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Genetic alterations in azoospermia patients may reveal potential biomarkers for male infertility: A bioinformatic study

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This study was approved by the Local Ethics Committee of the Ankara University (Document number: 39-837).
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Abstract

Background/Aim: Azoospermia is defined as the absence of sperm in semen and is one of the most common causes of male infertility, with a prevalence of 10-15% in infertile men. Conventional methods for semen analysis do not provide a clear understanding of the etiology of azoospermia. Although testicular biopsy may exclude obstructive cases, non-obstructive azoospermia (NOA) treatment is limited due to a limited understanding of the underlying molecular mechanisms. Analysis of genetic alterations in azoospermia patients compared to the fertile population may be a valuable tool for determining diagnostic biomarkers for male infertility. This study aims to use bioinformatic tools to determine the top candidates in certain pathways altered in azoospermia.

Methods: Expression data (GSE108886) of the differential testicular transcriptome in patients with NOA was selected from the Gene Expression Omnibus (GEO) database. Testicular RNA was harvested from azoospermia patients (n=11) and healthy controls (n=1, pooled sample). The differentially expressed genes (DEGs) were examined using GEO2R software. Biological pathways were identified through the Kyoto Encyclopedia of Genes and Genomes (KEGG). Construction of the protein network and detection of hub genes were conducted in the STRING database. Data validation was performed via ELISA assay for the FOXO3 gene in obstructive and NOA patients. Significance was set at *P*-value <0.05.

Results: In NOA patients, 2115 genes were upregulated, and 1753 genes were downregulated compared to the control group. Ninety-one genes involved in spermatogenesis were downregulated. KEGG analysis revealed that the glucagon signaling, AMPK signaling, insulin and estrogen signaling, and oocyte meiosis pathways were upregulated, while the regulation of actin cytoskeleton, MAPK signaling pathway, focal adhesion, and chemical carcinogenesis – reactive oxygen species pathways were downregulated. Downstream genes with the highest score were PSMA4, PSMA6, PSMC1, PSME4, and UBA52, which are responsible for the ubiquitin-dependent protein degradation. The top hub genes with increasing expression were RPS18, RPS2, and RPS4X

Conclusion: Although hub genes selected within the altering pathways may serve as a diagnostic tool for NOA, further validation of the presented data is necessary, as protein-protein interactions may not reflect alterations in gene expression *in vivo*.

Keywords: male infertility, azoospermia, bioinformatic analysis, GEO, microarray

Introduction

The inability of a male to impregnate a fertile female following regular sexual intercourse for 12 months or more is defined as male infertility [1]. While congenital, systemic, and environmental risk factors exist, conventional semen analysis does not always provide information on male fertility potential or etiology. Azoospermia, which is simply defined as the absence of sperm in the semen, affects 1% of the male population and 10–15% of infertile men [2]. Obstructive azoospermia (OA) results from a blockage or missing connection along the reproductive tract, while non-obstructive azoospermia (NOA) may be due to impaired spermatogenesis or testicular dysfunction caused by genetic disorders [3]. While OA can be treated by surgery, both the diagnosis and treatment of NOA are limited [4]. Therefore, identifying altered genes involved in NOA pathology as potential diagnostic biomarkers for novel treatment options is crucial.

Several genetic factors have been linked to NOA, including karyotype abnormalities such as Klinefelter syndrome, translocations, and deletions, including Y chromosome microdeletions of the AZFa, AZFb, and AZFc subregions [5]. Other genetic factors include but are not limited to, Kallmann syndrome [6], mild androgen insensitivity syndrome [7], and mutations in genes involved in spermatogenesis, such as TEX11 [8,9] and FSHR [10].

In mammals, spermatogenesis involves over 40 stages, where the morphology, cellular components, genetics, and epigenetics of the male germ cell undergo significant changes [11,12]. During these stages, different protein groups are organized for stage-specific cellular events through sensitive genetic adjustments. Conventional semen analysis does not always provide information on fertility potential or etiology in idiopathic cases [13]. Therefore, examining semen should include protein-protein interactions and alterations in protein-coding genes at all stages of spermatogenesis for NOA patients, highlighting the urgent need for diagnostic markers.

