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The Association of *DAZ1* Gene Deletion with Azoospermia in Iraqi Infertile Men

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Abstract

A disease of the reproductive system known as infertility is characterized by a clinical pregnancy that cannot be obtained after twelve months or longer of unprotected, ordered sexual copulation. Infertility disorder has impacted the reproductive system when a clinical pregnancy cannot be obtained after twelve months or more of unprotected, ordered sexual interaction. The following investigation was conducted to examine the gene expression levels in Iraqi patients with azoospermia, 150 human blood samples were collected from different regions in Baghdad governorate include (private medicals Labs and the high institute for infertility diagnosis assisted reproductive techniques and Kamal Al- Samara'ay IVF Hospital). The control group consists of 50 sample males with a range of 22-51 years old, while the patient (infertile group) consists of 100 sample males with ages ranging between 25-51 years old, the variations in this gene's expression between patients and healthy controls were taken into consideration. The age correlation for azoospermia patient's occurrence compared with controls was studied after subdividing them into subgroups and between patients and controls, there were no discernible differences in terms of age for any of the study groups. The average level of gene expression decreased significantly in this study, according to the findings (ΔCt) of *DAZ1* in patient groups compared to the corresponding ΔCt means in the control group (0.12±0.91 vs. 1.45±0.25), also the folding of expression 2-ΔCt of *DAZ1* gene in azoospermia groups, reached to (0.39±0.21), which indicating upregulation of this gene in infertile Iraqi men.

Keywords: Infertility, DAZ I, age, patients.

العلاقه بين حذف جين ال*DAZ1* وفقد النطف في الرجال العراقيين المصابين بالعقم

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الخلاصة

العقم هو مرض يصيب الجهاز التناسلي يعرف بالفشل في تحقيق الحمل السريري بعد اثني عشر شهرًا أو أكثر من الجماع الجنسي المنظم غير المحمي .أجريت الدراسة التالية للتحقق من مستوى التعبير الجيني لجين DAZ1 في مرضى فقد النطاف العراقيين ، تم جمع مائة وخمسين عينة دم بشري من مناطق مختلفة في محافظة بغداد تشمل (مختبرات طبية خاصة ومعهد عالي لتشخيص العقم بتقنيات الإنجاب المساعدة وكمال السامرائي للاطفال الانابيب). تتكون المجموعة الضابطة من 50 عينة من الذكور تتراوح أعمارهم بين 22-

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51 سنة ، بينما تتكون المجموعة الضابطة من 100 عينة من الذكور تتراوح أعمارهم بين 22−51 سنة. متضمن. تمت دراسة الارتباط بين العمر لحدوث فقد النطاف مقارنة بالضوابط ولم تظهر فروق ذات دلالة إحصائية حسب العمر بين المرضى والمجموعة الضابطة. أظهرت نتائج هذه الدراسة انخفاضًا معنويًا في متوسط مستوى التعبير الجيني (ΔCt) لمجموعات المرضى مقارنة بمتوسط (ΔCt) المقابل في المجموعة الضابطة. كما يعكس طي التعبير الجيني (2−CtΔΔ) لجين DAZ1 الذي سجل (0.21±0.39)، وهذ يشير الى التنظيم فوق العالي للجين في الرجال الراقيين المصابين بالعقم.

1. Introduction

Infertility is a serious health issue that affects about 15% of couples. It is the inability to get pregnant after twelve months of unprotected sexual activity (Dissanayake et al., 2019). Infertility is "a condition of the reproductive system that is characterized by the inability to obtain a clinical pregnancy after 12 months or more of continuous, unprotected sexual activity without any other explanation." (WHO,2016).

Azoospermia is the medical term for when a man's sperm doesn't have any sperm in it. It's linked to not being able to have children. About 1% of men have azoospermia (Hwang, et al., 2018). Azoospermia comes in three forms: 1)Pre-testicular azoospermia- (non-obstructive) NOA happens when the hormones that make sperm don't work as well as they should (Staff et al., 2010) 2)Testicular azoospermia- (non-obstructive) NOA is caused by any change in the way the testes are built or how they work (Esteves et al., 2013) 3)Post-testicular obstructive azoospermia (OA) is caused by problems with ejaculation caused by some kind of blockage in the reproductive system (Hwang et al., 2018; Saud and Rasool, 2022).

