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An in silico analysis of human sperm genes associated with asthenozoospermia and its implication in male infertility

Xue Xia Liu, MD[a], Li Cai, MD[b], Fu Jun Liu, MD[a,*]

Abstract

Asthenozoospermia is the most common clinical symptom of male infertility. Molecular markers associated with asthenozoospermia spermatozoa are scarcely identified. The objective of this study was to screen the differentially expressed genes (DEGs) in asthenozoospermia spermatozoa and assess the underlying bioinformatics roles in regulation of sperm quality.

Based on gene expression omnibus (GEO) database, the GSE22331, GSE1133, and GSE4193 expression profile data were downloaded. The DEGs of asthenozoospermia spermatozoa were identified. Germ cell specific genes in DEGs were further screened. Then, gene ontology (GO) and over-representation analysis of DEGs were performed, followed by protein–protein interaction (PPI) network analysis. Expressions of selected genes of *TEX11*, *ADAMTS5*, *ASRGL1*, *GMCL1*, *PGK2*, *KLHL10* in normozoospermia and asthenozoospermia spermatozoa were identified using real time Reverse Transcription-Polymerase Chain Reaction (RT-PCR).

A total of 1323 DEGs were identified, including 1140 down-regulated genes. Twenty one and 96 down-regulated genes were especially expressed in spermatogonia and round spermatids, suggesting their testicular origins and influences on sperm quality. Bioinformatics analysis showed enriched functions of ubiquitin-like protein transferase or protein binding activities in down-regulated genes. Expressions of selected genes were validated by RT-PCR, which was consistent with bioinformatical results.

The present study provided a novel insight into the understanding of sperm quality, and a potential method and dataset for the diagnosis and assessment of sperm quality in the event of male infertility.

Abbreviations: cDNAs = complementary DNAs, CT = threshold cycle, DEGs = differentially expressed genes, DEPC = diethypyrocarbonate, GEO = gene expression omnibus, GO = gene ontology, PBS = phosphate-buffered saline, RT-PCR = Reverse Transcription-Polymerase Chain Reaction, Ub = Ubiquitin, WHO = World Health Organization.

Keywords: asthenozoospermia, bioinformatics, male infertility, sperm, sperm quality

1. Introduction

Infertility has been a worldwide health problem, affecting 10% to 15% of couples with child-bearing age. Male factors have been deemed to 50% causes for infertility.[1] Clinically, male infertility is often manifested as poor sperm quality. Mammalian spermatozoa are produced in testis by experiencing complex spermatogenesis process, and get maturation characteristics in epididymis mainly by post-translational modifications. Abnormality of any steps in spermatogenesis or sperm maturation can cause poor sperm quality, leading to male infertility.[2,3]

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XXL and LC have contributed equally to this work.

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Mammalian spermatogenesis is a complex cell proliferation and differentiation process, including spermatogonia mitosis, renewal meiosis, and terminal differentiation of spermiogenesis.[4,5] The completion of this complex process requires the spatiotemporal expression of different specific testis genes. Numerous studies have reported the discovery of the testis-specific genes, which was proved as the key molecules to affect sperm quality through the analysis of knockout mice model. Our previous studies identified testis-specific genes and characterized their functions in sperm quality regulation.[6–11] For instance, PGK2 was exclusively expressed in human testis and significantly related to sperm quality. It maybe served as a biomarker for assessment of sperm quality.[11] Increasing evidence showed that testis specific genes are key molecules in sperm quality evaluation, an aberrant expression of any key genes will affect the outcome of spermatogenesis, which leads to altered ejaculated sperm quality or male infertility.

Asthenospermia is the common clinical case, which was characterized with poor sperm motility.[12] Underlying mechanisms of its poor sperm quality at protein or gene levels is deserved to be studied. Many proteomic analysis on asthenozoospermic spermatozoa have been reported.[13–19] A group of proteins were identified and functionally analyzed in silico. However, limited proteins were identified at proteomics levels, which can't fully explore the underpinning functions or pathways.

