

Downregulation of KIF2C and TEKT2 is Associated with Male Infertility and Testicular Carcinoma

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Research

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Abstract

Background:

Genes have an important role in spermatogenesis and the maintenance of fertility, and may act as a potential biomarker for the clinical diagnosis of infertility. However, a comprehensive understanding of how these biological processes of infertility are regulated at the molecular level remains to be illustrated.

Methods:

In the present study, we sought to identify associated genes by reanalyzing separate studies from GEO datasets (GSE45885, GSE45887, and GSE9210) and validation dataset (GSE4797). DEGs were used the limma package. GO and KEGG pathway enrichment analyses were performed using the clusterprofier package. The STRING database was used construct a protein-protein interaction network. The interaction between mRNA and TF was predicted by using miRWalk. At last, the expression levels of hub genes were determined by TCGA data in GEPIA.

Results:

The results showed that several shared genes significantly associated with azoospermia. Finally, we effectively screen out two genes (KIF2C and TEKT2) for validation by GSE4797 in spermatozoa of infertile men with Johnsen score. Among these two genes, KIF2C and TEKT2 significantly down-regulated in spermatozoa of infertile men. The regulatory network of TF-miRNA-target gene was established, we found KIF2C-miRNAs(has-miR-3154,6075,6760-5p,1251-5p,186-sp)-TFs(EP300,SP1) might work in spermatozoa of infertile men.

Conclusions:

Our study might help to improve our understanding of the mechanisms in azoospermia and provide diagnostic biomarkers and therapeutics targets.

1. Introduction

Infertility is that couples can't have children after one year's normal sexual intercourse without protection, to influence 10–15 percent of couples.[1–4] From the latest WHO statistics, nearly 50–80 million persons suffer from infertility.[5, 6] A few researches demonstrate that nearly 50% of all cases of infertility occur because of female factors, 20% – 30% male factors, and 20% – 30% couples.[6–8] Male infertility is a multifunctional disease involving multiple phenotypes, which affects about 7% of men all the world .[9]Male infertility is a complex multi-functional pathological state, showing highly heterogeneous phenotypic phenomena, from complete absence of sperm in testis to significant changes in sperm quality.[10, 11] Genetic factors account for at least 15% of male infertility and contribute to the three main causes of male infertility: spermatogenic function defects; ductal obstruction or disorder; hypothalamic–pituitary axis disorder. Patients with azoospermia are at the highest risk of carriers of genetic abnormalities.

With technological development of microarray and high-throughput sequence technology, we can immediately identify expression diversifications at the transcription level, which beneficially contribute to infertile men. A lot of researches concentrated on differently expressed gene (DEG) analysis have found some potential molecular targets and diagnostic biomarkers at the transcription level in infertility. Agnieszka et al find that genes such as gametogenetin (GGN), germ cell associated 1 (GSG1), adenylate cyclase 10 (ADCY10), and gametocyte-specific factor 1 like (GTSF1L) are down regulated in men with azoospermia. Zhang et al recognize ribosomal protein S3 (RPS3), RPS5, RPS6, RPS16 and RPS23 were downregulated in teratozoospermia.[12, 13]

As the study of male infertility is still insufficient, our aim is to analyze whether there are rare potential disease-related variants in candidate genes associated with infertility, and to offer clinical proof.[14]In order to better reveal the complex molecular system of spermatogenesis and study the related genes of spermatogenesis, comprehensive bioinformatics method was used in this study.

2. Methods And Materials

2.1 Fetching testicular tissue microarray data sets from GEO

The data sets were obtained from GEO (<http://www.ncbi.nlm.nih.gov/geo/>) in the National Center for Biotechnology Information Database (NCBI) using the accession numbers GSE45885, GSE45887, and GSE9210. Azoospermia associated data sets GSE45885 and GSE45887 were submitted by Agnieszka Malcher and based on the GPL6244 platform ([HuGene-1_0-st] Affymetrix Human Gene 1.0 ST Array [transcript (gene) version]). The information of testicular samples in GSE45885 and GSE45887 was obtained from the published literature (Table 1). The study procedure showed in Fig. 1.

Table 1
Characteristics of GEO sample.

GSE NO.	Patients	GPL NO.	Experiment type	Organism	Title	Description
GSE45885	31	GPL6244	Expression profiling by array	homo sapiens	Potential biomarkers of non-obstructive azoospermia identified in microarray gene expression analysis	Control group:4; NOA group:27; Age:28–54(yrs).
GSE45887	20	GPL6244	Expression profiling by array	homo sapiens	The gene expression analysis of paracrine/autocrine factors in patients with spermatogenetic failure compared to normal spermatogenesis	Control group:4; NOA group:16; Age:28–54(yrs).
GSE9210	58	GPL887	Expression profiling by array	homo sapiens	A testicular gene expression profile for NOA patients, and ART3 as a genetic susceptibility gene for NOA	47 non-obstructive azoospermia (NOA) and 11 obstructive azoospermia (OA) patients
GSE4797	28	GPL2891	Expression profiling by array	homo sapiens	Microarray analysis of human spermatogenic dysfunction	full spermatogenesis (Johnsen Score 10, 12 samples), arrest at the spermatid stage (Johnsen Score 8, 6 samples), arrest at spermatocyte stage (Johnsen Score 5, 5 samples) and Sertoli-cell-only syndrom (Johnsen Score 2, 5 samples).
Abbreviation:NOA,non-obstructive azoospermia;OA, obstructive azoospermia.						

