spermatogenesis dysregulation

Hyperprolactinemia is a condition of excessive prolactin hormone in the blood. In men, >15ug/ml of serum prolactin is considered aberrant which could be due to pathological and physiological conditions [1]. In about 11% of oligospermia-affected males, the cause of infertility is hyperprolactinemia [2]. The most common cause of hyperprolactinemia are prolactinomas (also called lactotroph adenomas) [3]. In hyperprolactinemia conditions, the pulsatile secretion of GnRH is inhibited which causes a decrease in the synthesis of glycoprotein hormones namely, Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH). Hence, the spermatogenesis process gets altered leading to impairment in sperm motility and alteration in sperm quality [2].

Previous studies on genes associated with hyperprolactinemia-induced male infertility show that the prolactin hormone may act on specific hypothalamic neurons namely, Kiss1, and leads to the modulation of the reproductive axis. Kisspeptins (encoded by the kiss1 gene) are upstream regulatory elements of GnRH involved in the regulation of gonadotropin secretion by gonadal hormones. Hence, prolactin may influence kiss-1 expressing neurons and affect spermatogenesis [4]. Another afferent pathway is also involved similarly, in which the γ- aminobutyric acid (GABAergic) neurons get modulated similarly by prolactin and impact fertility [5]. Several studies reveal the stimulation of prolactin mediates pathways such as Janus Kinase (JAK)-Signal Transducer and Activator of Transcription proteins (STAT) pathway and Mitogen-Activated Protein Kinases (MAPK) pathway [6]. Previous findings did not involve the analysis of biomarker genes and pathways involved in the spermatogenesis process of hyperprolactinemia patients. Hence, this study aimed to elucidate the differentially expressed genes along with biomarker genes and pathways associated with the reproductive axis of male hyperprolactinemia patients.

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MATERIALS AND METHODS

Microarray data

The Gene Expression Omnibus (GEO) database, National Center for Biotechnology Information (NCBI), was used to retrieve the gene expression profile with the series accession number of GSE36314, based on the platform of GPL8300 (Affymetrix Human Genome U95 Version 2 Array), which was previously reported by Tong *et al*. [7]. The data for analysis were downloaded in raw CEL format individually. The gene expression profile downloaded was limited to male patients and male controls only.

Processing the data

The data were primarily subjected to log 2 transformations and then pre-processed using affy package (Bioconductor software package) in R language [8] before being imported to BRB-Array Tools (v4.6.0 stable, National Cancer Institute, USA). An aggregate of over 12,625 probes was obtained. The threshold intensity was set to the minimum value of 10 and quantile normalization was used to center each array. The genes which showed minimal variation across the array set were not included in the analysis. The genes were excluded if their expression were <20% and the fold change in either direction from the gene's median value was <1.5 or if the percentage of missing data was greater than 50% [9].

Identification and comparison of DEGs

In the BRB-Array tools, a random-variance t-test was run to determine the DEGs between the two groups where P 0.01. Additionally, an FDR of 0.05 and an at least a 2.0-fold change in the dataset were required for admission [10].

Hierarchial clustering analysis

The samples that have comparable levels of gene expression were categorized collectively. Based on probe information from datasets, the expression values of DEGs in various samples [10] were evaluated using hierarchical clustering analysis using BRB-Array tools.

Gene ontology and pathway enrichment analysis

The gene enrichment analysis tool, DAVID (The Database for Annotation, Visualization, and Integrated Discovery) was deployed to understand the biological meaning of genetic discoveries [11]. The DEGs obtained in the current study were subjected to GO and KEGG pathway enrichment analysis [12].

Protein-protein interaction analysis

PPI networks were constructed using the online platform, The Search Tool for the Retrieval of Interacting Genes (STRING, version 10.5) [13].

RESULTS AND DISCUSSION

Determination of DEGs

The raw data consisting of 12,625 probes were filtered by threshold intensity (minimum value) and fold change and then quantile normalization was applied. A total of about 1,757 DEGs were obtained which were subjected to a univariate t-test at $P < 0.01$ between the hyperprolactinemia group and controls with a criterion of FDR < 0.05 and at least a fold change of 2 was used to identify significant DEGs. The outcome of the test was 152 DEGs out of which 138 genes were upregulated (Table 1) and 14 genes were downregulated (Table 2).

