CYLD Gene Expression as a Molecular Biomarker in Infertile Men with Obstructive and Non-Obstructive Azoospermia: A Comparative Study

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Abstract

Background and objectives: It is widely accepted that estimation of success and risks associated with infertility treatment via TESE method, which involves sperm extraction from the testes, is significantly important. While various studies have assessed the success of this estimation method, carboxyl-terminal hydrolase (CYLD) gene expression has not yet been evaluated as a biomarker. Therefore, this study aimed to evaluate CYLD gene expression in infertile men with obstructive and non-obstructive azoospermia (OA and NOA). Materials and methods: Selected by simple random sampling, 30 and 15 men with NOA and OA were allocated to patient and control groups, respectively. After isolating and delivering the sperm to the laboratory for ICSI, the textural residues were used for RNA extraction. In addition, the level of CYLD gene expression was measured in both groups using RT-qPCR method. Moreover, we compared the research groups in terms LH and FSH hormone levels. Data analysis was performed in Graph Pad Prism 7.0 using non-pairing t-test by estimating the odds ratio and 95% confidence interval. Results: While CYLD gene expression decreased in patients, compared to healthy individuals, the difference was not statistically significant. On the other hand, a significant difference was observed between the patient and control group regarding the level of LH and FSH hormones. Conclusion: According to the results, there was an insignificant decrease in the level of CYLD gene expression, which indicated the lack of ability of the gene to predict the success of TESE. Nevertheless, the difference in the LH and FSH hormone levels in the subjects of the control and patient groups demonstrated the possibility of using these hormones to predict the success of TESE.

Keywords: Infertility, CYLD Gene, Real-time PCR, Gene Expression

INTRODUCTION

Infertility is defined as not being able to get pregnant despite having frequent, unprotected sex for at least a year for most couples. Infertility problem has been detected in 10-15% of couples. According to the latest world health organization's report, about 50-80 million people around the world are affected by infertility [1]. Large-scale studies have showed that female, male and both-gender infertility is found in approximately half, 20-30%, and 20-30% of cases, respectively [2]. In sexual reproduction new organisms are produced from the fusing of the nucleus of a male sex cell with the nucleus of a female sex cell. Reproductive organs are considered to be either primary or secondary organs; the primary reproductive organs are the gonads, which are responsible for gametes and hormone production, whereas secondary organs aid in the growth and maturation of gametes and developing offspring. The testicles are primary male reproductive organs, and male germ cells, spermatozoa, are produced in a unique process known as spermatogenesis. The term spermatogenesis describes the development of male gametes in the ferrous semen covering tissue from diploid spermatogonia, which leads to the release of differentiated haploid germ cells into the seminiferous tubules. Several studies have shown the crucial presence of testosterone and FSH for successful completion of spermatogenesis [3].

While infertility can be caused by many reasons, semen analysis is a vital part of diagnosing male infertility. Unusual semen parameters are related to sperm concentration,

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appearance, and motility. In the world health organization handbook, semen quality is divided into six groups, including azoospermia (a lack of sperm in seminal fluid), oligospermia (sperm concentrations of less than 20 million sperm/ml), asthenozoospermia (less than 32% of the sperm are able to move efficiently), teratozoospermia (less than 4% of the sperm have a normal appearance), oligoasthenospermia (the parameters related to appearance, motility and density of sperm are impaired), and cryptozoospermia (no semen in ejaculate). In cryptozoospermia, sperm may be observed after centrifugation [4].

Disorders of this classification may have a genetic cause. About 14% of men with azoospermia and 5% with oligospermia have chromosomal abnormalities, which is much higher than the general population, reported at 0.6%[5]. Some of the gene mutations associated with pathological syndromes can also be linked to male infertility. One of these syndromes is congenital bilateral absence of the vas deferens (CBAVD), which results in obstructive azoospermia in 80-90% of the cases. CBAVD is caused by cystic fibrosis transmembrane conductance regulator (CFTR) gene mutations [6]. Infertile men need to have sperm retrieved directly from the testis or epididymis. Azoospermia can be divided into obstructive (OA) and non-obstructive (NOA) categories. Occurring because of a reproductive tract obstruction, azoospermia can be either congenital (CBAVD) or acquired (as a result of infection, vasectomy, or physical injury to the genital tract).