We fetched Level 3 gene expression profile (level 3 data) for Testis carcinoma patients from the TCGA data portal (<https://tcga-data.nci.nih.gov/tcga/>).

2.2 Microarray data preprocessing

In this study, the original CEL data was imported into R (version 3.5, <https://www.rproject.org/>), and the data background was corrected and normalized using the Affy R-package (Bioconductor version 3.6). Affybatch's

mas5calls method returns expression sets corresponding to specific genes through multiple probes.

2.3 Differentially expressed gene selection.

DEGs were used the limma package (version 3.6). $P < 0.05$ and $|\log_2 \text{fold change}| > 1$ were selected as the cutoff values.

2.4 Functional annotation and pathway analysis of DEGs.

GO analysis consisted of biological process (BP) and cellular compartment

(CC) and molecular function (MF) are common methods for functional annotation of large-scale genomic data. To learn more the mechanism of DEGs involved in infertility, we used clusterprofiler R-package to analyze the enrichment of GO and KEGG pathways (version 3.16, <http://www.bioconductor.org/packages/release/bioc/html/clusterProfiler.html>). $P < 0.05$ was considered to be a statistically significant difference in these analyses.

2.5 Protein interaction network and module analyses.

The STRING database (<http://string-db.org>), A protein-protein interaction network composed of up-regulated and down-regulated DEGs was constructed, with a cutoff score more than 0.4. Using the clusterone plug-in of Cytoscape v3.6.1 to select the important modules from the PPI network ([cytoscape.org/plugins HTML](http://cytoscape.org/plugins/HTML)) with $P < 0.01$ indicated statistical significance. The degree/betweenness/closeness centrality and K-core analysis were performed using two plug-ins CentiScaPe and Molecular Complex Detection (MCODE) in Cytoscape to illuminate the most important nodes and modules in the network.

2.6 TF-miRNA-target gene network construction

Interactions between differentially expressed miRNAs and differentially expressed mRNAs were predicted using miRWalk 3.0 (<http://mirwalk.umm.uni-heidelberg.de/>), and a score 0.95 was considered as the cutoff criterion for the prediction analysis in miRWalk. Only the target mRNAs included in all of these databases were selected for the further analysis. The interaction between mRNA and TF was predicted by using miRWalk 3.0 and the score 0.4 was considered as cutoff criterion for the prediction analysis in the experimental module of LncBase. After the predicted targets were intersected with DEGs, miRNAs, TFs and mRNAs were selected for further analysis. Cytoscape software (version 3.6.1) was used to visualize the regulatory network.

2.7 Validation and expression of Hub-gene in male infertility and testicular carcinoma

At last, the expression levels of hub genes were determined by TCGA data in GEPIA (<http://gepia.cancer-pku.cn>). Next, validation of hub-gene in male infertility by GSE4797 in GEO (Table 1). $P < 0.05$ was considered to indicate a statistically significant difference in these analyses.

3. Result

3.1 Analysis of DEGs.

The expression profile data were pre-processed and then analyzed with the Affy package in R language. The whole gene expression was screened. All RNA expression levels are presented in Fig-2. Hierarchical cluster analysis indicated that patients with azoospermia and the normal samples exhibited differing distributions. The results revealed that grouping was reasonable, and the data successfully underwent further analysis. Microarray data from the normal samples were compared with those from the azoospermia samples and a total of 1396 DEGs were identified. Figure 2-F showed 27 DEGs co-expression in three GEO data sets.

3.2 Functional annotation and pathway analysis of DEGs.

A total of 1396 genes were conducted by enrichment analysis, with $P \leq 0.05$ used to determine statistical significance. The top 10 GO terms enriched by up and downregulated genes were primarily enriched in “tubulin binding”, “cyclin-dependent protein serine/threonine kinase regulator activity”, “steroid dehydrogenase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor”, “motor activity”, “protein binding involved in protein folding”, “microtubule binding”, “glutathione transferase activity”, “dynein heavy chain binding”, “steroid dehydrogenase activity”, “extracellular matrix structural constituent” (Fig. 2-D). Figure 2-A,B,C displayed that GO-MF,CC,BP. The KEGG pathways of DEGs were primarily enriched in “Oocyte meiosis”, “Human T-cell leukemia virus 1 infection”, “cell cycle”, “progesterone-mediated oocyte maturation”, “glucagon signaling pathway”, “foxo signaling pathway”, “staphylococcus aureus infection”, “aldosterone synthesis and secretion”, “toxoplasmosis”, “carbon metabolism”.

3.3 Protein interaction network and module analysis

To investigate the relationship of shared genes between modules, PPI networks were constructed using Cytoscape software based on the STRING database. In addition, the k-core analysis was performed to identify the key clusters and hub genes of PPI networks. Through Cytoscape-MCODE analysis, we set parameters as follows: Degree Cutoff: 5, Node Score Cutoff: 0.2, K-Core: 5. We identified 10 clusters of 338 spermatogenesis-associated genes (Fig. 3). The hub genes in cluster 1 (Fig. 3) that exhibited the highest scores (52.59) were TOP2A, CDT1, KPNA2, TACC3, TEKT2,NUF2, ATAD2, PBK, DLGAP5, TYMS, KIF18A, PTTG1, SPAG5, CDCA8, AURKA, EZH2, CCNB2, EXO1, NCAPG, SMC2, BUB1, KIF15, CDK1, CDC45, ZWILCH, KNTC1, NEK2, KIF20A, MCM4, MAD2L1, CDCA5, CDC20, CCNB1, CENPK, CKS2, OIP5, HMMR, PLK4, ASPM, CDKN3, CEP55, RAD54L, TTK, KIF2C, CENPF, CDCA2, SKA3, SGOL2, RAD51, SPC25, RFC4, MND1, CENPM, CENPU, CASC5, BIRC5, CDC25C, GMNN, RACGAP1, ANLN, UHRF1.