Genetic causes of male infertility are discovered in 15% of instances. These elements can be divided into single-gene mutations and chromosomal abnormalities. (Stouffs et al., 2014). Chromosomal abnormalities are any lack of or re-arrangements of genetic material as unusual at the chromosomal level. This is one of the main genetic reasons why men can't have children. Chromosome issues affect 2% of men with oligospermia and 14% of men with azoospermia, respectively, which is a lot more than the general population (about 0.6%). (Pylyp et al., 2013 and Abed et al., 2023).

Some chromosomal disorders are passed down from parent to child, while others are learned. Klinefelter syndrome is the aneuploid sex chromosome is one of the most frequent hereditary causes of azoospermia. 14% of cases of male infertility are caused by it. Defects in the 47 XYY chromosome can prevent spermatogenesis due to elevated FSH and Y chromosome disomy going wrong (Groth et al., 2013). Due to an increase in FSH, men with Noonan syndrome can lose their hair and have trouble making sperm. 3 percent of people with severe Oligozoospermia have translocations. Robertsonian and bilateral translocations are the most important (Choi et al., 2013). Infertile men are eight times more likely to have an inversion, which happens when a piece of a chromosome is broken off and moved (Neto et al., 2016 and Altaee et al., 2021).

Even though these changes are balanced, they can lead to severe azoospermia in some cases. Zofardi and his colleagues found the role of examining deletions in the long arm of the Y chromosome in six males who were unable to conceive. They called the deletion region an "Azoospermic factor" (AZF). There are three zones in this area: AZFa, AZFb, and AZFc (Pan et al., 2019).

Microdeletions follow the recombination of comparable parts of palindromic sequences Microdeletions on the Y chromosome affect 10% of infertile men, while only 7 percent of

men with low sperm counts have them. Most microdeletions happen in the AZFc region for about 80% of all reported cases. The Sertoli cell phenotype is caused by deletions that include the entire AZFa area. Azoospermia is typically the outcome of intra-AZFb deletion (Pan et al., 2019).

Azoospermia, Sertoli cell syndrome, and Oligozoospermia can all result from deletions in the AZFc area, which are all forms of infertility. Infertility can result from some gene abnormalities that induce pathological disorders. For example, Obstructive azoospermia is seen in 80–90% of those who have "Congenital Bilateral Absence of Vas Deferens (CBAVD)." This issue is caused by a change in the "Cystic Fibrosis Transmembrane Regulator (CFTR)". When eyelashes don't function properly, an autosomal recessive heterogeneous condition known as primary ciliary deficiencies results. About half of all men with Asthenospermia have this problem. So far, not much is known about infertility that does not have a clear cause (azoospermia) (Pan et al., 2019; Yenzeel,2021). In many males with non-obstructive azoospermia, a Y chromosome microdeletion is present, which means they don't make sperm. Many of these cases are unrelated (Michael and Eugene, 2011).

When these deletions were found, the "Azoospermia Factor" (AZF) was proposed as a genetic cause of some cases of infertility. The AZF area has been broken up into three possible regions: AZFa, AZFb, and AZFc (Vogt, 1998). Dazl is needed for the development of all germ cells, and DAZ works in male germ cells like pro-spermatogonia/gonocytes, spermatogonia, spermatocytes, and elongated spermatids. Boule is expressed in meiotic cells in most species but in the germline founders of some invertebrates (Fu et al, 2015).

This study aimed to find the relationship of DAZ1g between deletion with Azoospermia in infertile Iraqi men.