On the other hand, testes' lack of ability to produce sperm is the main cause of sperm absence in the ejaculate. In this regard, male infertility is not due to the obstruction of the vas deferens, detected in 60% of men with azoospermia [7]. Testicular sperm extraction (TESE) and intracytoplasmic sperm injection are the best treatments of choice for men with NOA. In a common TESE, spermatozoa are carried out through testicular biopsy under anesthesia or local anesthesia. Another method is applying MicroTESE. However, sperm extraction by MicroTESE is very complicated. In general, the logic of MicroTESE is recognizing core regions of sperm production in the testes based on the size and appearance of seminiferous tubules with the aid of a microscope. Spermatozoa can be retrieved from dilated seminiferous tubules, and all of the process can be observed under a microscope [8]. Recently, some reports on the successful use of radius of seminiferous tubules for accurate estimation of sperm recovery have been published.

However, sperm is not found as a result of TESE in half of azoospermia. Serum hormones such as FSH and Inhibin B and noninvasive tests (e.g., testicular volume assessment) have not significantly predicted testicular recovery [9]. In these cases, only testicular histopathology can be used as a predictor of successful sperm recovery. This has increased the importance of molecular methods in successful prediction of this type of pregnancy. In fact, diagnostic biomarker design is recognized as a valuable molecular method.

In spermatogenesis, many morphological changes occur in spermatid from the spermatid stage to the spermatozoa stage. At this stage, additional organelles (e.g., mitochondria, Golgi apparatus, endoplasmic reticulum and cytoplasm) must be removed to create compact spermatozoa [10]. The ubiquitin proteasome pathway contributes to the analysis and elimination of these compounds. The mentioned system is essential for spermatogenesis in mammals[11, 12]. Ubiquitination is a type of post-translational modification that regulates biological and cellular processes, including transcriptional regulation, immune response, embryo development and intracellular signaling pathways.

Ubiquitin protein has 76 amino acids and is highly conserved among eukaryotes [13]. This molecule binds to its substrate as monomers and polymers in lysine amino acid. Monoubiquitin plays a role in DNA repair, vesicular categorization, signal transduction and endocytosis, whereas polyubiquitination plays a role in protein breakdown and signal transduction. Polyubiquitination in lysine 63 results activation of protein or transfer of signal [14, 15]. Ubiquitin molecules bind covalently to their target proteins. Ubiquitinated proteins are usually degraded by protease 26s or lysozyme, and ubiquitin molecules are released by the activity of DUB deubiquitinases, which play an important role in spermatogenesis. Ubiquitin carboxy-terminal hydrolases (UCHs) are among the enzymes involved in this path [16]. In fact, UCHs are the most important regulators of ubiquitin proteasome system and a proteasome system that can separate the peptide chain from the end of ubiquitin carboxy [17]. One of the members of this family is carboxyl-terminal hydrolase (CYLD).

In related to familial cylindromatosis, the CYLD gene is first observed in patients experiencing skin tumors in head and neck areas. Another name of the gene is USPL2 and is placed on chromosome 16 at position q12.1. The largest transcript of this gene has 8503 nucleotides containing 18 exons. CYLD protein is translated from this gene that has 956 amino acids. In addition, CYLD has deubiquitinase activity and specifically cuts the lysine (K)-63-linked polyubiquitin [18]. This protein is a type of tumor inhibitor with a deubiquitinase activity, involved in the regulation of various paths by removing ubiquitin chains from proteins [19]. To date, no research has been conducted to assess the expression of the gene and its association with infertility. With this background in mind, this study aimed to use CYLD as a proper biomarker to predict sperm retrieval from male testicular tissues using the TESE technique.

Method

Sample collection

This experimental-descriptive study was performed on 30 NOA men as patient group and 25 OA men as control group, selected by simple sampling. Inclusion criterion was referral to the infertility center at Yazd Institute for Reproductive Sciences.

The participants underwent a microTESE surgical procedure to assess their spermatogenesis status and draw sperms by an urologist. During the surgery, testicular tissue samples were collected under general anesthesia and assessed under a microscope (model, manufacturing country). The available sperms in the sample (if there was any) were isolated and delivered to the laboratory for ICSI, and the remaining tissue samples were used for RNA extraction. In addition to giving consent to the urology center for undergoing a microTESE surgery, the participants expressed their consent for using the remaining testicular tissue samples to evaluate CYLD gene expression. It is notable that the exclusion criterion was unwillingness of patients to cooperate with the researcher.