3.4 TF-miRNA-target gene network construction

A total of 42 miRNAs could bind to shared genes predicted by miRWalk. Four genes did not have any binding miRNA. The related TF and the TFs regulatory network are shown in Fig. 4. The regulatory network of TF-miRNA-target gene was established, involving 18 TFs and 23 hub genes, such as KIF2C-miRNAs(has-miR-3154,6075,6760-5p,1251-5p,186-sp)-TFs(EP300,SP1).

3.5 Expression of Hub-gene in testicular carcinoma

A total of 12 genes (ANLN, CCNB1, CENPF, COIL, CYCS, KIF2C, KNSTRN, LELP1,OAZ3,SRPK2,TEKT2,WBTC1) were identified as hub genes. We used TCGA data of testis cancer to validate the hub gene expression with the

online tool of GEPIA. All of the hub genes are expressed differently in normal and cancer tissues of testis cancer by the criterion of $|\log FC| > 1$ and $p < .01$ (Fig. 5).

3.6 Validation of hub gene in male infertility

Through validation of hub-gene (A total of 12 genes were identified as hub genes in testicular carcinoma) in male infertility by GSE4797 set, we found expression of KIF2C between more than or equal to John score 5 and less than 5 existed significant difference. The expression of TEKT2 decreased with reduction of John score. Furthermore, we found KIF2C might act significantly in infertility and testis cancer through DEG, functional annotation and pathway, protein interaction network and module analysis.

4. Discussion

With the development of science and technology, the molecular mechanism of azoospermia has aroused great interest. The study of azoospermia depends on human studies, animal models, organ culture models and cell lines [9, 15]. The increase in the number of genes / proteins associated with male infertility has been confirmed [9, 16]. However, how these biological processes are regulated at the molecular level remains to be elucidated. Therefore, it is necessary to further study the pathogenesis of azoospermia at the molecular level. In this study, we identified the genes associated with azoospermia and systematically constructed a comprehensive framework of genes and miRNAs.

In the present study, we sought to identify associated genes by reanalyzing

separate studies from GEO datasets (GSE45885, GSE45887, and GSE9210) and validation dataset (GSE4797) [13, 17, 18]. The results showed that several shared genes significantly associated with azoospermia. Finally, we effectively screen out two genes (KIF2C and TEKT2) for validation by GSE4797 in spermatozoa of infertile men with Johnsen score. Among these two genes, KIF2C and TEKT2 significantly down-regulated in spermatozoa of infertile men. The regulatory network of TF-miRNA-target gene was established, we found KIF2C-miRNAs(has-miR-3154,6075,6760-5p,1251-5p,186-sp)-TFs(EP300,SP1) might work in spermatozoa of infertile men. Interestingly, the relative expression levels of KIF2C and TEKT2 had a negative correlation with Johnsen score, which showed potential role of spermatogenesis.

KIF2C (also known as the mitotic centromere-associated kinesin, MACK) is a member of the kinesin-13 family of microtubule (MT)-depolymerizing kinesins, which is critical in the regulation of microtubule dynamics. During cell division, KIF2C plays an important role in inhibiting the wrong connection between MT and chromosome [19, 20]. The function of kif2c in interphase cells is not obvious, although its main localization in nucleus suggests that kif2c may play a role in nuclear processes. Kif2c promotes the formation of DNA damage foci, which may involve the migration and aggregation of DSBs (DNA Double Strand Break) [21–25]. We found KIF2C might play a significant role on testis cancer and azoospermia.

Tektins (TEKTs), the proteins of the microtubules in Cilia, Flagella, Basal bodies and centrioles [26–28], have been found in various animals, including Filariae, including silk-worms [29], mice [26, 30] and humans [31, 32]. They were originally isolated from sea urchins and are a group of proteins: TEKT-A, -B and -C [33, 34]. On the other hand, five types of TEKTs have been identified in mammals. TEKT2, which is similar to Tektin-t, locates

in the main part of human spermatozoa but no immune signal was detected in the middle or at the end of the human sperm. Tektin2, a membrane protein, is responsible for sperm flagellum movement. Previous studies show that CatSper and tektin are associated with male infertility because they play an important role in sperm motility[35]. Tektin2 is essential for the integrity of motilin arm in sperm flagellum. Lack of tektin2 can lead to impaired sperm motility and male infertility [36]. The low expression of tektin2 mRNA was observed in frozen spermatozoa, suggesting that the decrease of sperm motility after cryopreservation may be due to the transcriptional damage of some sperm motility related genes [37].

The miRNAs play an important role in infertility. In 2009, for the first time, expression of miRNAs in a testicular sample of NOA patients compared to fertile control samples evaluated by microarray technology, identified 19 upregulated and 154 downregulated miRNAs [38]. Hsa-miR-141, hsa-miR-429, hsa-miR-7-1-3p, hsa-miR-34b, hsa-miR-34c-5p, hsa-miR-122 expression levels were significantly different in azoospermia [39, 40]. Through luciferase experiments, miR-525-3p which targets SEMG1 gene and hsa-miR-210 which targets insulin-like growth factor II (IGF2) [41, 42]. The lower expression of hsa-miR-188-3p results in higher expression of MLH1 gene in azoospermia patients and leads to apoptosis in spermatozoa[43].