Recent studies have shown the presence of mRNAs in human sperm, and indicated that sperm RNAs were associated with sperm quality. They were supposed to have clinical values to be

mRNA biomarkers for assessment of sperm quality and fertility potential, and also reflect spermatogenesis status. Transcriptomic analysis based on gene expression omnibus (GEO) repository provides a useful tool for comprehensively analyzing gene expressions. Herein, by combining with bioinformatics analysis and verification by molecular biology methods, we performed a comprehensive in silico analysis to find spermatogenesis genes involved in sperm quality assessment.

2. Materials and methods

2.1. Ethics statement

This study was approved by the ethics committee of YantaiYuhuangding Hospital. Written informed consent was signed by participants or their relatives.

2.2. Sample preparation

Semen samples were collected from 8 healthy young adults men (aged 28–36 years old) who were ruled out of varicocele, and 8 asthenozoospermic patients (aged 25–36 years, progressive motility <32%). Semen was obtained by masturbation after 7 days of sexual abstinence. Collection and processing of semen samples was conducted in accordance with the guideline of World Health Organization's (WHO) Laboratory Manual for the Examination and Processing of Human Semen (*5th* edition, 2010). All donors gave their written informed consent with donating the sperm ejaculates for the purposes of the research project. All procedures were approved by the Ethics Committee of YantaiYuhuangding Hospital.

The semen samples were liquefied at 37 °C for 30 minutes, then were separated by Percoll on a discontinuous density gradients with 45% and 85%, that could rule out the contamination of round cells including germ cells leukocytes, etc. The spermatozoa were collected from 85% underlayer, and washed three times with phosphate-buffered saline (PBS). Meanwhile, a microscopic examination was performed to check the quality of purified spermatozoa.

2.3. Real-time RT–PCR

Each sperm pellet was homogenized in 500 μL of TRIzol reagent at room temperature for 30 minutes. Then 200 μL of chloroform was added and shaken for 15 seconds, following centrifugation at 12,000 × *g* for 15 minutes at 4 °C. The upper supernatant was collected and precipitated using 500 μL isopropanol. After centrifugation of 12,000 × *g*, pellet was washed by 70% ethyl alcohol and resolved in sterile diethypyrocarbonate (DEPC) water. Complementary DNAs (cDNAs) were synthesized according to the instructions provided using avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI). Primers were designed using Primer Premier 7.0 software (PREMIER Biosoft International, Palo Alto, CA) and each primer was submitted into an National Center for Biotechnology Information (NCBI) BLAST search to ensure specificity for the target mRNA. Real-time RT-PCR was performed under conditions of 2 minutes at 95 °C, followed by 15 seconds at 95 °C and 50 seconds at 65 °C for 40 cycles. Data were analyzed using the GeneAmp5700 Sequence Detection System software (version 1.1; Applied Biosystems, Foster City, CA) and were converted into threshold cycle (CT) values. All samples were normalized according to b-actin content. The formula $2^{-\triangle Ct}$ was used to calculate the relative mRNA levels.[20]

2.4. Data mining

The microarray-based, high-throughput gene expression data of sperm were obtained from the GDS DataSet of the GEO repository in the NCBI archives (www.ncbi.nlm.nih.gov/geo). To analyze differential gene expressions between spermatozoa samples from normal and asthenozoospermia patients, data from GSE22331 were downloaded and re-analyzed. To analyze gene expression patterns in human tissues and human germ cells, datasets of GSE1133 and GSE4193 were downloaded and examined. Gene expression profiles of normal human tissues were included in GSE1133. Gene expression profiles of 4 spermatic subpopulations of mouse type A, type B spermatogonia, pachytene spermatocytes, and round spermatids were included in GSE 4193.

2.5. Gene ontology (GO) analysis

The general functions of DEGs were broadly classified according to the GO annotation (www.geneontology.org) and protein class annotation in Panther (http://www.pantherdb.org).