2. Material and methods

2.1 Subjects

One hundred and fifty human blood samples were collected from both fertile and infertile men at different regions in Baghdad governorate/Iraq; including (private medicals Labs and high institute for infertility diagnosis assisted reproductive techniques and Kamal Al-Samara'ay IVF Hospital). The control group (fertile group) consist of 50 sample males aged ranging between 22-51 years old, while the patient (infertile group consists of 100 sample male with ages ranging between 22-51 years old.

2.2 Blood collection

To conduct an RT-qPCR study, samples were obtained from each patient as well as a control group and placed in tubes containing TRIzol TM Reagent.

2.3 Total RNA Extraction with TRIzol

RNA was isolated from the sample according to the procedure of TRIzolTM Reagent as the following:

Sample lysis

0.5 mL of blood and 0.5 mL of TRIzol TM Reagent were added to each tube, and the lysate was homogenized by pipetting up and down numerous times.

Three phase's separation

The lysate was added to each tube and the lid was then sealed with 0.2 mL chloroform. The interphase, colorless, and lower organic phases were separated by centrifuging for 10 minutes at 12,000 rpm. The aqueous phase containing the RNA was put into a new tube.

RNA precipitation

The aqueous phase received 500 microliters of isopropanol, which was introduced and left for 10 minutes before being centrifuged at 12,000 rpm for 10 minutes. A white gel-like pellet of total RNA developed at the tube's bottom. Throw away the excess liquid.

RNA washing

Each tube was filled with 0.5mL of 70% ethanol, vortexed, then centrifuged at 10,000 rpm for 5 minutes. The pellet was aspirated in ethanol and dried by air. Solubility Pellets were rehydrated in 100 l of nuclease-free water and incubated at 55–60 °C for 10-15 minutes.

RNA purity and concentration measurements:

The test has a strong RNA selectivity and is accurate for initial sample concentrations between 10 ng/µL and 100 ng/µL. The test is run at room temperature, and the signal is consistent for three hours.

2.4 Primer Design

DAZ1 and GAPDH cDNA sequences were obtained from the NCBI Gen Bank database. As indicated in Table 1, Using melting temperatures between 60 and 65 degrees Celsius, RT-qPCR primers were made using Premier 3 software, 18 to 23 nucleotide primers, and 75 to 150 base pair PCR amplicons are recommended.

Table 1: Sequence of primers used in the study**.**

Primer Name	Primer Sequence	Product Size
GAPDH-F	5′-GTCTCCTCTGACTTCAA-3′	101 pb
GAPDH-R	5′-ACCACCCTGTTGCTGTA-3′.	
GAPDH-F	(GAAGGTCGGAGTCAACGGATT)	
GAPDH-R	(TGAAGGGGTCATTGATGGCAA)	
DAZ1-F	(CTCCTCCTCCACCACAGTTTC)	92 pb
DAZ1-R	(CTGAGTTACAGGATTCGGCGT)	

2.5 Detection of Gene Expression for *DAZ1* Genes

The total reaction from cDNA synthesis to PCR amplification was done by using the kit of GoTaq® 1-Step RT-qPCR Systems (from Promega, USA), which occurs in a single tube, where convert RNA to cDNA in the one-step method. The procedure was conducted in a reaction volume of 10µl and the RNA total volume to be transcribed reversely was 1µl. A primer designed for *DAZ1* genes in addition to the GADPH (housekeeping gene) which considers an endogenous control gene to normalize mRNA level for *DAZ1* genes.

Quantitative Real-Time PCR (qRT–PCR):

The expression levels of the DAZ1 gene were assessed using reverse transcription-quantitative polymerase chain reaction (qRT-PCR), a sensitive technique for the determination of steady-state mRNA levels. Quantitative real-time PCR with SYBR Green was performed to verify target gene expression.

Primer preparation

TaqMan fluorescent oligonucleotide probes and specific primers for the genes of the study were manufactured by Macrogene Company (Korea). For the preparation of the stock solution at a concentration of 100 pmol/µl, each lyophilized probe and primer was dissolved in

nuclease-free water. After that, the working solution is prepared by adding 10µl of the stock solution to free nuclease water (90µl) and stored at -20 °C until use as shown in Table 2.