Recently, several genes coding for cell junction proteins, transcription factors, cytokines, proteases, and protease inhibitors have been proposed as markers of NOA in numerous animal studies [14]. Despite the guidance of these studies, the excessive number of proteins involved in spermatogenesis limits the prediction of target genes. Thus human studies have been limited [15-17]. In the last decade, bioinformatics has transformed the field of reproductive medicine by providing a powerful tool for analyzing and interpreting large-scale genomic data. This study aims to use bioinformatic tools to reveal genetic alterations in NOA patients and identify specific genes and pathways that may be involved in sperm production. Through this approach, potential biomarkers for male infertility may be identified for diagnosing and treating the disease.

Materials and methods

Data acquisition

The study was designed as a bioinformatic investigation to identify genetic alterations in azoospermia patients and determine potential biomarkers for male infertility. Total testicular transcriptome data was selected from the Gene Expression Omnibus (GEO) database for various types of azoospermia. The GSE108886 dataset was obtained by testicular biopsy, and total testicular RNA from 11 azoospermia patients and one pooled control testicular RNA sample were analyzed via Illumina HumanHT-12 V4.0 expression chip. The differentially expressed genes (DEGs) between groups were analyzed using the GEO2R online tool, and statistically significant DEGs (P<0.01, log2FC ≥0 or ≤0) were compared using the Venny program.

Functional enrichment analysis

The alterations in molecular pathways and biological processes in the dataset were analyzed using the DAVID (https://david.ncifcrf.gov/) online tool. Gene Ontology (GO) was determined for biological processes (BP), cellular components (CC), and molecular functions (MF) subgroups. The Kyoto Encyclopedia of Genes and Genomes (KEGG) was used to identify biological pathways for DEGs. Terms with P-value <0.01 were considered statistically significant.

Protein-protein interactions

The DEGs were imported into the STRING database to determine protein-protein interactions (PPI). A confidence limit of >0.4 was set for constructing the protein interaction network. The network's topological properties were analyzed using Cytoscape software (Cytoscape v3.9.2), and the most interacted proteins within the defined network were selected as the hub genes. Protein clusters were determined using MCODE analysis in highly interconnected regions.

Data validation

Data was confirmed via ELISA assay for testicular tissues from one obstructive and one NOA patient. Following diagnosis, signed consent forms were collected from each patient for the further use of the remaining testicular tissue. Ethical approval was granted by the Ankara University Local Ethics Committee (Document number: 39-837). Protein extraction was performed using tissue lysis buffer (Thermofisher Scientific, Waltham, MA, USA) on ice for 1 h. 100 µl of standard or samples were added to a 96-well plate and incubated for 90 min at 37°C. Following a brief wash and a 1-h incubation in biotinylated FOXO3 antibody (MyBioSource.com Inc., San Diego, CA, USA) at the same temperature, horseradish peroxidase (HRP) and substrate reagents were added. Absorbance was detected at 450 nm.

Statistical analysis

Normally distributed data were evaluated using a one-way ANOVA (Analysis of Variance) test, and non-parametric data were analyzed using Sidak's multiple comparisons test in GraphPad Prism Software version 9.0.0. A P-value of <0.01 was considered statistically significant.

Results

Identification and functional analysis of DEGs

The GSE108886 dataset was analyzed using GEO2R to calculate P and log2FC values for conditions where P<0.05 and log2FC values are ≥0 or ≤0, respectively. The results showed that 2115 genes were upregulated and 1753 genes were downregulated in azoospermia patients compared to the control group.