Functional classification of the miRNA/mRNA pairs using bioinformatics tools indicated that they play a role in spermatogenesis, cell meiosis, cell cycle. We found KIF2C-miRNAs(has-miR-3154,6075,6760-5p,1251-5p,186-sp)-TFs (EP300,SP1) might work in spermatozoa of infertile men.

Our study also has some limitations. First, more samples could be included in this study and we assessed our results based on published observations. Further in vitro and/or in vivo experiments would need to be carried out to test reliability of our results. This might reduce the error caused by individual differences of patients.

5. Conclusions

We applied DEG analysis to identify genes associated with azoospermia in this study. Then, through a systems biology framework for a comprehensive and systematic biological function- and network-based analysis of azoospermia, we found 27 hub genes and test on the expression of Hub-gene in testicular carcinoma (found 12 hub genes were significantly different in testicular carcinoma). Furthermore, we made the validation of hub-gene (A total of 12 genes were identified as hub genes in testicular carcinoma) in male infertility by GSE4797 set and found TEKT2 and KIF2C were significant in infertility. The regulatory network of TF-miRNA-target gene was established and we found KIF2C-miRNAs(has-miR-3154,6075,6760-5p,1251-5p,186-sp)-TFs (EP300,SP1) might work in spermatozoa of infertile men. Our study might help to

improve our understanding of the mechanisms in azoospermia and provide diagnostic biomarkers and therapeutics targets.

Abbreviations

GEO, Gene Expression Omnibus database

TCGA, The cancer genome atlas

DEG, differently expressed gene

GGN, gametogenetin

GSG1, germ cell associated 1

ADCY10, adenylylate cyclase 10

GTSF1L, gametocyte-specific factor 1 like

NCBI, National Center for Biotechnology Information Database

BP, biological process

CC, cellular compartment

MF, molecular function

GEPIA, Gene Expression Profiling Interactive Analysis

FC, fold change

GO, Gene Ontology

KEGG, Kyoto Encyclopedia of Genes and Genomes

TF, Transcript factor

KIF2C, Kinesin Family Member 2C

TEKT2, Tektin 2

MACK, mitotic centromere-associated kinesin

DSBs (DNA Double Strand Break

NOA, Non-obstructive azoospermia

MT, microtubule

IGF2, insulin-like growth factor II

Declarations

Authors' contributions

Xiaofeng LI and Jianquan Hou have made substantial contributions to conception and design. WQ Xu downloaded the data. HM Cao wrote the manuscript. Fei Wang edited the article for spelling, grammar. Haiming Cao, Weiqiang Xu, Xiaofeng LI, Jianquan Hou reviewed and and intellectual content.

Competing interest

The authors declare there are no conflicts of interest in this work.

Available of data and materials

The datasets analyzed for this study can be found in the GEO datasets (<https://www.ncbi.nlm.nih.gov/gds>) and TCGA.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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Figures