Table 1 Top 10 upregulated genes

ProbeSet	Name	Fold-change	P-value
1332_f_at	Growth Hormone 1	93.57	$< 1 \times 10^{-7}$
32243_g_at	Crystallin Alpha B	10.92	3×10^{-7}
33711_at	Proopiomelanocortin	72.11	7×10^{-7}
35879_at	Galanin and GMAP prepropeptide	64.44	1.2×10^{-6}
38299_at	Interleukin 6	5.52	3.3×10^{-6}
34777_at	Adrenomedullin	4.36	3.4×10^{-6}
36784_at	Chorionic Somatomammotropin Hormone like 1	25.02	5.9×10^{-6}
230_s_at	Follicle Stimulating Hormone Beta subunit	6.52	7.9×10^{-6}
37024_at	Lipopolysaccharide-Induced TNF factor	7.07	8.1×10^{-6}

Statistical criterion – Fold change > 2, a false discovery rate < 0.05 and P-value < 0.01

Table 2 Top 10 downregulated genes

ProbeSet	Name	Fold-change	P-value
37184_at	Syntaxin 1A	0.48	4.26×10^{-4}
39572_at	Glutamate Ionotropic Receptor Kainate subunit 2	0.37	5.16×10^{-4}
37450_r_at	GNAS complex locus	0.46	7.48×10^{-4}
35017_f_at	Major Histocompatibility Complex, Class I, J	0.45	8.67×10^{-4}
32566_at	Chondroitin Polymerizing Factor	0.48	1.44×10^{-3}
36892_at	Integrin subunit Alpha 7	0.28	1.93×10^{-3}
428_s_at	Beta-2-Microglobulin	0.46	2.26×10^{-3}
37489_s_at	Solute Carrier Family 4-member 3	0.47	2.37×10^{-3}
201_s_at	Beta-2-Microglobulin	0.33	3.83×10^{-3}
37156_at	ETS Variant 1	0.49	4.18×10^{-3}

Statistical criterion – Fold change <0.05, a false discovery rate < 0.05 and P-value <0.01

Hierarchical clustering analysis of DEGs

The dataset's expression values were used as the basis for hierarchical clustering analysis. The samples were split into the control group and the group with hyperprolactinemia. Heat maps were used to depict the gene expression data to interpret the findings (Fig 1). These findings indicate that the DEGs' expression patterns in the hyperprolactinemia group and controls were varied.

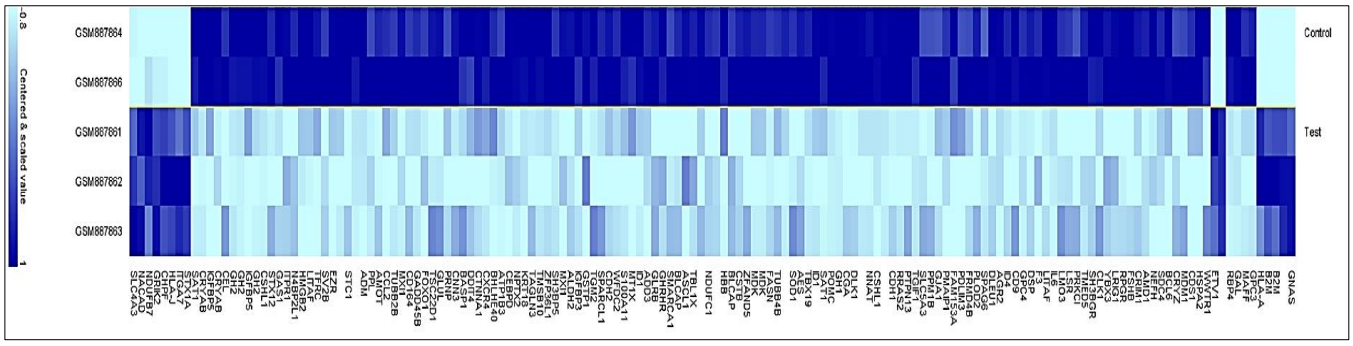


Fig 1 The heatmap of 152 DEGs

GO clustering enrichment

The DEGs were substantially enriched in biological processes (BP), cellular components (CC), and molecular function, according to GO word enrichment analysis (MF). The GO functional annotation clustering analysis of the DEGs revealed the following- (a) In BP, the enriched genes were mostly involved in cell-cell signaling, adhesion junction structure, and the growth hormone signaling pathways JAK-STAT cascade. (b) the CC of enriched DEGs was mainly associated with the Extracellular region, Fascia adherens, and Intercalated discs, (c) the MFs were mainly related to hormone activity, structural constituent of the cytoskeleton, and Beta-catenin binding.