Additional analysis of DEGs was performed using the DAVID software to investigate pathway enrichment. The results revealed that the DEGs were significantly enriched in the 'Molecular Function (MF)' group, followed by the 'Biological Process (BP)' and 'Cellular Component (CC)' groups (P<0.01). Among the subgroups, the most notable changes in gene numbers were observed in the mitochondrion, spermatogenesis, cell differentiation, motile cilium, and microtubule. Table 1 shows the GO terms and the list of important genes that increased or decreased.

The KEGG pathway analysis identified 16 downregulated and 11 upregulated gene clusters. Among them, the regulation of the actin cytoskeleton, MAPK signaling, and glucagon signaling pathways showed the most significant changes (Table 2).

Table 1: GO analysis of altered genes in azoospermia patients.

Category	Term	Count
Downregulated genes		
Biological process	GO:0000398~mRNA splicing, via spliceosome	14
	GO:0002181~cytoplasmic translation	9
	GO:0030968~endoplasmic reticulum unfolded protein response	8
	GO:0006412~translation	13
	GO:0051496~positive regulation of stress fiber assembly	6
	GO:0045454~cell redox homeostasis	5
	GO:0030433~ubiquitin dependent ERAD pathway	6
Cellular component	GO:0005739~mitochondrion	58
	GO:0005681~spliceosomal complex	12
	GO:0005840~ribosome	13
	GO:0022627~cytosolic small ribosomal subunit	7
	GO:0022626~cytosolic ribosome	8
	GO:0005769~early endosome	16
	GO:0071013~catalytic step 2 spliceosome	8
	GO:0031234~extrinsic component of cytoplasmic side of plasma membrane	6
Molecular function	GO:0019843~rRNA binding	6
	GO:0003735~structural constituent of ribosome	12
	GO:0003779~actin binding	16
	GO:0051015~actin filament binding	12
Upregulated genes		
Biological process	GO:0007283~spermatogenesis	91
	GO:0030154~cell differentiation	72
	GO:0060285~cilium-dependent cell motility	11
	GO:0007018~microtubule-based movement	17
	GO:0060294~cilium movement involved in cell motility	7
	GO:0036158~outer dynein arm assembly	8
	GO:0008152~metabolic process	6
	GO:0018095~protein polyglutamylation	5
	GO:0061621~canonical glycolysis	5
	GO:0018105~peptidyl-serine phosphorylation	18
	GO:0006096~glycolytic process	7
	GO:0006457~protein folding	16
Cellular component	GO:0031514~motile cilium	50
	GO:0005858~axonemal dynein complex	9
	GO:0036157~outer dynein arm	8
	GO:0005874~microtubule	42
	GO:0030286~dynein complex	10
Molecular function	GO:0051959~dynein light intermediate chain binding	11
	GO:0008569~ATP-dependent microtubule motor activity, minus-end-directed	8
	GO:0003796~lysozyme activity	7
	GO:0045505~dynein intermediate chain binding	11
	GO:0003777~microtubule motor activity	12
	GO:0015631~tubulin binding	11
	GO:0004674~protein serine/threonine kinase activity	36
	GO:0051082~unfolded protein binding	15

GO: Gene ontology

Table 2: Altered KEGG pathways in azoospermia patients.