RNA extraction and measurement

In order to extract RNA, we first collected testicular tissue samples from NOA and OA subjects and kept the samples in a freezer at -80°C. Given the significant important of extracting high-quality RNA with proper quantity for gene expression analysis via Real-time PCR, RNA was extracted using Qiagen kit based on kit instruction protocol. Afterwards, the amount of RNA was measured by optical density (OD) determination method, whereas the level of absorbance at 260 nm was estimated by a NanoDrop™ 1000 spectrophotometer (made in Germany). The impurities in the RNA solution were determined by estimating absorbance of 260/230 and 260/280, which showed protein and DNA contamination, respectively.

cDNA synthesis

Since the RNA quality must be ensured before cDNA synthesis, we extracted two µl RNA and placed it on 1.5% agarose gel with 110 V voltage in electrophoresis tank for 45 minutes. The samples were used for cDNA synthesis in case of healthy RNA and observing smear and two bonds in 822 and 1522 positions that showed 18 S and 28 S ribosomal RNAs, respectively.

A thermos scientific kit was used following RNA extraction based on kit instruction. We applied oligo dT primer and random hexamer to construct cDNA. In this regard, we needed 0.2-5 µg of RNA, where the concentration of RNA used varies with the concentration of other reactants within this range. In cDNA synthesis method, a required amount of RNA, water, primer, reaction buffer, RNase inhibitor, dNTP and reverse transcriptase were pours in a 0.2-ml tube and all compounds were completely mixed with piping. In the next stage, the mixture was entered into a thermocycler machine based on a specific schedule. In the end, cDNA construction was accompanied by cDNA synthesis with GAPDH and PCR primers to evaluate accuracy and observing its product on gel electrophoresis.

Real-time PCR Reaction

We first evaluated the sequence of primers to carry out real-time PCR. After the synthesis of primers, we prepared and used 10 picomol stock primers dilution in each reaction. It is notable that the primers were kept at -20°C. Table 1 shows primer sequences and characteristics, according to which beta-actin gene was a housekeeping gene used as internal control.

Table 1: Primer characteristics and sequences

primer	Melting point	Length	Sequence
Beta-actin forward	59.2	18	AGCACAGAGCCTCGCCTT
Beta-actin reverse	57.8	18	CACGATGGAGGGGAAGAC
CYLD-forward	56.4	20	CAAATAGACGTGGGCTGTCC
CYLD-reverse	57.3	20	CGCCACAATCTTCATCACAC

Data analysis

Data analysis was performed using Graph Pad Prism 7.0. After being collected by Real-time PCR, the data was measured by ΔΔCT equation and entered into Excel software. In the end, non-pairing t-test was used to evaluate the data by measuring odds ratio and 95% confidence interval.

Results

Molecular Evaluation Results of RNA Extraction

The quality of the extracted RNAs was evaluated by quantitative and qualitative methods and the absorbance at 260-280 nm of the samples ranged from 1.7 to 2.2. The ratio of 230-260, indicating protein contamination, varied from 1.6 to 1.9, which was favorable. The gel electrophoresis was also performed for RNA integrity assessment (Figure 1). If extraction yielded results beyond the above evaluation, the test was repeated for RNA extraction.

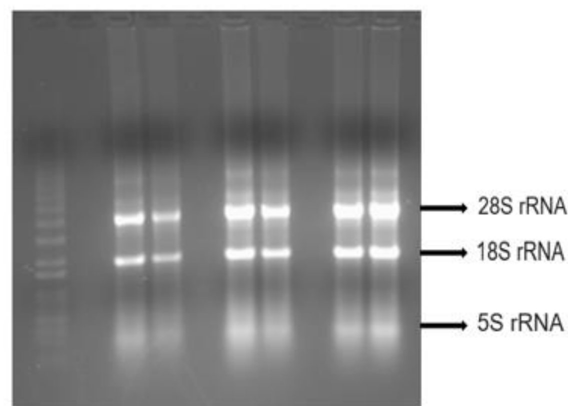

Figure 1: Evaluation of quality of RNA extracted by gel electrophoresis

cDNA Synthesis and q-RTPCR Reaction

In this study, we used 200 ng RNA for cDNA synthesis. The ACTB gene was also applied as a housekeeping gene. Moreover, the gene expression level was determined in Real-time PCR by Ct, which is the minimum number of cycles in which the amount of fluorescent absorbed by the device reaches a detectable level (Figure 2).