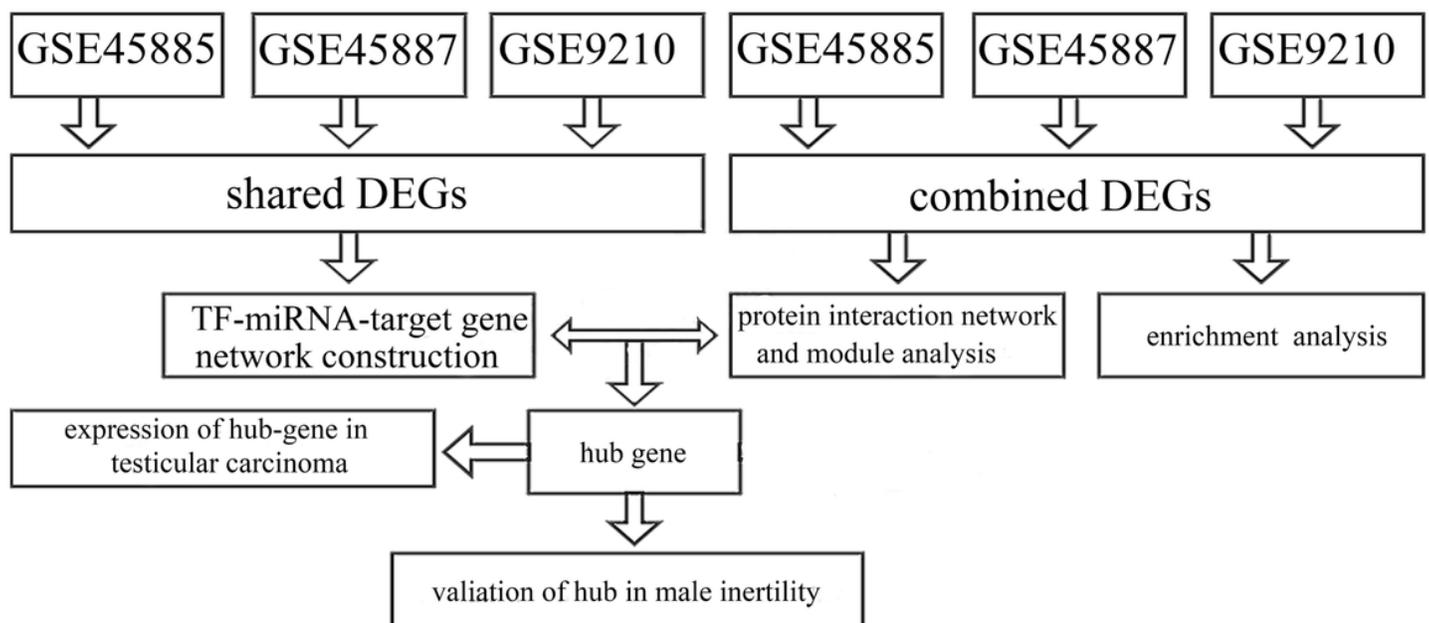


Figure 1

The study procedure.

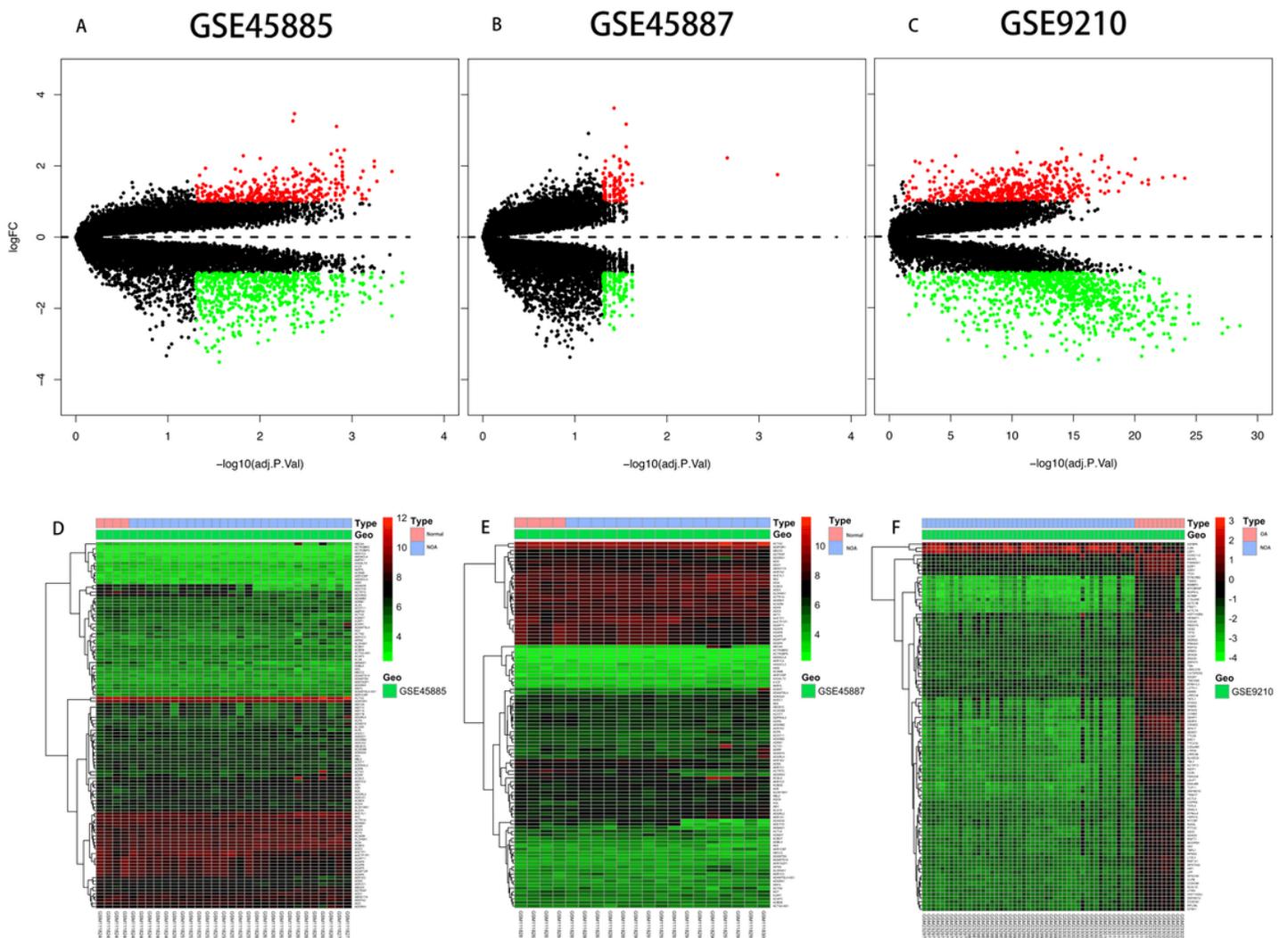


Figure 2

Differential expression analysis. (D,E,F) Heat map presenting the expression pattern across different samples. The horizontal axis represents sample names. The left vertical axis presented clusters of DEGs, and the top horizontal axis presents clusters of samples. Red represents upregulated genes and green represents downregulated genes. (A,B,C) Volcano plot of DEGs. The y axis is logFC and the x axis represents \log_{10} (adjusted P value). The red dots represent the DEGs upregulated and the green dots represent the DEGs downregulated while the black dots represent genes that were not differentially expressed. DEGs, differentially expressed genes; FC, fold change.