KEGG Pathway	Co	Downregulated genes
hsa04810:Regulation of actin cytoskeleton	17	ITGB1, CYFIP1, ROCK2, MSN, RHOA, SLC9A1, CRKL, CXCL12, ARPC3, PDGFC, GNA12, MYH9, PIP4K2A, RAC1, PFN1, RAF1, CRK
hsa04612:Antigen processing and presentation	9	HLA-DRB4, HSPA5, NFYC, PSME3, PSME1, RFXANK, CALR, B2M, LGMN
hsa04722:Neurotrophin signaling pathway	11	SHC1, ARHGDIB, GRB2, RAC1, FOXO3, RAF1, CAMK2G, CRK, RHOA, ATF4, CRKL
hsa03010:Ribosome	12	MRPL4, RPS4X, MRPL20, RPS4Y2, RPS18, RPLP1, RPL12, RPS3, RPL27, RPS2, RPS4Y1, RPL6
hsa04110:Cell cycle	10	CCND2, YWHAB, CDK4, GADD45A, MYC, SKP2, GADD45G, CDC25B, CDC14B, YWHAH
hsa04510:Focal adhesion	13	ITGB1, SHC1, ROCK2, CAV2, VEGFC, RHOA, CRKL, CCND2, PDGFC, GRB2, RAC1, RAF1, CRK
hsa04210:Apoptosis	10	CASP7, TUBA1A, GADD45A, CTSK, HTRA2, FADD, RAF1, LMNB2, GADD45G, ATF4
hsa04530:Tight junction	11	ITGB1, TUBA1A, ROCK2, CDK4, ARPC3, MYH9, PARD6G, MSN, RAC1, RHOA, AMOT
hsa05208:Chemical carcinogenesis - reactive oxygen species	13	COX7B, NDUFA6, NDUFA10, NDUFA1, AKR1A1, FOXO3, COX5B, COX7A1, UQCRH, GRB2, RAC1, RAF1, ACP1
hsa04218:Cellular senescence	10	PPP3CB, CCND2, TRAF3IP2, CDK4, GADD45A, MYC, FOXO3, RAF1, SQSTM1, GADD45G
hsa04012:ErbB signaling pathway	7	SHC1, MYC, GRB2, RAF1, CAMK2G, CRK, CRKL
hsa04010:MAPK signaling pathway	15	GADD45A, SRF, VEGFC, GADD45G, CDC25B, CRKL, PPP3CB, MYC, PDGFC, GNA12, GRB2, RAC1, RAF1, CRK, ATF4
hsa00010:Glycolysis/ Gluconeogenesis	6	LDHB, PDHA1, PGAM1, AKR1A1, PGK1, PGAM4
hsa04670:Leukocyte transendothelial migration	8	ITGB1, CXCL12, ROCK2, CTNNA1, MSN, RAC1, RHOA, RAPGEF4
hsa04062:Chemokine signaling pathway	11	GNG10, CXCL12, SHC1, ROCK2, GRB2, RAC1, FOXO3, RAF1, CRK, RHOA, CRKL
KEGG Pathway	**Co**	**Upregulated genes**
hsa04922:Glucagon signaling pathway	16	ATF2, PDHA2, PGAM2, CALML3, CPT1B, ACACA, LDHC, G6PC2, CREB1, PPP3CC, PPP3R2, PRKACG, PHKG2, AKT3, PFKP, PCK2
hsa00010:Glycolysis/ Gluconeogenesis	11	LDHC, GPI, HK3, PDHA2, G6PC2, PGAM2, GAPDHS, PGK2, PFKP, PCK2, HK1
hsa04152:AMPK signaling pathway	13	STRADA, CAB39L, TSC1, CPT1B, ACACA, G6PC2, CREB1, PPP2R1B, PPP2R3C, PPP2R2B, AKT3, PFKP, PCK2
hsa04066:HIF-1 signaling pathway	12	LDHC, HK3, PDHA2, FLT1, EGLN2, NOS2, NOS3, AKT3, CUL2, PGK2, PFKP, HK1
hsa04114:Oocyte meiosis	13	PLK1, CUL1, SPDYE4, CALML3, SPDYE6, PPP1CC, SPDYE2B, PPP3CC, PPP3R2, PLCZ1, PPP2R1B, PRKACG, PGR
hsa04910:Insulin signaling pathway	13	BRAF, CALML3, TSC1, ACACA, HK1, MAPK10, HK3, PPP1CC, G6PC2, PRKACG, PHKG2, AKT3, PCK2
hsa04915:Estrogen signaling pathway	13	ATF2, HSPA1L, NOS3, KRT23, KRT34, CALML3, KRT33A, CREB1, PRKACG, KRT15, AKT3, RARA, PGR
hsa04146:Peroxisome	9	NOS2, PEX11A, AGPS, ACSL6, FAR2, PEX11G, CRAT, PEX13, PAOX
hsa04920:Adipocytokine signaling pathway	8	MAPK10, G6PC2, AKT3, ACSL6, ACSBG2, CPT1B, PCK2, NFKBIB
hsa05230:Central carbon metabolism in cancer	8	LDHC, HK3, PDHA2, NTRK3, AKT3, PGAM2, PFKP, HK1
hsa03320:PPAR signaling pathway	8	GK, ACSL6, AQP7, DBI, ACSBG2, CPT1B, PCK2, GK2

