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

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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 spermatozoa 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 = diethylypyrocarbonate, 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 spermatozoa are produced in testis by experiencing complex spermatogenesis, which leads to altered ejaculated sperm quality.[2,3] Mammalian spermatogenesis is a complex cell proliferation and differentiation process, including spermatogonia mitosis, renewal meiosis, and terminal differentiation of spermiogenesis. 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 mouse model. Our previous studies identified testis-specific genes and characterized their functions in sperm quality regulation.[4–11] 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.

Asthenozoospermia 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

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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 consent informed 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 diethylycarbontane (DEPC) water. Complementary DNAs (cDNAs) were synthesized according to the instructions provided using avian myeloablastosis 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^−ΔΔCT 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 spermatid 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
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 spermocyte 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
meiotic phase, leading to male infertility.\cite{21} As shown in Fig. 2, ADAMTS5, TEX11 were specifically expressed in spermatogonia, and had no significant difference in testis after Ubb deletion. While ASRG1 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 tests 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, ASRG1, 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.\cite{22} Spermatozoa

**Table 1**

| Overrepresentation enrichment analysis of differential expressed genes in asthenozoospermia spermatozoa. |
|---------------------------------------------------------------|
| **ID**              | **Name**       | **P value** | **ID**              | **Name**       | **P value** |
| 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:00098631         | Protein binding involved in cell adhesion | 9.09E–05 |
| GO:0044389          | Ubiquitin-like protein ligase binding      | 2.10E–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:0096631          | 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 |                   |                       |             |
| 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                 | 3.46E–09 | GO:0009141          | Nucleotide triphosphate metabolic process | 9.43E–07 |
| GO:0022412          | Cellular process involved in reproduction  | 1.81E–08 | GO:0009213          | Nucleotide 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     | 1.58E–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 |                   |                       |             |
| GO:00019233         | 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                               | 1.16E–05 | GO:0044455          | Mitochondrial membrane part            | 7.61E–09 |
| GO:0009813          | Centrosome                                 | 9.61E–05 | GO:0008978          | 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                          | 1.16E–03 | GO:0030055          | Cell-substrate junction                | 2.29E–04 |
| GO:0048770          | Pigment granule                            | 1.91E–03 | GO:1900204          | Oxidoreductase complex                 | 2.44E–04 |
| GO:0019867          | Outer membrane                             | 2.99E–03 | GO:0044445          | Cytosolic part                        | 3.85E–04 |
| GO:0009529          | Oligo                                    | 3.10E–03 | GO:0070069          | Cytochrome complex                     | 5.75E–04 |
are well-known to be highly specialized cells with compartmentalized functional regions, which endow them complex functions in fertility processes.\cite{23} The key genes were orderly expressed to determine the successful completion of spermatogenesis.\cite{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 asthenozoospermia 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 2. Expression of Tex11, Adam5, Asrgl1, Gmcl1, Pgk2, Klhl10 in the testis from wild type and Ubb deletion mouse testis.
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. 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. 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. 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. 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. When compared with fertile control group, Aydos et al. 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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