2.6. Over-representation analysis of DEGs

Over-representation analysis of the GO terms, including biological processes and molecular functions, was conducted using ConsensusPathDB-human (http://cpdb.molgen.mpg.de/CPDB), which is a molecular functional interaction database. GO level 2 and 3 categories were selected, and the *P*-—value cutoff was set as .01. The PPI network was established by the STRING (search tool for recurring instances of neighboring genes) (released 10.5, 2017–05–14) (http://string-db.org/).

2.7. Statistical analysis

Data are reported as means ± SD. Means of 2 groups were analyzed using the Student *t* test. GraphPad Prism 7 (La Jolla, CA) was used to perform the statistical analysis. A value of $P < .05$ was considered to be significant.

3. Results

3.1. Identification of differentially expressed genes in asthenozoospermia spermatozoa

Differentially expressed genes (DEGs) of asthenozoospermia spermatozoa may be served as biomarkers for assessment of sperm quality. Comparison of expressions of genes in spermatozoa from normal and asthenozoospermia patients revealed that 1323 gene transcripts were differentially expressed. 1140 genes (86%) were down-regulated in asthenozoospermia spermatozoa, and only 183 genes (14%) were upregulated in asthenozoospermia spermatozoa. A broad functional classification showed that DEGs were related to various molecular functions. A large number of genes had activities of catalysis and binding. Compared with up-regulated genes, more percentage of down-regulated genes were mainly related to the ones of transporter activity, structural molecular activity, and protein and chromatin binding (Fig. 1A). Prominent biological processes of localization, biogenesis, and biological regulation were performed by down-regulated genes (Fig. 1B). A group of down-regulated genes were related to well-known reproductive processes of mitotic cell cycle, spermatid differentiation, sperm-egg recognition and binding (Fig. 1C, D).

Over-representation analysis showed that down-regulated genes in asthenozoospermia spermatozoa significantly functioned as