KEGG pathway analysis

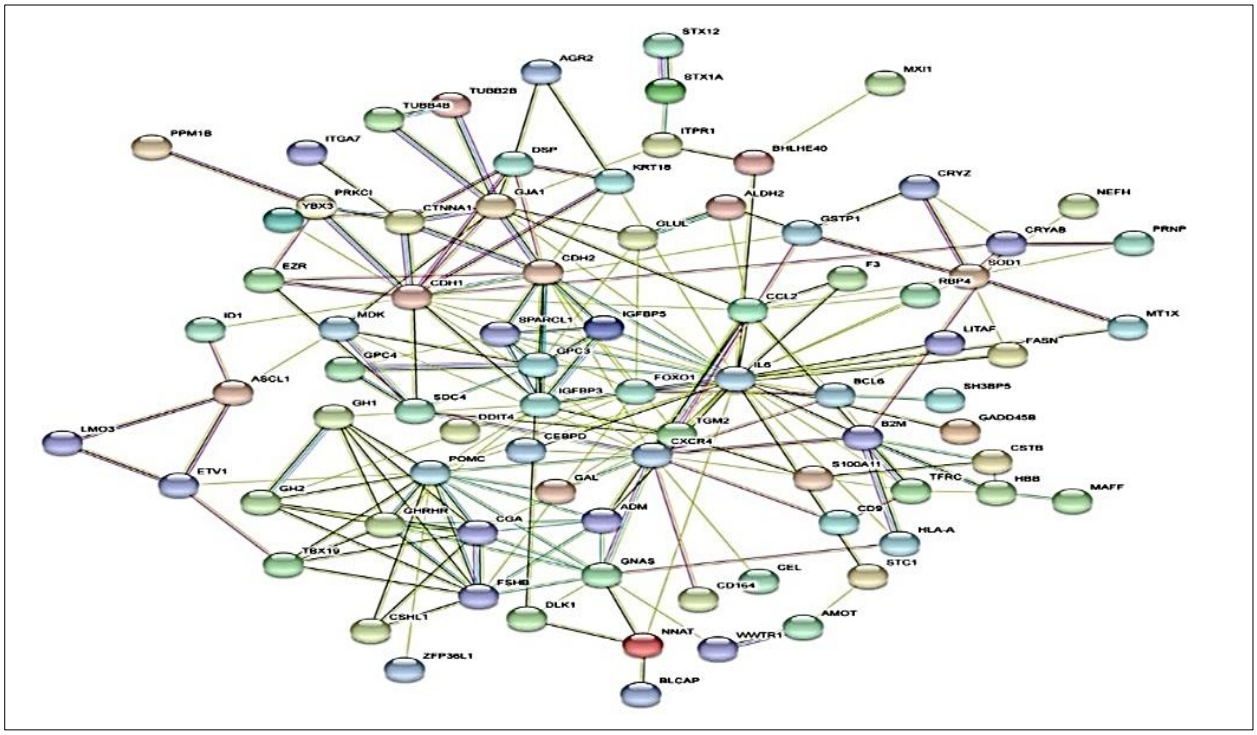
A total of 17 pathways were identified in which about 60 DEGs were enriched. Out of the 17 pathways, the Hippo signaling pathway, Neuroactive ligand-receptor interaction pathway, and GnRH signaling pathway were identified as key pathways that are involved in the dysregulation of the spermatogenesis process. The pathways and the respective enriched genes are presented in (Table 3).

Table 3 Top results from KEGG pathway analysis

KEGG IG	Term	Gene name	Count	P-value
hsa04390	Hippo signaling pathway	WWTR1, ACTB, AMOT, CDH1, CTNNA1, ID1, ID2, PRKCI	8	1.5×10^{-3}
hsa04080	Neuroactive ligand-receptor interaction	CH1, FSHB, GRIK2, GLRB, CGA, GH1, GH2, GHRHR	8	3.7×10^{-2}
hsa04912	GnRH signaling pathway	GNAS, FSHB, CGA, ITPR1	4	8.4×10^{-2}

Construction of PPI network

PPI network of the DEGs was constructed using the online server STRING, version 10.5. The PPI network as shown in (Fig 2) was obtained after excluding the disconnected nodes. To identify the hub genes, a statistical analysis was performed on each node gene in the network. As a result, POMC and IL-6 genes were retrieved as key genes. Further, the genes associated with the 3 key pathways obtained from KEGG enrichment, were influenced by both POMC and IL-6 genes.