Co: Count

PPI network analysis and identification of hub genes

The integrated PPI Network was analyzed using the STRING database, resulting in 60 nodes and 112 edges for decreasing genes and 58 nodes and 86 edges for increasing genes (P<0.01). The core genes were ranked according to their predicted scores using the network analyzer embedded in Cytoscape software. The hub genes were analyzed using cytoHubba, and the top 20 genes with the highest score for decreasing (Figure 1) and increasing (Figure 2) gene expressions were listed. The decreasing hub genes with the highest score were RPS18, RPS2, and RPS4X, while the increasing hub genes were PSMA4, PSMA6, PSMC1, PSME4, and UBA52. According to the GO analysis, the decreasing hub genes with the highest score were related to ribosomal translation, while the increasing hub genes were not enriched in a specific pathway.

Figure 1: Top 20 hub genes with the highest score according to STRING analysis within the identified PPI network for decreasing profiles (P<0.01). The scores decrease from red to yellow.

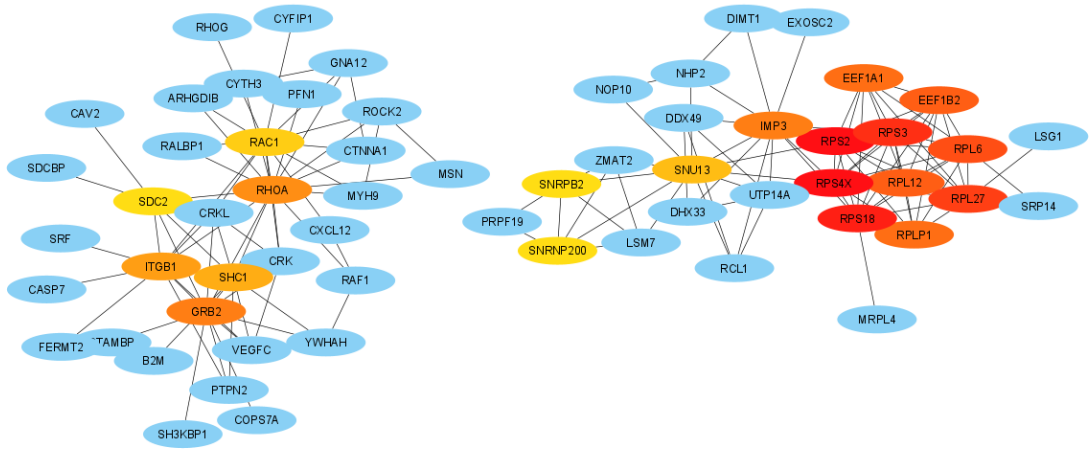
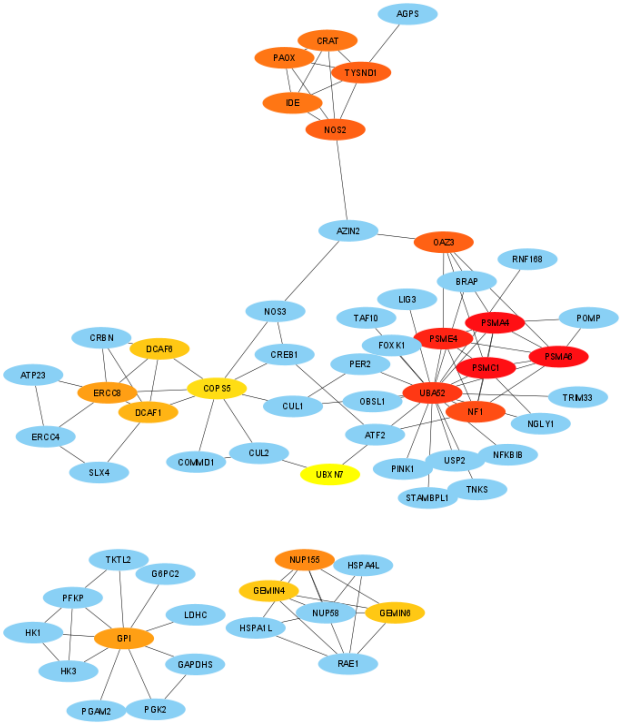