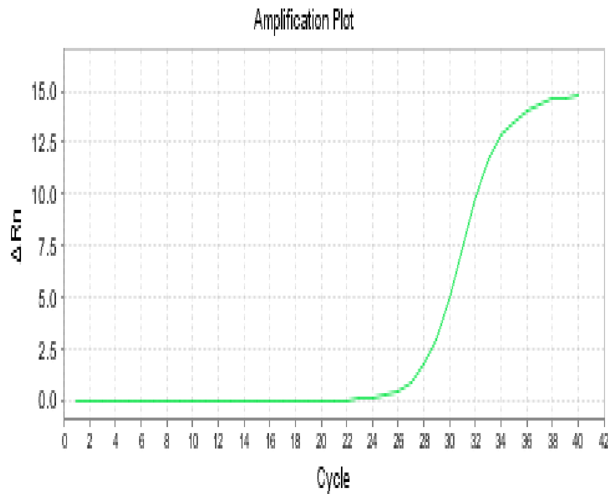

Figure 2: Melt curve related to CYLD gene

For this experiment, the cDNAs were diluted at a ratio of 1:10, and standard curve at various dilutions and related Cts are illustrated below (figures 3 & 4).

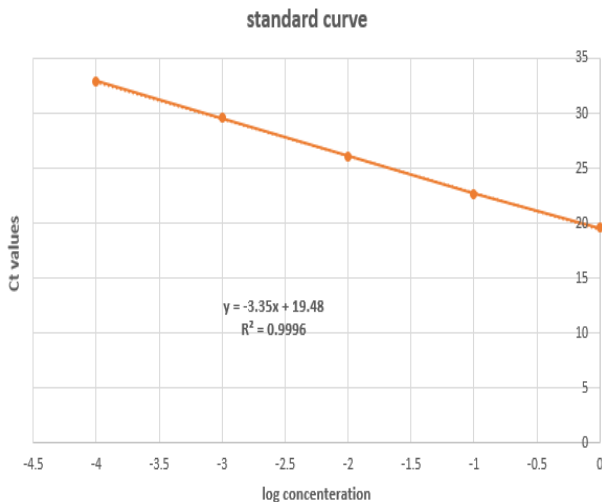

Figure 3: Standard curve related to CYLD gene

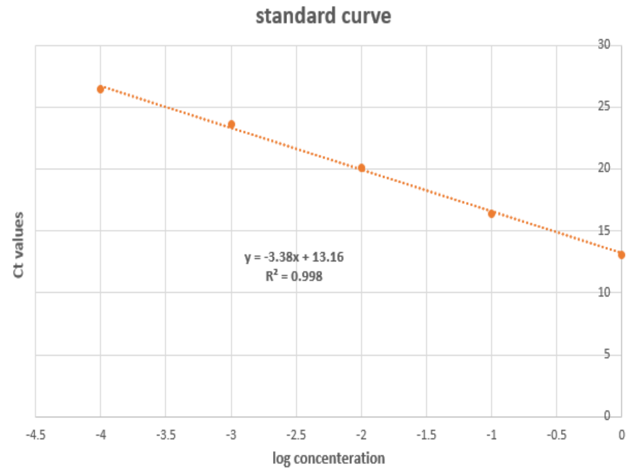

Figure 4: Standard curve related to ACTB gene

The melt curve of genes has a significant importance in Real-time PCR technique. The melt curve of ACTB and CYLD is shown in Figure 5.