The circle represents the gene and colored lines denote varying protein-protein interaction evidence

Fig 2 Protein-Protein Interaction of both upregulated and downregulated DEGs

POMC and IL-6 genes were identified to play a crucial role in the spermatogenesis process of hyperprolactinemia patients.

The POMC neurons, located in the ventero-lateral subdivision of the arcuate nucleus regulate GnRH neurons which directly control reproduction [14]. The precursor protein, POMC (expressed by POMC neurons) gives rise to three different opioid neuropeptides namely, the alpha-Melanocyte Stimulating Hormone (α-MSH), β-endorphins and Adrenocorticotropic hormone (ACTH) [15]. Few experimental evidence states that the neuroactive peptide, β- endorphin (opioid peptide) is involved in the direct inhibition of GnRH and LH secretion in rats [16-17]. Thus, the augmentation of POMC protein results in negative steroid feedback [18]. In the hyperprolactinemia patient group, the POMC gene was highly upregulated which revealed that the GnRH and LH secretion were confined.

Adrenocorticotropic Hormone (ACTH), a 39 amino acid long peptide, a resultant from the POMC precursor activates GNAS (Guanine Nucleotide binding protein- Alpha Stimulating) gene which mediates steroidogenesis via stimulation of steroidogenic acute regulatory protein (StAR) [19]. ACTH migrates to the adrenal glands and leads to the secretion of glucocorticoids. These glucocorticoids primarily inhibit ACTH secretion via negative feedback [20]. Hence, ACTH interaction with GNAS is limited and that causes GNAS downregulation in hyperprolactinemia patients. Further, GNAS mediates cAMP pathway and that induces the common alpha-glycoprotein subunit gene (CGA) which is common to heterodimeric glycoproteins namely LH, FSH and Thyroid-stimulating hormone [21-23]. The downregulation of GNAS gene directionally causes interference with the CGA gene. Collectively, POMC neurons plays a major role in altering the GnRH signaling pathway, which leads to the disturbance in hormonal activities related to the spermatogenesis process. Furthermore, intervention with opioids involved in the neuroactive ligand-receptor interaction pathway intrudes the FSH, LH and TSH functioning as derived from the KEGG pathway analysis.

The results of GO analysis showed that the interaction between the extracellular matrix and the cytoskeleton possibly triggers the activation of signaling platforms which potentially stimulates or represses the steroidogenic potency of ACTH (involved in GnRH signaling) [24].

InterLeukin-6 (IL-6) is a multifunctional cytokine [25] which can be produced by endocrine cells [26]. The production of IL-6 in the mammalian testis has stage specificity in the seminiferous epithelial cycle, during spermatogenesis [27]. IL-6 mediates the regulation of Sertoli cells and spermatogenic cell development [4]. Recent findings suggest IL-6 is involved in the disruption of the blood-testis barrier (BTB) and testicular tight junctions [28]. IL-6 is also found to inhibit testosterone secretion in Leydig cells [29]. Hence, elevated IL-6 has negatively influenced the neuroactive peptide ligand-receptor interaction pathway leading to disruptive functioning of gonadal hormones.

Recent data has revealed the hippo pathway's importance in the control of steroidogenesis, which is crucial for sexual maturation [30-31]. Another research has demonstrated that the YAP1 was expressed in the middle part of spermatozoa and that the hippo signaling cascade is involved in the process of capacitation and fertilization [32]. ACTH stimulates glucocorticoids and acts as hormonal activators of Yes Associated Protein (YAP) [33]. In addition to it, stimulation of IL-6 was found to repress protein expression of the Epithelial-Mesenchymal Transition (EMT) marker, E-cadherin which triggers YAP [34]. With reference to hyperprolactinemia patients in this study, the modification in YAP and E-cadherin has led to divergent changes in the functioning of the hippo signaling pathway.

Comprehensively, the research suggests that both POMC and IL-6 neurons may provide novel targets in understanding the mechanism underlying hyperprolactinemia-induced male infertility.

CONCLUSION

The present study revealed that the Hippo signaling pathway, Neuroactive ligand-receptor interaction pathway, and GnRH signaling pathways play key roles in altering the spermatogenesis process of hyperprolactinemia patients. The biomarker genes namely, POMC and IL-6 genes were also identified to have a profound impact on the spermatogenesis process of hyperprolactinemia group, which can be taken as targets to design novel therapeutics for the respective condition. However, a definitive molecular study on animal groups and human patients are required to validate the exact significance of the identified genes and pathways.

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