Figure 2: Top 20 hub genes with the highest score according to STRING analysis within the identified PPI network for increasing profiles [P<0.01]. The scores decrease from red to yellow.



Highly interconnected regions were analyzed with MCODE, and the cut-off score was set to 3 (Table 3). Protein clusters with the highest score included RPL12, RPS4X, RPL6, RPL27, RPLP1, RPS18, RPS3, EEF1A1, RPS2, and EEF1B2 for decreasing profiles, and PSME4, OAZ3, PSMA6, PSMC1, PSMA4, UBA52, and NF1 for increasing profiles, respectively.

Data validation

To validate the bioinformatic analyses, testes tissues from one obstructive and one NOA case were examined for the levels of FOXO3 protein. As predicted, the levels of FOXO3 protein were significantly decreased in the NOA sample (P=0.004).

Table 3: MCODE analysis for protein clusters in the identified PPI network.

Score	Nodes	Edges	Downregulated genes
9,778	10	44	RPL12, RPS4X, RPL6, RPL27, RPLP1, RPS18, RPS3, EEF1A1, RPS2, EEF1B2
5	5	10	SNRNP200, SNU13, ZMAT2, SNRPB2, LSM7
4	4	6	ITGB1, SDC2, RHOA, SHC1
3,6	6	9	NDUFA10, NDUFA6, ATP5H, ATP5L, NDUFA1, COX5B
3,333	4	5	RCL1, DHX33, DDX49, UTP14A
3,333	4	5	STX6, VAMP3, STX7, STX10
3,333	4	5	VCP, HSPA5, OS9, DERL1
3,333	7	10	COMMD7, COMMD6, COPS7A, COMMD10, GRB2, VEGFC, CRK
3	3	3	RGS19, RGS1, RGS10
3	7	9	THRA, PRDX5, PRDX1, NCOA1, RXRA, TXNRD1, TXN
3	3	3	HIST3H2A, MORF4L2, MEAF6
3	3	3	FOXO3, YWHAH, YWHAB
Score	**Nodes**	**Edges**	**Upregulated genes**
6,333	7	19	PSME4, OAZ3, PSMA6, PSMC1, PSMA4, UBA52, NF1
6	6	15	PLAT, F2, EDN1, PLAU, THBS1, VWF
5	5	10	PAOX, NOS2, IDE, TYSND1, CRAT
4,5	5	9	COPS5, DCAF6, DCAF1, ERCC8, CRBN
4	4	6	HK3, HK1, GPI, PFKP
4	4	6	CATSPERD, CATSPERB, CATSPER1, CATSPERG
3,333	4	5	GEMIN4, NUP58, RAE1, GEMIN6
3,333	4	5	POLR2I, GTF2A2, METTL14, POLR2D
3,333	4	5	SPA17, ROPN1L, ROPN1, AKAP3
3	3	3	UBE2V1, UBE2D3, UBE2U
3	3	3	SPAG6, SPAG16, MEIG1
3	3	3	TRAF3IP1, CLUAP1, TTC26
3	3	3	DNAH2, CFAP70, DNAH17
3	3	3	PPP2R1B, PPP2R2B, BRAF
3	3	3	BBIP1, BBS12, BBS5
3	3	3	ADSSL1, ASRGL1, IL4I1