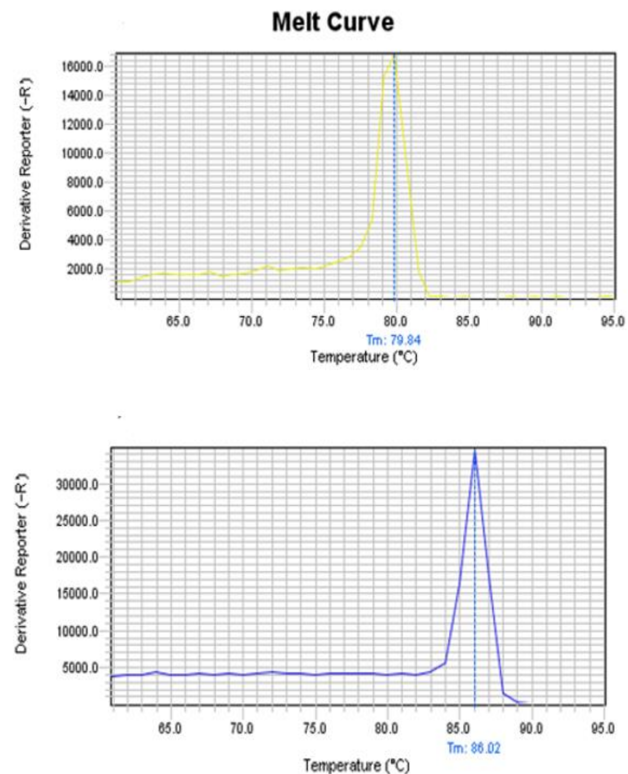

Figure 5: Melt curve related to CYLD (above) and ACTB (below) genes

CYLD Gene Expression

At this stage, we applied the 2^−ΔΔCT equation to measure CYLD gene expression level. In this respect, the CYLD gene expression level decreased in patients, compared to healthy individuals, as shown in Figure 6. Nonetheless, the difference was not significant (P>0.05).

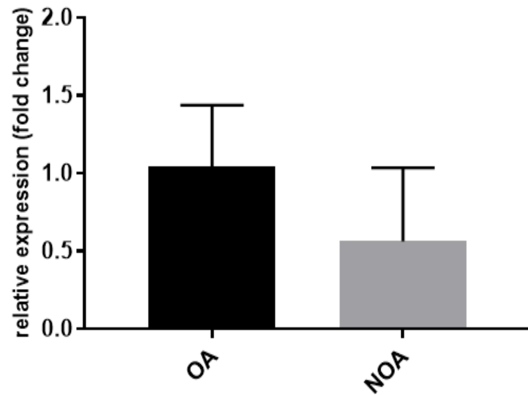

Figure 6: CYLD gene expression level in the control and patient groups

Hormone Level Evaluation

The fertile and infertile groups were compared in terms of LH and FSH hormone levels. In this regard, the results were indicative of a significant difference between the patient and control group (P<0.05).

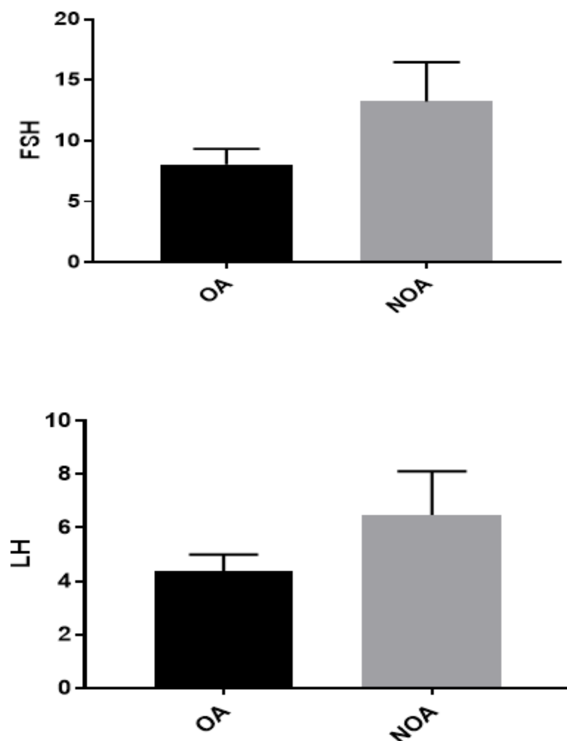

Figure 7: Levels of LH and FSH in the Patient and Control Groups

Discussion

Estimation of costs and hazards related to treatment of infertility, which mainly involves sperm retrieval from testes by surgery, is significantly important for infertile men with a genetic background. In addition to possible risks and expenses, the method assesses the level of success of sperm recovery. On the other hand, the method acts inefficiently in predicting sperm presence [20]. The only non-invasive method useful in identifying infertile patients for surgery with this method is the microdeletion test Y. Including the complete elimination of AZFa and AZFb regions, Y microdeletion cannot produce sperm [21].