Table 2: Component of the reaction of quantitative real-time PCR

Component	20 µl Reaction
qPCR Master Mix	10 µl
Forward primer (10 µM)	1 µl
Reverse primer (10 µM)	1 µl
Template DNA	5 µl
Nuclease-free Water	3 µl

The heat profile as indicated in Table 3 was used to program the cycling protocol for the following optimum cycles.

Table 3: PCR conditions for amplification of DAZ 1 and GAPDH gene expression by RT-qPCR.

Cycle Step	Temperature	Time	Cycles
Enzyme Activation	37	15:00	1
Initial Denaturation	95	05:00	
Denaturation	95	00:20	40
Annealing	60	00:20	
Extension	72	00:30	

Each sample's threshold cycle (CT) was determined using the real-time cycler program. The mean values of each sample were computed after being run in duplicate. - Selected gene expression data were standardized against housekeeping.
The ΔΔCt method by (Kenneth, J. and M. Thomas 2001), as advised, to analyze the data, and the outcomes were expressed as folding changes in gene expression as follows:
The difference between each target gene's CT value and the housekeeping gene's CT value for each sample was computed. "Ct (control) = CT (gene)-CT" (HKG) Ct (patient) = CT (gene) - CT (HKG). For the genes of interest, the variation in Ct values expressed as (Ct) was calculated as follows: Ct = (patient) Ct - Ct (control) To compute the fold-change in gene expression as follows: Fold change=2-ΔΔCt

3. Statistical Analysis

Using the available statistical tool SPSS-28, data analysis was done (Statistical Packages for Social Sciences- version 28).

4. Result and Discussion

All of the samples were taken from people of different ages who have nothing to do with each other. For age and medical condition, the differences between patients and controls were shown in Tables (4 and 5). The age results showed no significant differences, while Diabetes mellitus record significant differences.

Table 4: The age of patients and controls.

		Azoospermia patients		Controls		P value
		No.	100	No.	50	
Age (years)	20---29	16	16.0	10	20.0	0.579
	30---39	50	50.0	27	54.0	
	40---49	34	34.0	13	26.0	
	Mean±SD (Range)	36.3±6.0 (25-48)		34.6±6.2 (22-49)		0.094

Table 5: Medical illness of patient and control.

		Azoospermia patients		Controls		P value
		No	%	No	%	
Smoking	Yes	6	6.0	6	12.0	0.202
	No	94	94.0	44	88.0	
Alcohol drinking	Yes	6	6.0	-	-	0.077
	No	94	94.0	50	100	
Hypertension	Yes	16	16.0	5	10.0	0.318
	No	84	84.0	45	90.0	
Diabetes mellitus	Yes	12	12.0	-	-	0.011*
	No	88	88.0	50	100	
Heart disease	Yes	5	5.0	-	-	0.108
	No	95	95.0	50	100	
Renal failure	Yes	3	3.0	-	-	0.216
	No	97	97.0	50	100	

4.1 Quantitative Expression of *DAZ1*

The results of this study showed that both the patient samples and the control samples had very pure RNA (1.8 to 2.00). All samples were able to have their total RNA extracted. The amount of total RNA in the patient samples ranged from 20 to 30 ng/l, while the amount in the control samples was between 40 and 50 ng/l. A high concentration of total RNA depends on the conditions of the extraction, which must be done in a very clean way. It is well known that TRIzol is used to get the total RNA out of blood (Li et al., 2008).

4.2 cDNA reverse transcription

Reverse transcription of complementary DNA was done on the second day of RNA extraction. Since cDNA for both the DAZ1 gene and the housekeeping gene had to be made, a common primer reaction was used. Later, the effectiveness of qPCR was used to figure out how well cDNA was being concentrated. All of the steps had a perfect yield, which shows that reverse transcription worked well. Based on the Tm given in the manufacturer's instructions for each primer, the following equation was used to figure out the optimal annealing temperature: The melting point (Tm) is equal to 2 (A+T) + 4 (G+C).