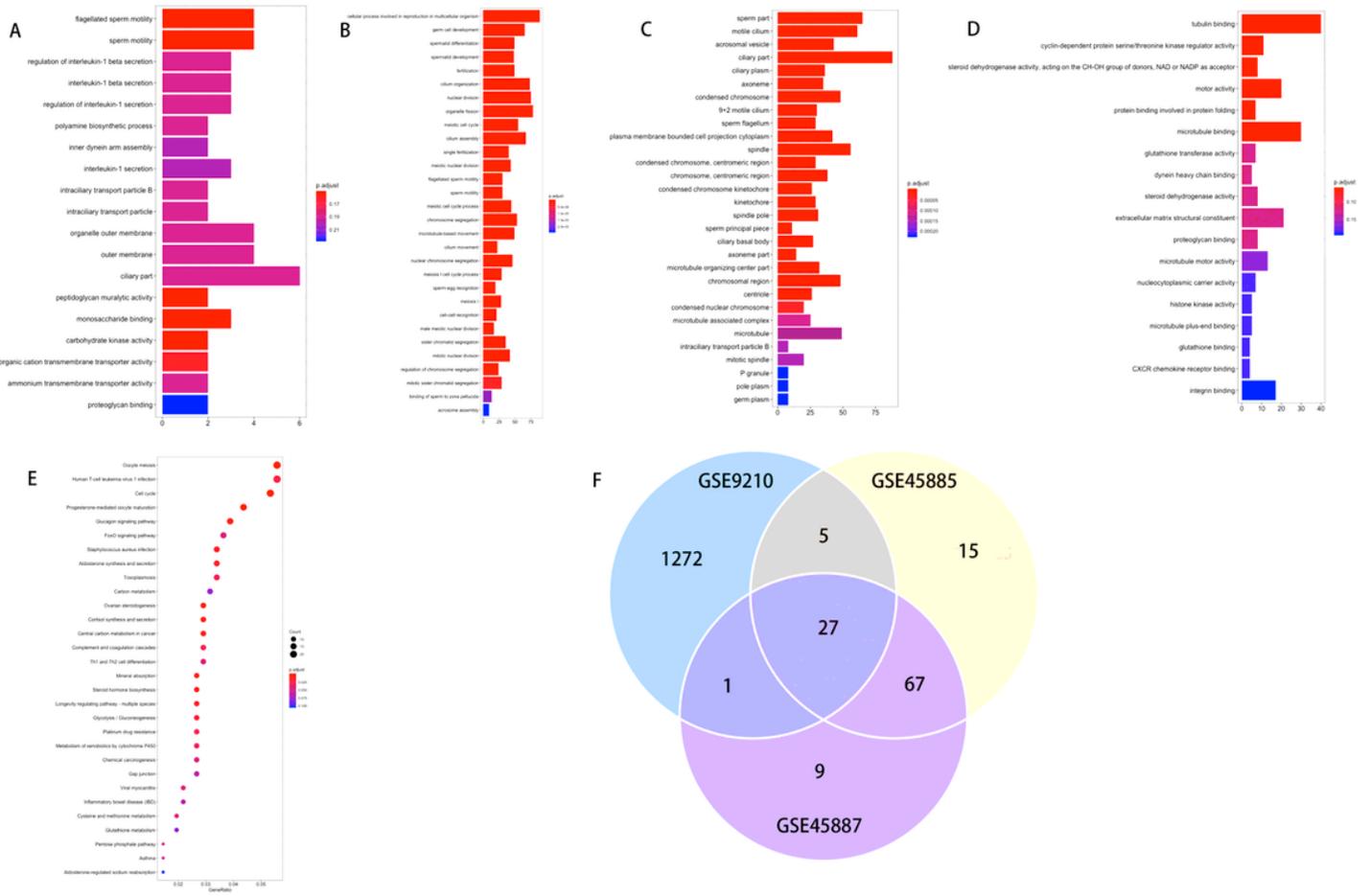


Figure 3

(A-E) GO and KEGG results of DEGs. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes;(F) Venn plot of candidate hub genes commonly owned in GSE45885, GSE45887 and GSE9210.