In the present study, we evaluated the level of CYLD gene expression in men with OA and NOA. According to the results, gene expression decreased in the infertile subjects, compared to fertile and healthy individuals. However, this decrease was not statistically significant (P>0.05). According to reports, the CYLD has deubiquitinase activity. This protein is a type of tumor inhibitor with deubiquitinase activity, involved in the regulation of different paths by eliminating ubiquitin chains in involved proteins [16]. The protein affects the ability to regulate multiple members of the NF-kB pathway, which plays an important role in apoptosis regulation.

In a study by Ranganathan et al. (2002), CYLD protein expression was directly related to the mobility concentration and sperm morphology [22], which is in line with our findings. In another research by Pentikäinen et al., increased expression of protein was related to the amount of apoptosis in human testicular germ cells [23]. Moreover, the gene negative regulates RIP1, which results in the inhibition of apoptosis and disruption of spermatogenesis[24].

In 2014, Ni et al. realized that 362-5p microRNA induces metastasis in carcinoid liver cells by inhibiting CYLD gene expression [25].

In 2014 Dang Feng et al. evaluated papillary thyroid cancer, reporting a decrease in CYLD due to being targeted by b181, which increased apoptosis feature [26]. Homozygous knockout mice are infertile for the CYLD - / - gene, but heterozygous CYLD mice are +/- fertile. While spermatogonia and spermatocytes are easily found in seminiferous tubules, postsynaptic germ cells such as spherical and elongated spermatids are rarely found in these tubes [27].

Follicle-stimulating hormone (FSH) plays an important role in reproduction of mammals [28]. By affecting sertoli cells, the hormone facilitates the transfer from spermatogonia to spermatocytes [29]. In the present study, a significant difference was observed between the patient and control groups regarding the level of FSH hormone (P<0.05). Increased expression of the hormone has been reported in infertile men in other studies [30, 31]. Moreover, the LH hormone regulates spermatogenesis by affecting leydig cells, and decrease of this hormone is observed in infertile men [31]. This hormone also maintains secondary sexual characteristics by increasing testosterone stimulation from Leydig cells [32]. Furthermore, a significant reduction was observed in the patient group, compared to the control group (P<0.05).

Conclusion

The present research aimed to assess the relationship between CYLD gene expression and FSH and LH hormone levels with male infertility. According to the results, which gene expression decreased in infertile individuals, the level of reduction was not significant. Moreover, a significant difference was observed between the control and patient groups regarding LH and FSH hormone levels.