The annealing temperature (Ta) ranges from Tm minus 2 to 5 C. The aforementioned equation was also used to determine the melting points of the reverse primer and the forward primer. Comparison of the annealing temperatures for forward and reverse primers led to the identification of the lowest temperature (^{o}C). The housekeeping gene GADPH and the DAZ1 gene expression levels were quantified using the relative quantification method of Polymerase Chain Reaction in Real Time. The amount of gene expression was measured and compared to

the level of a housekeeping gene using the Ct value and folding (2 Ct) technique., as shown in Figures 1, 2, and 3. (1).

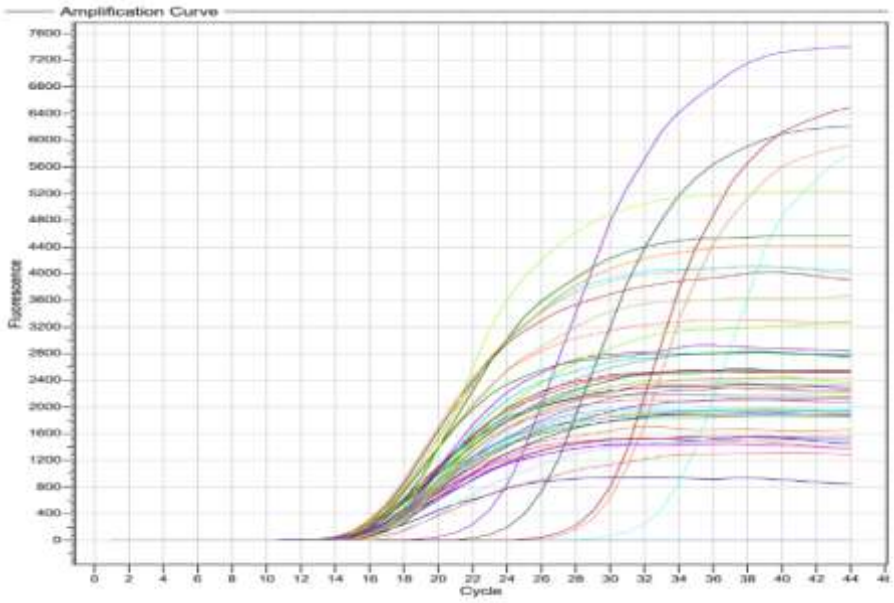

Figure 1: Amplification plots of *DAZ1* gene by RT-qPCR.

Figure 2 shows a typical melt curve for the DAZ1 gene for samples analyzed by RT-PCR.

The amplicons were seen to have a single peak. The melt curve showed that each sample had a single, pure amplicon, and the intercalating dye assay was thought to have a high level of specificity for amplification.