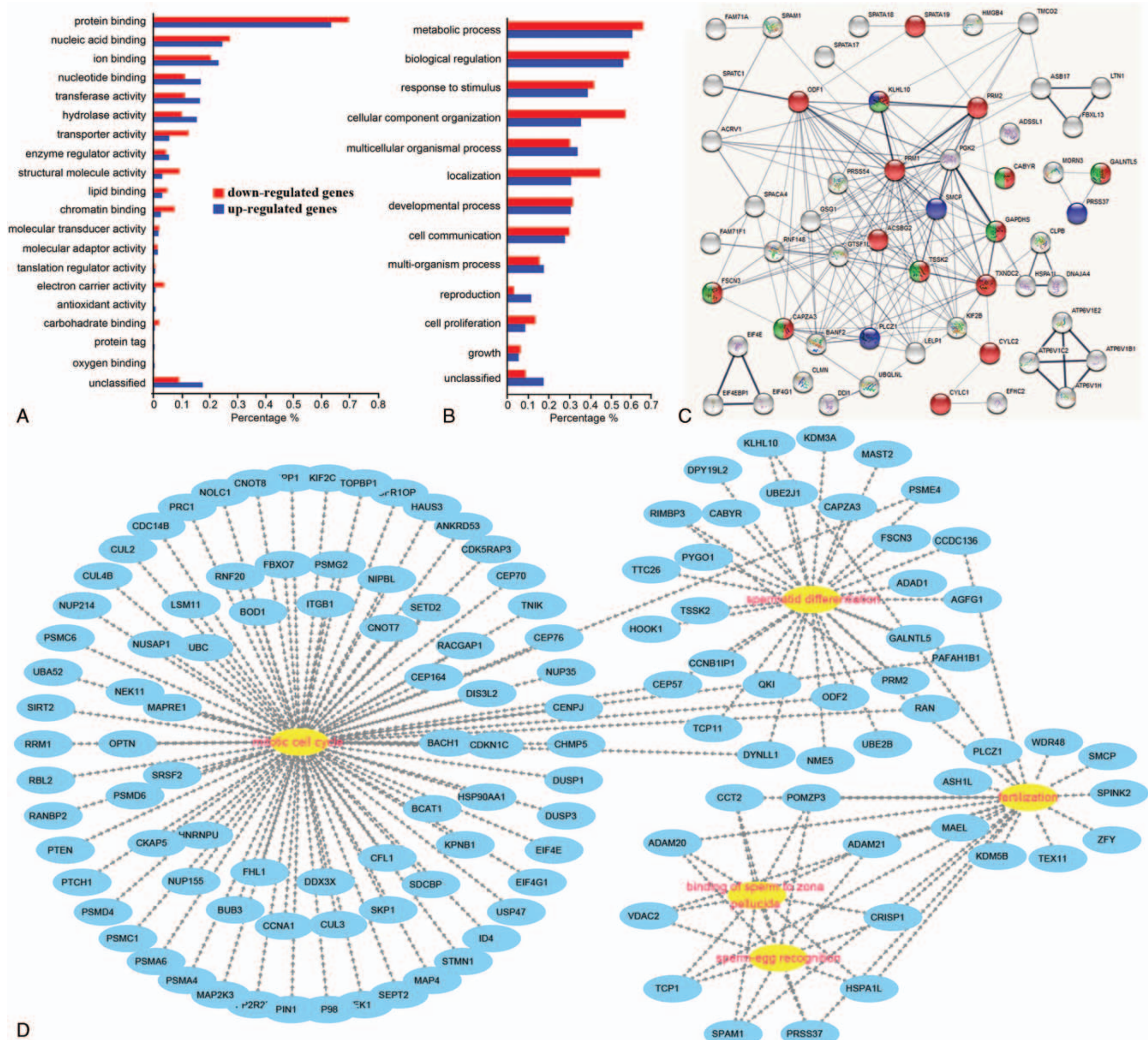

Figure 1. Functional classification of differentially expressed genes in asthenozoospermia spermatozoa. GOSlim summary were listed including molecular function (A) and biological process (B); the height of the bar represented percentage of the number of genes observed in the category. Protein–protein interactions generated by analysis of round spermatid specific DEGs (C), Red circles indicated the biological process of spermatogenesis, green circles indicated germ cell development, and blue circles indicated fertilization processes. Network of down-regulated genes involved in biological processes of male reproduction (D). DEGs = differentially expressed genes.

ubiquitin-like protein transferase or protein binding activities (Table 1).

3.2. Identification of germ cell-specific genes associated with asthenozoospermia

Germ cell-specific genes are potential key genes involving in regulation of sperm quality. By comparing the gene expressions in mouse spermatogonia, spermatocyte and round cells, 845, 105, and 448 genes exhibited specific expressions in round cells, spermatocyte, and spermatogonia cells, respectively.

Comparison between germ cell-specific genes and DEGs in asthenozoospermia showed that 96 (11.6%) round spermatid specific genes and 21 (6.3%) spermatogonia specific genes were down-regulated in asthenozoospermia. Notably, only 2 spematocyte specific genes of GMCL1 and ASRGL1 were down-regulated in asthenozoospermia. Nine germ specific genes (7 spermatogonia and 2 round spermatid specific genes) were up-regulated in asthenozoospermia (Supplementary Table 1, http://links.lww.com/MD/C653).

A functional enrichment analysis of 96 round spermatid specific genes showed that these genes were mainly involved in the processes of spermatogenesis and fertilization. They were the main components of spermatozoa (Fig. 1C).

3.3. Validation of the mRNA expression in asthenozoospermia

Ubiquitin (Ub) plays important roles in germ cell development, and its deletion resulted in mouse spermatogenesis arrestion at

Table 1

Overrepresentation enrichment analysis of differential expressed genes in asthenozoospermia spermatozoa.