References

1. Hamada AJ, Esteves SC, Clinics AA, undefined. A comprehensive review of genetics and genetic testing in azoospermia. SciELO Brasil.
2. Ishikawa T. Surgical recovery of sperm in non-obstructive azoospermia. Asian journal of andrology. 2012;14(1):109.
3. Origin, migration, and proliferation of human primordial germ cells. 2013:19--37.
4. Jungwirth A, Giwercman A, Tournaye H, Diemer T, Kopa Z, Dohle G, et al. European Association of Urology guidelines on Male Infertility: the 2012 update. 2012;62(2):324-32.
5. Assche EV, Bonduelle Ma. Cytogenetics of infertile men. academicoupcom.
6. Bobadilla JL, Macek M, Fine JP, Farrell PM. Cystic fibrosis: A worldwide analysis ofCFTR mutations?correlation with incidence data and application to screening. Human Mutation. 2002;19(6):575--606.
7. Reproduction PNS, and Development F, undefined. Causes of azoospermia and their management. Csiro.
8. Carpi A, Sabanegh E, and Sterility JMF, undefined. Controversies in the management of nonobstructive azoospermia. Elsevier.
9. Reprod SCEA, undefined. Male infertility due to spermatogenic failure: current management and future perspectives. cbraorgbr.
10. Hecht NB. The making of a spermatozoon: a molecular perspective. Developmental genetics. 1995;16(2):95--103.
11. Baarends WM, der Laan R, Grootegoed JA. Specific aspects of the ubiquitin system in spermatogenesis. Journal of endocrinological investigation. 2000;23(9):597--604.
12. Sadowski M, Suryadinata R, Tan AR, Roesley SNA, Sarcevic B. Protein monoubiquitination and polyubiquitination generate structural diversity to control distinct biological processes. IUBMB Life. 2012;64(2):136--42.
13. Sun L, opinion in cell Biology ZJCC, undefined. The novel functions of ubiquitination in signaling. Elsevier.
14. Ramanathan HN, in b, molecular Biology YY, undefined. Cellular strategies for making monoubiquitin signals. Taylor \& Francis.
15. Sigismund S, Polo Sa. Signaling Through Monoubiquitination. 2004:149--85.
16. Eletr ZM, Wilkinson KD. Regulation of proteolysis by human deubiquitinating enzymes. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research. 2014;1843(1):114--28.
17. Wilkinson KD. Ubiquitination and deubiquitination: targeting of proteins for degradation by the proteasome. 2000;11(3):141--8.
18. Sato Y, Goto E, Shibata Y, Kubota Y, Yamagata A, Goto-Ito S, et al. Structures of CYLD USP with Met1-or Lys63-linked diubiquitin reveal mechanisms for dual specificity. Nature structural \& molecular biology. 2015;22(3):222.
19. Massoumi R. Ubiquitin chain cleavage: CYLD at work. Trends in biochemical sciences. 2010;35(7):392--9.
20. Carpi A, Sabanegh E, Mechanick JJF, sterility. Controversies in the management of nonobstructive azoospermia. 2009;91(4):963-70.
21. Stahl PJ, Masson P, Mielnik A, Marean MB, Schlegel PN, Paduch DAJF, et al. A decade of experience emphasizes that testing for Y microdeletions is essential in American men with azoospermia and severe oligozoospermia. 2010;94(5):1753-6.
22. Ranganathan P, Kattal N, Moustafa MH, Sharma RK, Thomas AJ, Agarwal AJF, et al. Correlation of nuclear factor kappa B (NFKB) with sperm quality and clinical diagnoses in infertile men. 2002;78:S95.
23. Pentikäinen V, Suomalainen L, Erkkilä K, Martelin E, Parvinen M, Pentikäinen MO, et al.
24. Wright A, Reiley WW, Chang M, Jin W, Lee AJ, Zhang M, et al. Regulation of early wave of germ cell apoptosis and spermatogenesis by deubiquitinating enzyme CYLD. Developmental cell. 2007;13(5):705--16.
25. Ni F, Zhao H, Cui H, Wu Z, Chen L, Hu Z, et al. MicroRNA-362-5p promotes tumor growth and metastasis by targeting CYLD in hepatocellular carcinoma. Cancer letters. 2015;356(2):809--18.
26. Li D, Jian W, Wei C, Song H, Gu Y, Luo Y, et al. Down-regulation of miR-181b promotes apoptosis by targeting CYLD in thyroid papillary cancer. International journal of clinical and experimental pathology. 2014;7(11):7672.
27. Gao J, Huo L, Sun X, Liu M, Li Da. The tumor suppressor CYLD regulates microtubule dynamics and plays a role in cell migration. Asbmb.
28. Plant TM, Marshall GRJEr. The functional significance of FSH in spermatogenesis and the control of its secretion in male primates. 2001;22(6):764-86.
29. Zirkin BR, editor Spermatogenesis: its regulation by testosterone and FSH. Seminars in cell & developmental biology; 1998: Elsevier.
30. Eelaminejad Z, Favaedi R, Sodeifi N, Gilani MAS, Shahhoseini MJRbo. Deficient expression of JMJD1A histone demethylase in patients with round spermatid maturation arrest. 2017;34(1):82-9.
31. Medrano J, Marques-Mari A, Aguilar C, Riboldi M, Garrido N, Martinez-Romero A, et al. Comparative analysis of the germ cell markers c-KIT, SSEA-1 and VASA in testicular biopsies from secretory and obstructive azoospermias. 2010;16(11):811-7.
32. Ramaswamy S, Weinbauer GFJS. Endocrine control of spermatogenesis: Role of FSH and LH/testosterone. 2014;4(2):e996025.