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LINE-1 Gene Expression Profile in some Iraqi Patients with Oligozoospermia

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Abstract: *LINE-1* is a transposable element in the DNA of some organisms and belongs to the group of Long interspersed nuclear elements (LINEs). It consists of about 17% of human genomic DNA. Most of the *LINE-1* genes imprinting in the human genome are inactive; despite, some retained the ability to retro-transpose. In the present study, we examine 100 Iraqi patients [(50) Oligozoospermia infertile patients (OZ) and (50) normospermia fertile patients (NP)]. Samples were collected (100) serum blood sample to determine hormone levels using ELISA Kits and (100) seminal fluid samples for microscopic analysis and molecular study to estimate the *LINE-1* gene-expression level using the qRT-PCR technique. Levels of mRNA expression were measured by two steps reverse transcription-polymerase chain reaction (RT-PCR) technique. Results showed an increase in the expression levels of mRNA gene-expression was an up-regulation in gene expression level for *LINE-1* gene in the OZ group, besides, hormonal levels assay we observed that present elevation in serum hormone levels of FSH, LH and testosterone in Oligozoospermia group and this evolution responsible for male infertility and alteration of spermatogenesis.

Keywords: Hormonal assay, Gene Expression, *LINE-1*, Meal Infertility, Oligozoospermia, Semen parameters.

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Introduction

Long interspersed elements [LINEs], including *LINE-1* (L1) elements, are composing about 17% of human DNA that continues the diversify of human genomes (1). *LINE-1* is the only autonomous mobile element in the human genome. Its ability to mobilize, or retro-transpose, via an RNA intermediate generates additional copies of *LINE-1* within the genome (2). It encoded proteins (ORF1p and ORF2p) that can mobilize non-autonomous retro-transposons, other non coding RNAs, and messenger RNAs (mRNA), leading to the generation of processed pseudogenes (3,4). ORF1p serves as a nucleic acid chaperone, while ORF2p contains endonuclease and reverse transcriptase enzymatic domains active in retro-transposition (5). As a consequence, *LINE-1* is new heritable insertions and active *LINE-1*s are major drivers of somatic and germ line that can sporadically cause genetic diseases that are closely associated with the germ line predominant during embryogenesis (6, 7). The *LINE-1* elements expression patterns of endogenous it has complex processing of their mRNA (8). MicroRNA (miRNA) is small endogenous, single-strand RNA

molecules that regulate gene expression at a post-transcriptional level at the various stages of spermatogenesis starting from primordial germ cells to mature spermatozoa and in other cell types like Sertoli and Leydig cells regulation differentiation, proliferation, development, and apoptosis (9,10). Epigenetics is mechanisms involved in the regulation of gene expression include the regulation of non‑coding RNA, without altering the underlying nucleotide sequence (11,12). Mutation and/or aberrant expression of miRNAs have been associated with the progression of various disorders, including male infertility (13). Spermatogenesis a complex procedure of proliferation and differentiation of germ cells that leads undifferentiated diploid cells to differentiate into haploid men gamete cells (14,15). Therefore, the abnormality in the expression levels of a gene related to the cell division and the maturation of spermatozoa lead to affect the quality of spermatozoa (16). Oligospermia is a common cause of infertility in males, and it is less than the minimum required for natural pregnancy (sperm count <15×106 ml) according to 'the World Health Organization (17). The current study aimed to determine the prevalence of *LINE-1* gene expression analysis using qRT-PCR and associated risk factors with oligozoospermia infertility using semen analysis.

Materials and Methods

Study design

The study design case-control patient consists of 50 Oligozoospermia (OZ) (sperm count <15×106 ml) according to the World Health Organization guidelines (17) and 50 Normospermic (NP) as an apparently healthy control patient with normal seminal fluid analysis (SFA). Samples were collected from OZ and NP (100) seminal fluid samples by masturbation after 3-7 days of abstinence for analyzing seminal fluid profile, after liquefaction for 30 mint for microscopic analysis and molecular study. Blood samples (100) were collected (5ml vein blood) in gel tubes, and placed at 37°C for 10 mint for clotting and centrifuged at 1200 rpm for 5 mint, serum was separated and used for hormone assay using an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. All samples were carried out from Kamal Al-Samurai Specialized Hospital for Infertility and IVF, Baghdad, Iraq.