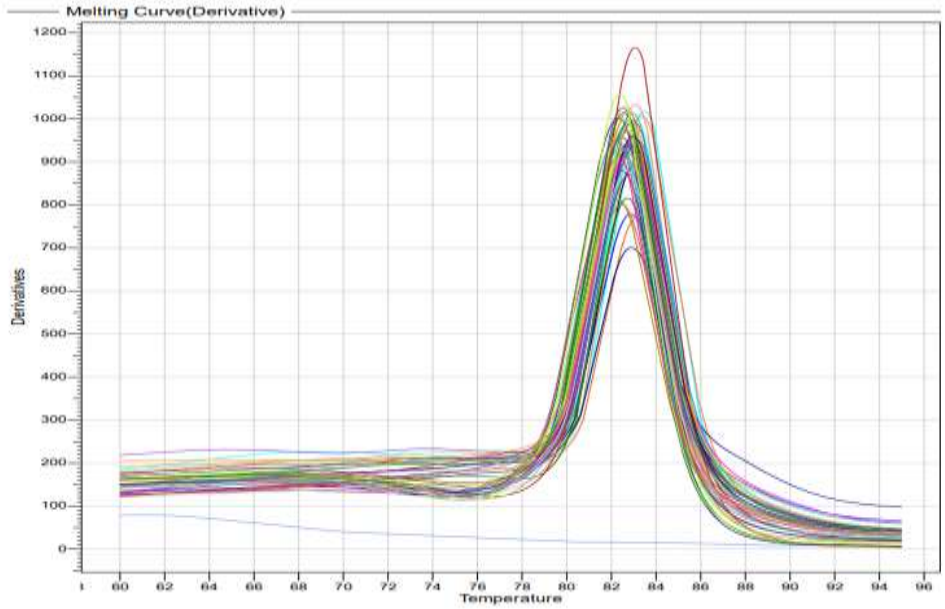

Figure 2: The melt curve of *DAZ* I gene amplicons after RT-qPCR analysis.

4.3 Expression level of *DAZ* I gene in the studied groups

The ΔCt of the *DAZ1* gene in blood samples of azoospermia groups (was 0.12±0.91), while the control group (was 1.45±0.25). There was a significant ($p < 0.05$) decrease in Δthe

Ct mean of patient groups compared to the corresponding ΔCt t means in the control group as shown in Table (6).

Table 6: The studied groups' expression level ΔCt of DAZ I.

	Patients	Controls	P value
ΔCt	0.12±0.91	1.45±0.25	0.0001#

The folding of expression $2^{-\Delta\Delta Ct}$ of the *DAZ1* gene in azoospermia groups, was (0.39±0.21), which indicates upregulation of this gene in infertile Iraqi men.
Gene expression of DAZ I was very different between azoospermia patients and controls.

This is in line with what other studies have found. More than half of all cases of infertility are caused by problems with sperm production. We don't know much about how this disease starts. Molecular studies have found that male infertility is caused by several etiopathogenetic factors, such as microdeletions of the long arm of the Y chromosome (Yq). Chromosome deletions are becoming a major genetic cause of male infertility, and Y chromosome microdeletions are more likely to happen in cases of ~~the~~ severe spermatogenic defect (Suganthi et al. 2014). In fResearchwed that the number of Y chromosome microdeletions was different in different parts of the world. We looked at how often Y happened. Chromosome microdeletions in Azoospermic infertile males Al-Janabi et al., 2020) and compared it with the rlts of this study. In this study, we looked at 100 males with azoospermia to see if our results were similar to those of the other studies. The number of AZF microdeletions was high. In fact, the higTheAZF microdeletions in this study is similar to what has been found in other studies of the Iraqi population. Khalaf et al. (2012) found that 65 percent of the Azoospermic group had a high frequency of Yq microdeletions. Hanoon et al. (2017) found that Yq microdeletions were very common (65 percent). In two more studies, which were done by Ghorbel et al (2012a,b). In the azoospermic group (1.3%), the frequency was lower. The results of our study on the Iraqi population showed that people with azoospermia had a much higher rate of Y chromosome microdeletions than people in other nearby countries. But this rate is higher than what has been seen in Turkey (3.3 and 6 percent) (Sargin et al. 2004; Cavkaytar et al. 2012), Kuwait (7.75 percent) (Alkhalaf and Al-Shoumer 2010), Iran (1.74 percent) (Saliminejad et al. 2012), and Egypt (36.7 percent) (Elhawary et al. 2010). In the end, we came to the conclusion thconcludedeletion is linked to azoospermia in men who can't have children. This means that Iraqi men with azoospermia should get a DAZ I marker test.

The ages of the men in this study were close to each other. The fact that there wasn't a difference between the age groups doesn't mean that there isn't an effect of age on male infertility; it just means that the age groups were different from each other. The same thing was said by Elbashir et al. (2018), who found that this age did not affect whether or not a woman was fertile. This study agrees with Samawi's 2019 Table (7)

Table 7: The relationship between gene expressions fold of *DAZ I* gene with age.

Azoospermia patients		The fold of expression of *DAZ I* gene		P value
		No	Mean±SD	
Age (years)	20---29	16	0.382±0.265	0.819
	30---39	50	0.406±0.236	
	40---49	34	0.377±0.148	

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