Down-regulated genes			Up-regulated genes		
ID	Name	*P* value	ID	Name	*P* value
Molecular function					
GO:0019787	Ubiquitin-like protein transferase activity	2.47E–12	GO:0003735	Structural constituent of ribosome	1.58E–07
GO:0032182	Ubiquitin-like protein binding	2.05E–07	GO:0098631	Protein binding involved in cell adhesion	9.09E–05
GO:0044389	Ubiquitin-like protein ligase binding	2.19E–06	GO:0015002	Heme-copper terminal oxidase activity	2.67E–04
GO:0015631	Tubulin binding	9.57E–05	GO:0016651	Oxidoreductase activity, acting on NAD (P)H	9.88E–04
GO:0031369	Translation initiation factor binding	1.85E–04	GO:0001085	RNA polymerase II transcription factor binding	1.32E–03
GO:0008135	Translation factor activity, RNA binding	5.61E–04	GO:0009055	Electron carrier activity	1.66E–03
GO:0098631	Protein binding involved in cell adhesion	7.29E–04	GO:0008013	Beta-catenin binding	1.95E–03
GO:0045502	Dynein binding	9.36E–04	GO:0050839	Cell adhesion molecule binding	3.61E–03
GO:0003727	Single-stranded RNA binding	2.86E–02	GO:0070491	Repressing transcription factor binding	4.22E–03
GO:0016874	Ligase activity	1.07E–03			
Biological process					
GO:0048232	Man gamete generation	3.14E–13	GO:0070972	Protein localization to endoplasmic reticulum	4.44E–08
GO:0010608	Posttranscriptional regulation of gene expression	3.22E–09	GO:0006091	Generation of precursor metabolites and energy	9.97E–08
GO:0000209	Protein polyubiquitination	3.48*E*–09	GO:0009141	Nucleoside triphosphate metabolic process	9.43E-07
GO:0022412	Cellular process involved in reproduction	1.81E–08	GO:0009123	Nucleoside monophosphate metabolic process	1.92E–06
GO:0006997	Nucleus organization	6.51E–07	GO:1901657	Glycosyl compound metabolic process	1.57E–05
GO:0042787	Protein ubiquitination involved in ubiquitin-dependent protein catabolic process	7.22E–07	GO:0006413	Translational initiation	1.72E–05
GO:0006403	RNA localization	1.10E–04	GO:0072657	Protein localization to membrane	2.61E–05
GO:0071166	Ribonucleoprotein complex localization	1.59E–04	GO:0010257	NADH dehydrogenase complex assembly	3.08E–05
GO:1903320	Regulation of protein modification by small protein conjugation or removal	1.59E–04			
GO:0010498	Proteasomal protein catabolic process	1.96E–04			
Cellular component					
GO:0097223	Sperm part	3.46E–07	GO:0070469	Respiratory chain	6.43E–11
GO:0000151	Ubiquitin ligase complex	8.59E–06	GO:0005743	Mitochondrial inner membrane	6.81E–09
GO:0016604	Nuclear body	1.18E–05	GO:0044455	Mitochondrial membrane part	7.61E–09
GO:0005813	Centrosome	9.61E–05	GO:0098798	Mitochondrial protein complex	1.31E–08
GO:0035770	Ribonucleoprotein granule	1.51E–04	GO:0005840	Ribosome	1.57E–05
GO:0005635	Nuclear envelope	1.52E–04	GO:0005913	Cell–cell adherens junction	8.84E–05
GO:1905368	Peptidase complex	1.18E–03	GO:0030055	Cell–substrate junction	2.29E–04
GO:0048770	Pigment granule	1.91E–03	GO:1990204	Oxidoreductase complex	2.44E–04
GO:0019867	Outer membrane	2.99E–03	GO:0044445	Cytosolic part	3.83E–04
GO:0005929	Cilium	3.10E–03	GO:0070069	Cytochrome complex	5.75E–04