The inclusion criteria men previously diagnosed with infertility by seminal fluid analysis (SFA) test and by specialist androgen doctor. The exclusion criteria were applied for patients with transmitted and infectious sexual diseases, autoimmune disease, cancer, diabetes mellitus, varicocele, and obesity. Each volunteer patients (OZ and NP) has been written-learned consent was obtained from each appropriate patient, and the residual semen following analysis was used in the present investigation. All experimental procedures were conducted according to the Declaration of the Human Ethics Committee of the Ministry of Health in Iraq.

RNA Isolation

Total RNA was extracted from semen samples using the genomic RNA extraction and purification kit (RIBO-prep) (AmpliSens, Russia) according to the manufacturer’s instructions.

Quantity and quality of the isolated RNA were measured by Nanodrop.

Quantitative Reverse Transcription PCR Analysis

The expression of *LINE-1* was determined by two steps RT-PCR technique. The extracted mRNAs were reversed to cDNA using Reverta-L (RT-reagents Kit) (AmpliSens, Russia) according to the manufactured protocol using thermal cycler qPCR (Eppendorf, Germany) with the following condition, 95 ºC for 1mint one cycle and 95ºC at 15 sec for denaturation, 55 ºC at 20 sec for annealing, 72ºC at 7 mint for an extension that repeats for 40 cycles. Quantitative Real-time PCR (qPCR) was performed by using of WizpureTM qPCR Master Mix SYBR Green (Wizbio Solution, Korea) by analyzer real-time PCR (Biometera, Germany) and reverse transcription primers specific for *LINE-1* forward primer F:5 ′ - AAATGGTGCTGGGAAAACTG-3 ′ and *LINE-1* reverse primer R:5 ′ - GCCATTGCTTTTGGTGTTTT-3 ′ (18). The *LINE-1* expression level was normalized according to endogenous control mRNA Internal Control βeta globin (*IC β-globin*) as a housekeeping gene (HKG) as a reference gene. The *β-globin* primer forward F:5 ′ - GAGCCATCTATTGCTTACA -3 ′and *β-globin* reverse primer R:5 ′ - CCAACTTCATCCACGTTC -3 ′ (19), then the *LINE-1* gene expression level has been calculated using the ΔCt method (20).

Statistical analysis

All data were analyzed using SPSS software Version 26, (21). Spearman's test was applied to assess the correlation coefficient and an independent t-test was used in this study with $p \leq 0.05$ was considered as significant.

Results and Discussion

The result of hormonal assay profile was listed in Table (1) that revealed a highly significant difference in FSH, LH, and testosterone hormone levels for the OZ group than the NP group, the elevated in serum hormone levels of FSH, LH, and testosterone were observed in the infertile group compared with the fertile group. A similar result reported by Afrim *et al*., (21) and Farah, (22) that FSH, LH, and testosterone evaluation of hormone were indicating and management of male infertility. Also, the current result agreed with Meeker *et al*., (23) as well as Zainab *et al*., (24) result in they were founded that increased levels of FSH, LH, and testosterone hormones were affected by reduction sperm parameters. Another result was revealed by Sheikh *et al*., (25) that the elevated level of FSH its reliable indicator for damage of germinal epithelial in infertile men Oligozoospermia patients, also Holger *et al*., (26) and AL-Haboubi, (27). was reported that the concentrations of FSH generally reflected on the testicular histology, and causing primary spermatocyte arrest or hypo spermatogenesis with normal FSH concentration in some patients. Whereas LH stimulates spermatogenesis indirectly via testosterone. Turek *et al*., (28), also found increasing levels of gonadotropins in serum make interrupted the spermatogenic process leading to the decline in sperm count and infertility.

Table (1): Hormonal profile for the studied groups:

Hormones	OZ group (n=50)	NP group (n=50)	*P-value*
FSH (mIU/ml)	9.29 ± 3.45	4.49 ± 1.42	0.001***
LH (mIU/ml)	8.21 ± 1.84	6.60 ± 0.75	0.001***
Testosterone (ng/ml)	11.17 ± 1.73	5.95 ± 1.99	0.001***

‡ **Independent t-test was used to test between groups, *** significant at 0.001 prob. Level**