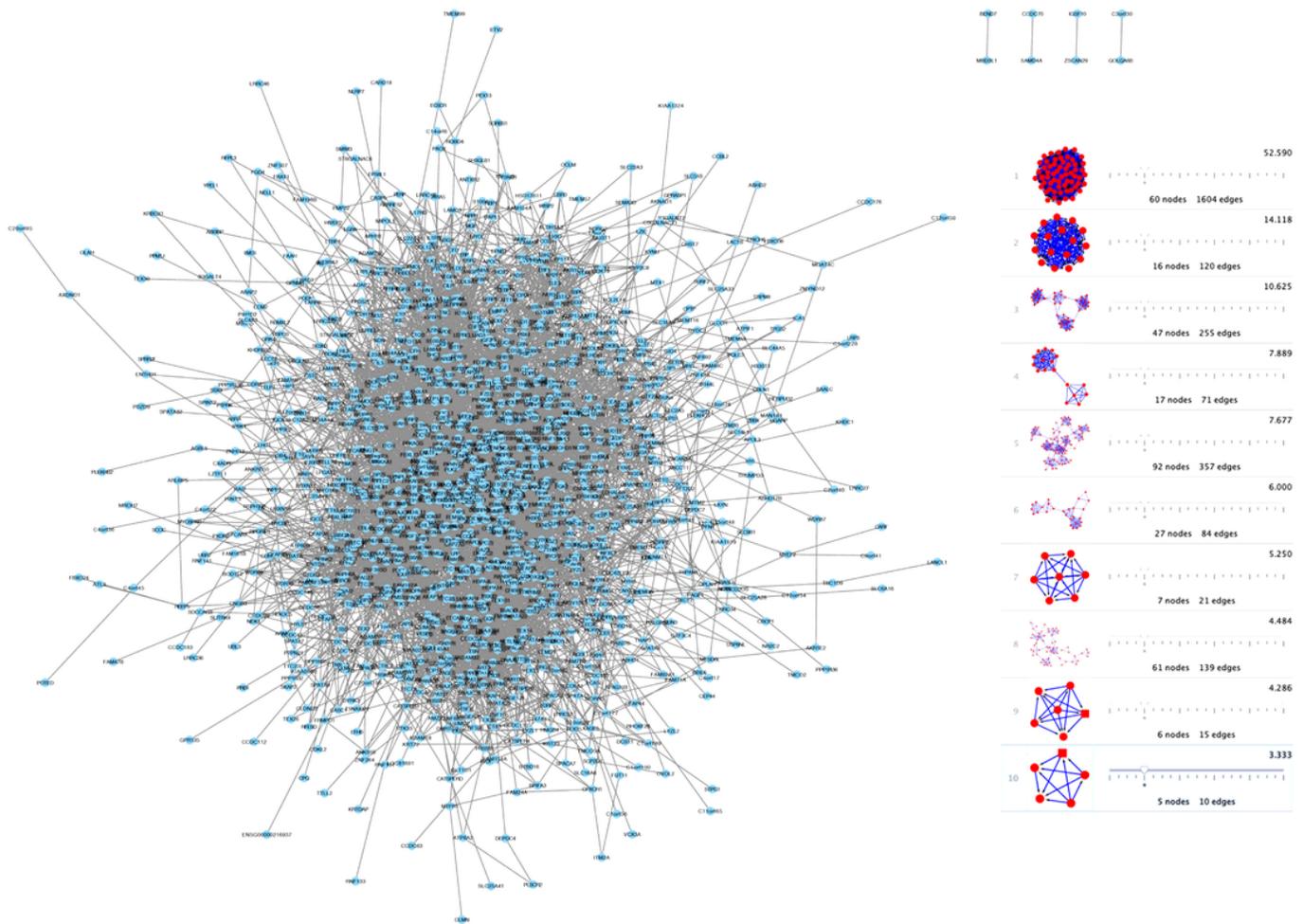


Figure 4

Network and module analysis of DEGs. (left) PPI network of DEGs obtained from the STRING database. (right) 10 clusters identified through Cytoscape-MCODE analysis. DEGs, differentially expressed genes; PPI, protein-protein interaction.