meiotic prophase, leading to male infertility.[21] As shown in Fig. 2, *ADAMTS5*, *TEX11* were specifically expressed in spermatogonia, and had no significant difference in testis after *Ubb* deletion. While *ASRGL1* and *GMCL1* were specifically expressed in spermatocytes, *PGK2* and *KLHL10* were specifically expressed in round spermatids. Deletion of *Ubb* resulted in the obviously decreased expressions of these genes in testis. Expressions of selected genes in Ubb deletion testis validated their germ cell specific expressions. Validation by Reverse Transcription-Polymerase Chain Reaction (RT-PCR) in spermatozoa from normozoospermia and asthenozoospermia was performed. As shown in Fig. 3, except for *TEX11*, *ADAMTS5*, *ASRGL1*, *GMCL1*, *PGK2*, and *KLHL10* had significantly decreased expression in asthenozoospermia spermatozoa.

4. Discussion

Spermatozoa are functional performer in male fertility, and produced by experiencing complex processes, including meiosis, mitosis, spermatogenesis, and spermiogenesis.[22] Spermatozoa

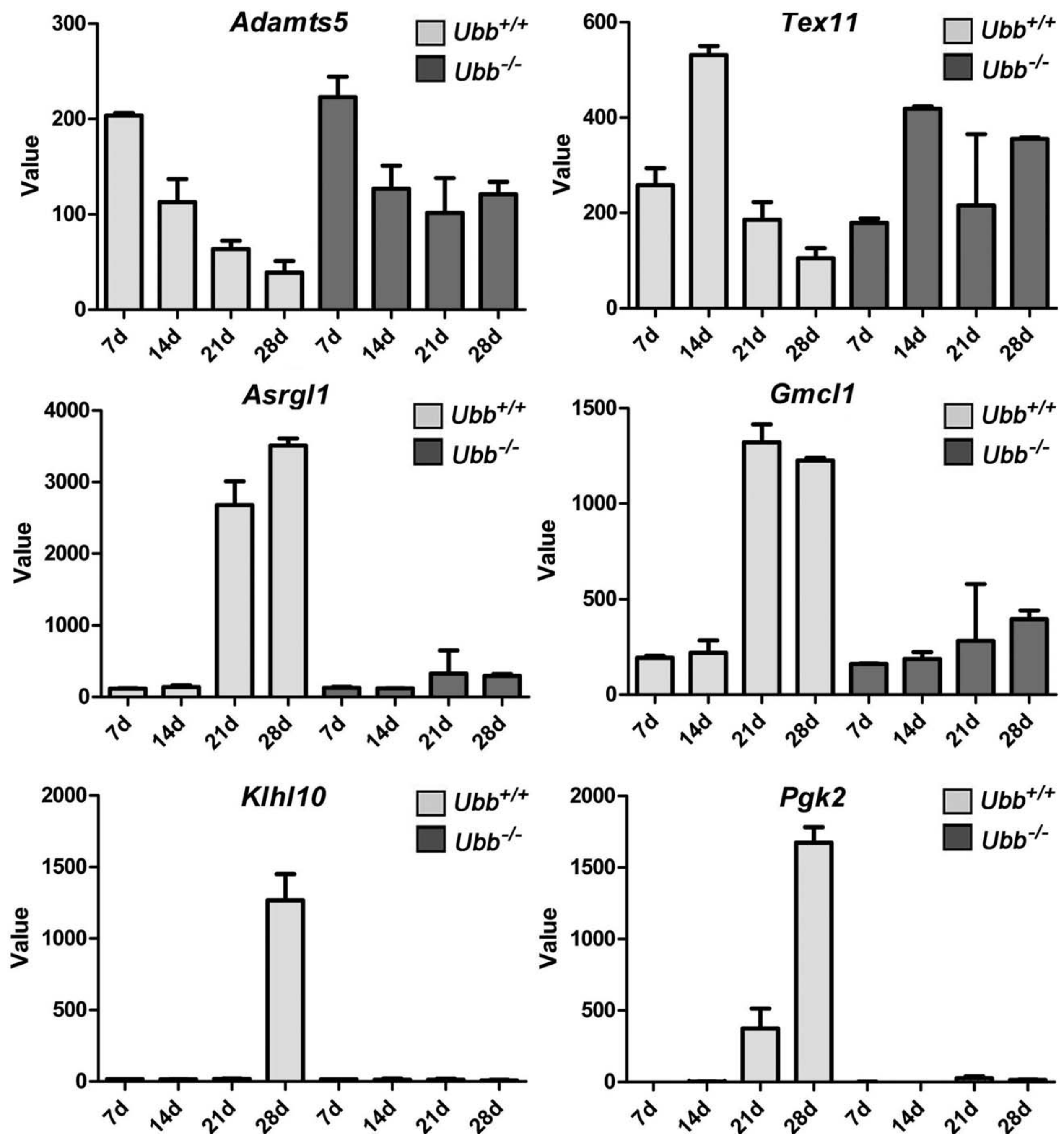

Figure 2. Expression of *Tex11*, *AdamTS5*, *Asrgl1*, *Gmcl1*, *Pgk2*, *Klhl10* in the testis from wild type and *Ubb* deletion mouse testis.

are well-known to be highly specialized cells with compartmentalized functional regions, which endow them complex functions in fertility processes.[23] The key genes were orderly expressed to determine the successful completion of spermatogenesis.[9,10] We hypothesized that alternative expressions of the key genes would contribute to poor sperm quality, and also defective spermatozoa could be traced back to abnormal gene expression. This study performed a bioinformatics dataming of genes associated with asthenozoospermia, and provided a novel insight into the understanding of sperm quality.

In the present study, we performed a systematic bioinformatics analysis of gene expression profile which was associated with asthenozoospermia. 86% of DEGs were down-regulated in asthenozoospermia spermatozoa, suggesting that decreased expression of key genes during spermatogenesis was the main cause of poor sperm quality. These genes covered broad functions, including metabolism, transport, binding, and catalysis activities. The down-regulated DEGs in asthenozoosperima were enriched in molecular function of ubiquitin related activities, which were involved in processes of protein ubiquitination and male gamete generation processes. A group of DEGs were well-known to be involved spermatogenesis or fertilization processes. The results demonstrated that these DEGs were key molecules involved in sperm physiological function. They