The result of the expression level of *LINE-1* gene as shown in Table (2) the Ct *LINE-1* mean ± SD for OZ was 3.61± 1.68, while the Ct *LINE-1* mean ± SD for NP group was 20.71±2.74, the result was a highly significant difference in mRNA expression levels in Ct levels of *LINE-1* gene between NP and OZ groups (P-value = 0.001). A similar result was reported by Victoria *et al*, (2010) that variation in gene expression levels of *LINE-1* mRNA production by qRT-PCR analysis in somatic human tissues testis for endogenous *LINE-1* gene comparable to those detected in cancer cells. The ΔCt *LINE-1* mean ± SD was 2.98±3.16 and19.9 ±3.73 for OZ and NP groups, respectively. The current result produces a higher significant difference between the OZ group and the NP group (P-value =0.00)1. On one hand, the result of ΔΔCt *LINE-1* mean ± SD for the OZ group was -0.63±3.67 and on the other hand, the result of $2^{\wedge-\Delta\Delta Ct}$ *LINE-1* was 1.55±3.5. The observed result in the present study of fold change was up-regulation on *LINE-1* gene expression profiles for OZ patients. The *LINE-1* gene determines as a biomarker for accurate diagnosis of male infertility on infertile males. It has been suggested that sperm mRNAs present in the ejaculated spermatozoa represent a genetic fingerprint, and could be considered to be a historical record of what happened in gene expression during spermatogenesis (29). Some studies have reported differences in the number of certain sperm transcripts between infertile and fertile men. A different expression signature was also determined related to the differences in sperm concentration, motility, and morphology (30, 31). This result disagrees with the result obtained by Holger *et al*., (2012) that not affect the difference between fertile patients than infertile patients in spermiograms (sperm concentration, motility, vitality, and morphology). Interestingly, it has been described that differences in the expression of a few hundreds of transcripts between fertile and infertile men with normal semen parameters (32). The *LINE-1* retrotransposons are silenced by multiple mechanisms: DNA methylation, histone modification, and piRNAs (piwi-interacting RNAs) (33). Higher *LINE-1* expression and retro-transposition caused by hypomethylation of *LINE-1* when correlated between infertile and fertile men were reported that affects gene expression during spermatogenesis (34,35). *LINE-1* de-silencing exhibits a binary expression pattern in up-regulation in spermatocytes but not in spermatogonia. The critical genes that play roles in the progression of spermatogenesis and aberrant expression of these genes may be responsible for the arrest of germ cell differentiation (36).

Table (2): Expression level of mean ± SD for Ct *LINE-1*, ΔCt *LINE-1*, ΔΔCt *LINE-1* and $2^{-\Delta\Delta Ct}$ *LINE-1* in semen of the studied.

Gene	Ct *LINE-1* NP	Ct *LINE-1* OZ	ΔCt *LINE-1* NP	ΔCt *LINE-1* OZ	ΔΔCt *LINE-1* OZ	$2^{\wedge-\Delta\Delta Ct}$ *LINE-1* OZ	Regulation
LINE-1	20.71 ±2.74	3.61± 1.68	19.9± 3.73	2.98±3.16	-0.63±3.67	1.55±3.5	Up-regulation
P-value‡	0.001^{***}		0.001^{***}				

Ct: threshold cycle, Independent t- test was used to test between groups * significant at 0.001 prob. Level.

According to Pearson correlation coefficient analysis between semen parameters and hormone profile for OZ group as shown in Table (3), the result revealed that there was a negative correlation coefficient between sperm count and sperm immotile (-0.39), in contrast, there was a positive correlation coefficient between sperm count and sperm motility (0.39). While there was a negative correlation coefficient between sperm motility and LH hormone level (-0.44). On one side hand, there was a great positive correlation coefficient between sperm immotile and LH hormone level (0.44). On the anther hand, there was a negative correlation coefficient between sperm normal morphology and testosterone as well as sperm abnormal morphology (-0.70, -1.00), respectively. In contrast result, there was a great positive correlation coefficient between sperm normal morphology and LH as well as FSH (0.74, 0.84), respectively. In addition, present a great positive correlation coefficient between sperm abnormal morphology and testosterone (0.70), but present a negative correlation coefficient between sperm abnormal morphology and LH and FSH (-0.70, 0.84), respectively. The relation between hormones in this study presented a great positive correlation coefficient between FSH and LH (0.63), but a great negative correlation coefficient between LH and testosterone ((-0.70). On another hand, there was present positive correlation coefficient between testosterone and $2^{\wedge-\Delta\Delta Ct}$ *LINE-1* gene expression level for the OZ group (0.39). In our investigation study as it clears the determining hormone profile FSH, LH, and testosterone for OZ men is a major step towards predicting infertility because the sperm parameters and hormonal assay serve as an indicator for medical personnel to determine male infertility (37). Similar findings by Wagner *et al*., (38) as identify that the results of qRT-PCR revealed a significant correlation between a percentage of total sperm parameters and the *LINE-1* gene expression level. In addition, a significant correlation was shown between sperm concentration, in regard these correlations, disagreed with other authors believe the motility of sperm can be influenced by the change in the expression levels of some genes expressed in testes (39,40). The current results of this study disagree with the result obtained from Sandra *et al*., (41) and Bahrehmand, (42) that found their no correlation was found between the

sperm parameters concentration or motility and the $2^{\wedge-\Delta\Delta Ct}$ *LINE-1* gene expression level. However, semen morphology was found positively correlated with transcript levels.