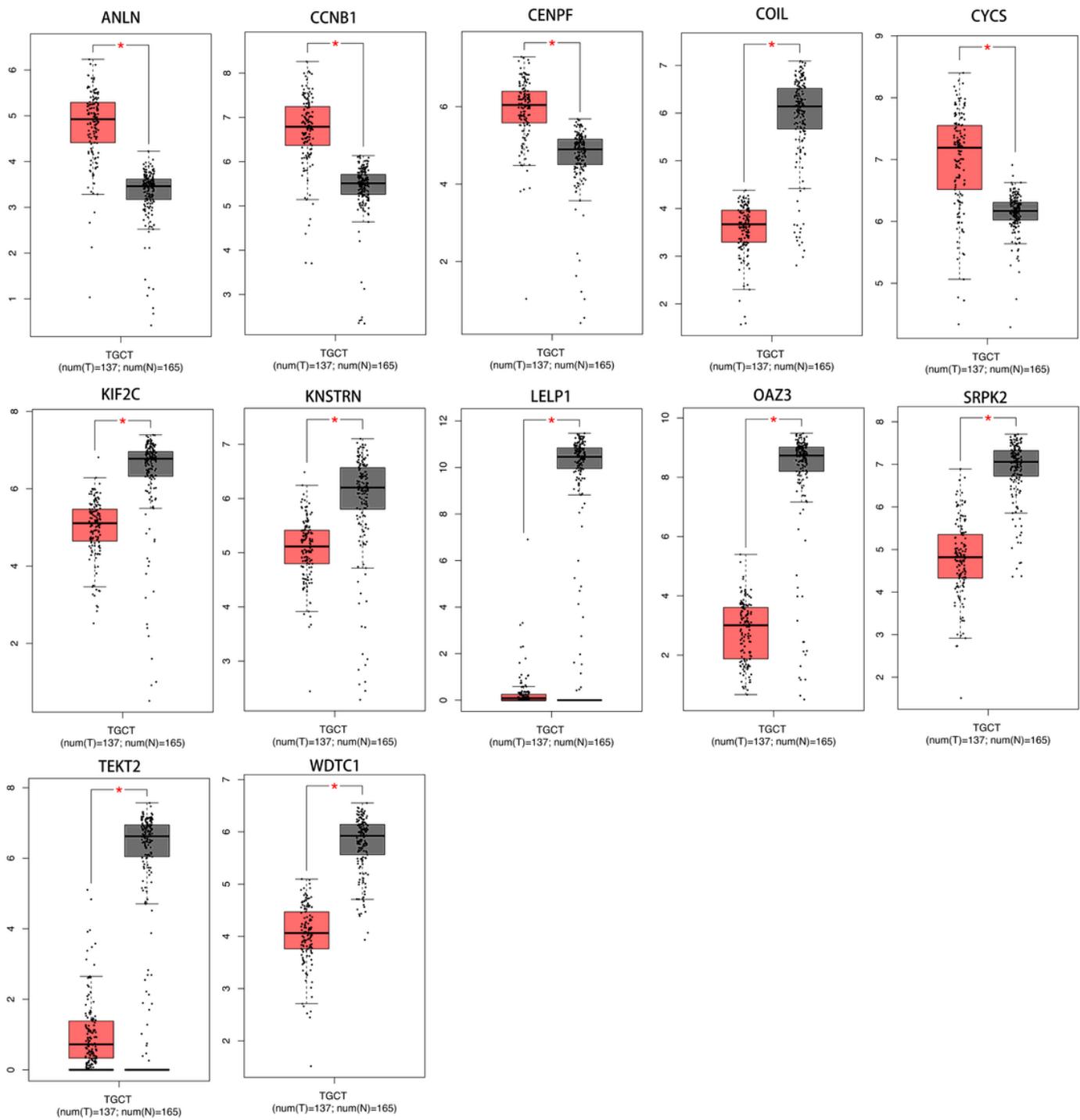


Figure 6

The transcriptional differences of hub gene levels between colon carcinoma tissues and the para-cancer tissues in TCGA. TCGA, The Cancer Genome Atlas ($*p < .001$)

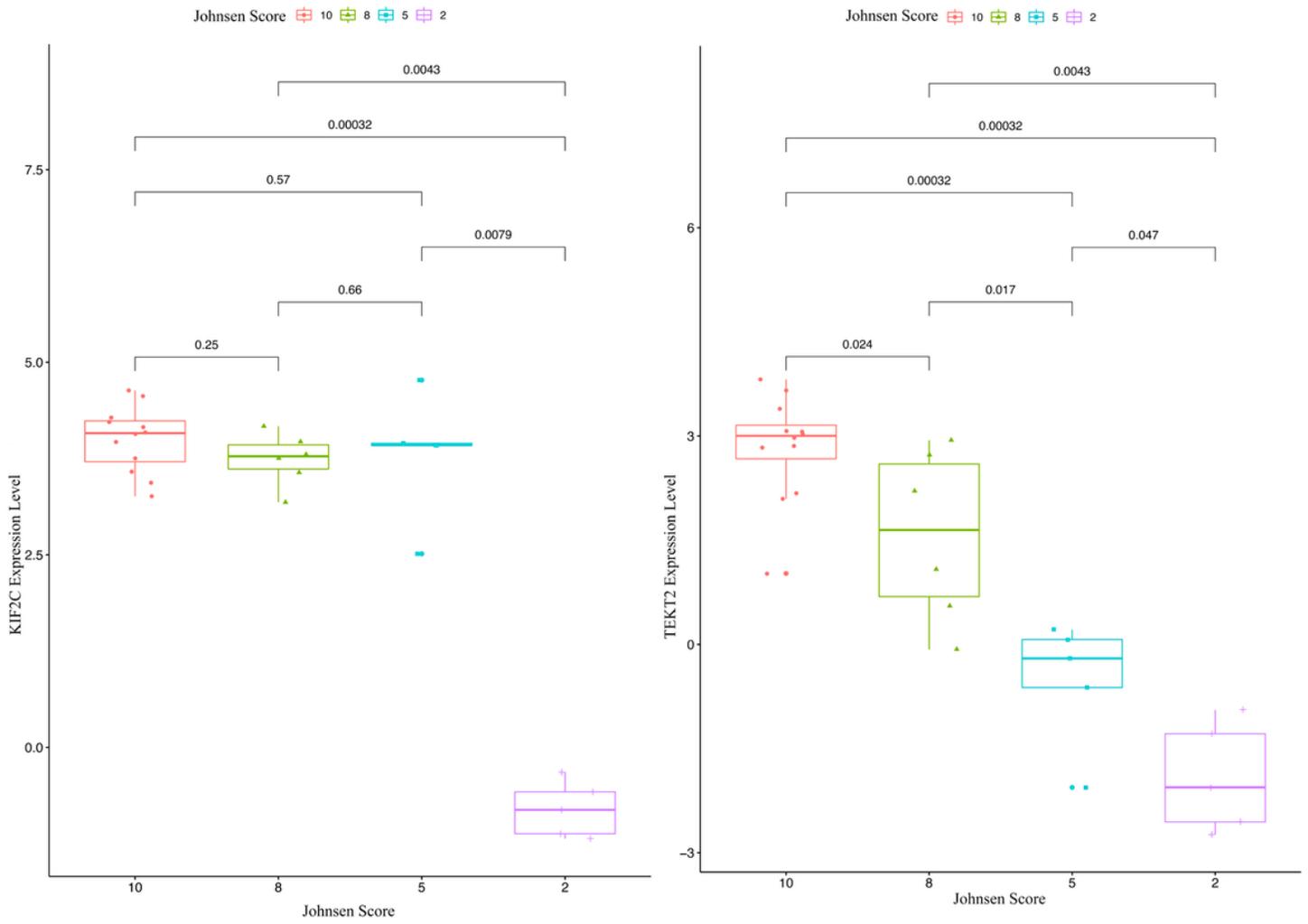


Figure 7

The validation of KIF2C and TEKT2 in GSE4797 associated with male infertility.