Discussion

Spermatogenesis is a complex biological process that relies heavily on the genetic regulation of protein synthesis and degradation. While genome-wide analyses of testicular tissue have shed light on protein synthesis in male reproduction, little is known about protein degradation in spermatogenesis [18,19]. This study aimed to address this gap in knowledge by examining 91 genes involved in spermatogenesis and identifying hub genes through STRING analysis. Interestingly, the highest-scoring hub genes were PSMA4, PSMA6, PSMC1, PSME4, and UBA52, which have not previously been linked to protein degradation in spermatogenesis. This study is the first to highlight the potential role of these genes in this critical process.

The ubiquitination-proteasome system (UPS) regulates protein activity by facilitating protein degradation [20,21]. In this study, gene ontology (GO) analysis identified six altered genes in the ubiquitin-dependent endoplasmic reticulum-associated degradation (ERAD) pathway. In mammals, sperm quality is determined in the epididymis, where sperm mature to their final developmental stage. Abnormalities on the sperm surface are detected by ubiquitin secreted by epididymal cells. Ubiquitin, a 76-amino-acid polypeptide, tags substrate proteins for proteolytic destruction via proteasomes [22]. Interestingly, our study found that UBA52, which is responsible for ubiquitin conjugation through the regulation of translation [23], was upregulated in NOA patients compared to controls. These findings suggest that UBA52 may regulate protein degradation during spermatogenesis and warrant further investigation.

Recent studies have identified ubiquitin-related proteasomes in seminal plasma, suggesting that they may regulate sperm function by modifying surface proteins [24,25]. Mutations in the PSMA4 gene have also been associated with NOA [26]. Additionally, the PSME4 protein, a proteasome responsible for histone exchange during spermatogenesis, is highly expressed in human testis according to the Protein Atlas database and has been linked to male infertility in various animal studies [27]. Interestingly, our study found that the spermato-proteasomes mentioned above were upregulated with the highest scores according to STRING analysis. Conversely, PSMA6, another proteasome found in higher concentrations in the sperm of infertile bulls [28], was also upregulated in NOA patients compared to controls. Given the relationship between sperm ubiquitination and sperm DNA defects found in the literature, our study provides reliable candidates for diagnosing NOA patients, all involved in ubiquitin-dependent protein degradation during spermatogenesis.

Our study utilized KEGG analysis to identify upregulated pathways such as glucagon signaling, AMPK signaling, insulin and estrogen signaling, and oocyte meiosis pathways. In contrast, downregulated pathways included regulation of actin cytoskeleton, MAPK signaling pathway, focal adhesion, and chemical carcinogenesis/reactive oxygen species pathways. While these pathways play a crucial role in spermatogenesis and/or spermiogenesis, genetic screening for a particular pathway is not currently available in humans. Nonetheless, our findings provide important insights into the molecular mechanisms underlying NOA and may pave the way for future studies aimed at identifying potential therapeutic targets.

Limitations

It is important to note that further validation of the presented data is necessary, as protein-protein interactions identified through STRING analysis may not reflect alterations in gene expression *in vivo*. Additional studies incorporating other approaches, such as functional assays or animal models, are needed to confirm the potential roles of these candidate genes and pathways in NOA.

Conclusion

The study demonstrated the upregulation and downregulation of genes that play important roles in mammalian reproduction. The expression of 91 genes involved in spermatogenesis was found to be decreased in patients with azoospermia when compared to controls. The study also identified hub genes, including PSMA4, PSMA6, PSMC1, PSME4, UBA52, RPS18, RPS2, and RPS4X, which were particularly clustered in the ubiquitin-dependent protein degradation pathway in spermatogenesis. These hub genes may serve as a diagnostic tool for NOA.

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