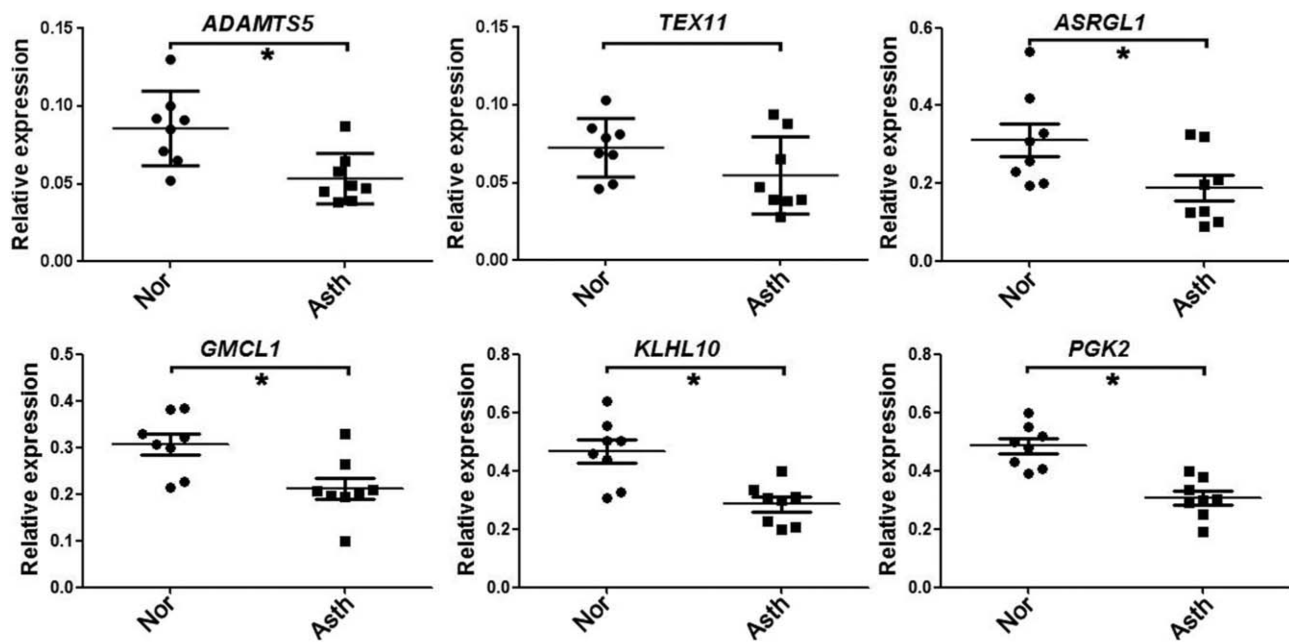

Figure 3. RT-PCR analysis of representative DEGs in normozoospermia and asthenozoospermia spermatozoa. Asth = asthenozoospermia, DEGs = differentially expressed genes, Nor = normozoospermia, RT-PCR = Reverse Transcription-Polymerase Chain Reaction.

provided a useful dataset for further mining fertility markers. It could supplement the current shortage of proteomic data. We previously suggested that germ cell-specific genes may be served as key molecular markers for assessing sperm quality.[11,12] Here we further identified germ cell specific genes according to strictest standard. Most germ cell specific genes were expressed in spermatogonia and round spermatids cells. Spermatogonia specific genes may play critical roles in germ cell proliferation and initiate of meiosis, while round spermatids specific genes were more correlated with sperm quality.[22] Our previous study has confirmed that PGK2 was specifically expressed in human testis, especially in round spermatids. Its coding product was decreased in spermatozoa from asthenozoospermia and elderly adults, that showed closely correlations with sperm quality.[12] In this study, we also found PGK2 mRNA showed similarly decreased tendency in asthenozoospermia spermatozoa, that was consistent with our previous study. KLHL10 was a high evolutionary protein in mammals, and was exclusively expressed in post-meiotic germ cells. Haploinsufficiency of Klhl10 could cause infertility in male mice.[24] TEX11 was an X-linked meiosis specific gene, and mutation was observed in infertile men with non-obstructive azoospermia.[24] ADAMTS5 belonged to metalloproteinase which were known to be responsible for the degradation of extracellular matrix. ADAMTS5 protein expression in semen was significantly related with sperm production.[25] When compared with fertile control group, Aydos et al[26] have revealed the expression of ADAMTS5 was statistically significant lower in nonobstructive azoospermia. Researchers thought low ADAMTS expression might have an important role in the etiology of male infertility and might use as a predictive marker for azoospermic patients. Validation by RT-PCR showed the decreased expression of selected genes in asthenozoospermia spermatozoa that was consistent with bioinformatics analysis. These genes may serve as biomarkers for assessment of human sperm quality.

Current knowledge about physiological and pathological aspects of spermatozoa is still limited. Each of spermatogenesis processes is characterized by its own gene expression patterns. Molecular markers of germ cells at various stages are useful for understanding the underlying mechanism of spermatogenesis. The study provided a useful dataset and new idea for further research of spermatogenesis and regulation of sperm quality, and also provided ideas and prospects for research in male contraception, diagnosis, and treatment of male infertility.

Author contributions

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Methodology: Li Cai.
Resources: Li Cai.
Validation: XueXia Liu.
Writing – original draft: FuJun Liu.
Writing – review & editing: FuJun Liu.

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