Table (3): The Pearson Correlation Coefficient Analysis of OZ group in Semen Parameters, Hormone Profiles and Gene expression:

Parameters OZ	Sperm Count (*10^6/ml)	Sperm motility (%)	Sperm Immotile (%)	Sperm Normal Morphology	Sperm Abnormal Morphology	FSH (mIU/ml)	LH (mIU/ml)	Testosterone (ng/ml)	$2^{-\Delta\Delta Ct}$ *LINE-1*
$2^{-\Delta\Delta Ct}$ *LINE-1*	0.10	-0.07	0.07	-0.30	0.30	0.11	-0.26	0.39*	1.00
Testosterone (ng/ml)	0.20	0.24	-0.24	-0.70**	0.70**	-0.28	-0.70**	1.00	
LH (mIU/ml)	-0.05	-0.44**	0.44**	0.74**	-0.74**	0.63**	1.00		
FSH (mIU/ml)	0.11	0.14	-0.14	0.84**	-0.84**	1.00			
Abnormal Morphology	-0.07	-0.81	0.81	-1.00**	1.00				
Normal Morphology	0.07	0.81	-0.81	1.00					
Sperm Immotile (%)	-0.39**	-1.00	1.00						
Sperm Motility (%)	0.39**	1.00							
Sperm Count (*10^6/ml)	1.00								

***Correlation is significant at the 0.05 level (2-tailed), **Correlation is significant at the 0.01 level (2-tailed).**

The result of correlation coefficient analysis for NP group between semen parameters and hormone profile as shown in Table (4), The result as it clears there was no correlation coefficient between sperm count and anther sperm parameters as well as between hormone profiles. In contrast, it presents a great negative correlation coefficient between sperm motility and normal morphology (-0.53), but a great positive correlation coefficient between sperm motility and abnormal morphology (0.53). Although, the present a great positive correlation coefficient between sperm immotile and normal morphology (0.54), but present a great negative correlation coefficient sperm immotile and abnormal morphology (-0.54). Whereas, the present negative correlation coefficient between sperm normal morphology and LH, FSH, and abnormal morphology (-0.42, -0.40, -1.00), respectively. On one side of the hand, the present negative correlation coefficient between FSH and testosterone hormone level (-0.68) and the positive correlation coefficient between FSH and LH, but negative correlation coefficient between LH and testosterone hormone level (-0.74). The current result in this study indicated that the study of hormonal levels related to the fertility of men. Similar results have been obtaining by Johannes *et al*., (43) they were reported that the LH, FSH, and testosterone within the normal reference range for men.

Table (4): The Pearson Correlation Coefficient Analysis of NP group in Semen Parameters, Hormone Profiles and Gene expression:

Parameters NP	Sperm Count (10^6/ml)	Sperm motility (%)	Sperm Immotile (%)	Sperm Normal Morphology	Sperm Abnormal Morphology	FSH (mIU/ml)	LH (mIU/ml)	Testosterone (ng/ml)
Testosterone (ng/ml)	0.02	-0.17	0.17	0.25	-0.25	-0.68**	-0.74**	1.00
LH (mIU/ml)	0.06	0.01	-0.01	-0.42*	0.42	0.84**	1.00	
FSH (mIU/ml)	0.10	0.27	-0.27	-0.40*	0.40	1.00		
Abnormal Morphology	0.09	-0.53**	0.54**	-1.00**	1.00			
Normal Morphology	-0.09	0.53**	-0.54**	1.00				
Sperm Immotile (%)	-0.24	-1.00	1.00					
Sperm Motility (%)	0.24	1.00						
Sperm Count (*10^6/ml)	1.00							

***Correlation is significant at the 0.05 level (2-tailed), **Correlation is significant at the 0.01 level (2-tailed).**

Conclusion

This study found the up-regulation level in gene expression of the *LINE-1* gene in Oligozoospermia patients and could be used as a biomarker for the detection of early infertility in males.

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