Iraqi Journal of Biotechnology, April 2020, Vol. 19, No. 1, 56-68

The Association of Mismatch Repair (*MLH1*) Gene Polymorphism with The Risk of Oligozoospermia in Iraqi Patients

Zaianab A. Kadhim , Ismail A. Abdul-Hassan

Institute of Genetic Engineering and Biotechnology / University of Baghdad / Baghdad / Iraq

Received: March 10, 2020 / **Accepted:** April 26, 2020 / **Published:** April 28, 2020

Abstract: Oligozoospermia is a type of male infertility in which the number of sperms is under 15 million/ml, it is caused by different factors but it is mostly associated with idiopathic disorders. MLH1 is a part of a genes system which codes for proteins responsible mainly for correcting post replicative DNA errors.rs1800734 is a single nucleotide polymorphism lies in the MLH1 CpG island at -93 from the translation start site while rs4647269 is an intron variant that lies in intron 9 .). Blood and semen samples were collected from Baghdad Specialist Fertility Centre through the period from 1st of August 2018 until the end of February 2019 and the study was carried out in the Laboratories of Institute of Genetic Engineering and Biotechnology for Postgraduate Studies - University of Baghdad .Genotyping of *MLH1* gene for SNPs rs1800743 and rs4647269 was done using Taqman genotyping assay by RT-PCR. Hormonal analysis was performed by using fluorescence immunoassay (FIA) on AFIAS autoanalyzer .The study showed that There were no significant differences between healthy and oligozoospermia patients in any of the genotypes of the SNP rs1800734, hence the SNP have no connection to the infertility incidence. As for rs4647269, The results showed that the CC genotype of rs4647269 was significantly higher ($p<0.05$) in healthy patient (15(30%) *versus* 9 (18%) in oligozoospermia patient contrary to TT genotype that was significantly higher in oligozoospermia patients15 (30%) *versus* 7(14%).The heterozygous genotype CT had no significance between the two groups.Seminal fluid analysis showed a significant($p<0.05$) decrease in sperm count (9.65 ± 0.89) ,motility(24.34 ± 2.072) ,activity8.06 ± 1.15 and normal sperms(4.60 ± 0.66) in the oligozoospermia patients .In oligozoospermia patients, mutant genotype TT of the SNP rs4647269showed a significance increase ($P<0.05$), compared to the wild genotype CC. Effect of SNP rs4647269 on FSH appear in the wild and heterozygous genotypes as an increase in oligozoospermia patients compared to the healthy ones.In rs4647269 there was a significant increase ($P<0.05$) in prolactin in both the wild and the mutant genotypes of the oligozoospermia patients compared to the control, only the heterozygous resulted in a significant ($P<0.05$) decrease in the oligozoospermia patients.only the wild genotype CC showed a significant decrease ($P<0.05$) in testosterone concentration of oligozoospermia patients.the results of rs1800734 showed that the only genotype that showed a significant difference in LH levels between oligozoospermia patients and control is GA genotype ($P<0.05$). FSH results were significantly higher ($P<0.01$) in oligozoospermia patients of the genotype GG than control of the same genotype.. The results show a significant increase ($P<0.01$) in PRL levels that accompanied the GG genotype in oligozoospermia patient. While the mutant genotype AA resulted in a significant decrease ($P<0.05$) in oligozoospermia patients. Only GG genotypes (the wild genotypes) showed a significant decrease ($P<0.05$) in testosterone concentration of oligozoospermia patients . This study have observed 4 possible haplotypes, GC hablotype was the most common in both groups (patients and controls) and it was significantly higher($p<0.05$) in control. Also the GT hablotype was higher($p<0.05$) in oligozoospermia patients than healthy controls. 5 of 8 possible haplotype combinations showed no statistically significant differences between oligozoospermia patients and control groups.This study concluded that 1. Both rs1800734 and rs4647269 SNPs of MLH1 gene have no role in the incidence of oligozoospermia in Iraqi men.

Keywoeds: Infertility, Oligozoospermia, Polymorphism, Hormones, *MLH1*.

Corresponding author: (Email: abdulsalamzainab842@gmail.com).

Introduction

Infertility is considered a complicated multifactorial disease that has been attracting a growing interest as the semen quality seems to deteriorate in young healthy men around the world (1). Several factors are associated with male infertility including both acquired factors and conditions of birth origin such as varicocele,infections,hormonal defffects, genetic defects, lifestyle and environmental causes (2).Investigation of male infertility is based on a thorough medical history, physical examination and at least two semen analyses (3) and yet infertility causality for half of the patients that finish the formal exams remain unknown(4) Testis is the site of elevated expression for 2200 genes across all human tissues which makes them extremely sensitive to genetic disruptions and several studies suggest a fundamental link between infertility and genetic factors but the exact architect that construct this relationship is not clear (5) and the number of genes that are conclusively linked to infertility by an obvious relationship are still low which is surprising considering the fast advancement in genetics techniques (6). Oligzoopermia is A type of male infertility in which the number of sperms is under 15 million/ml, its caused by different factors but it's mostly associated with idiopathic disorders (7). Mismatch repair system (MMR) is a group of proteins responsible for correcting post replicative DNA errors. MMR remove DNA mismatches that result of recombination between imperfectly matched sequences or from errors during DNA replication. It can also repair the damage of oxidative stress (8). MMR is studied profusely as a possible cause for cancers especially that Defective MMR increases mutation rates up to 1,000-fold which increase chances of malignancy(9). Several studies targeted MMR components as a possible suspect in male infertility and some of them were linked directly, for instance a *MLH3* gene variant was proved to negatively affect semen parameter, also another variant of the same gene increase female infertility risk(10 ; 11) .*MLH1* is an MMR element that's deficiency is found to cause sterility in Zebrafish by causing an arrest in spermatogenesis at metaphase I (12).Same observations were find in both genders of *MLH1*-deficient mice(13;14). The effect of the gene is less understood in human,in fact the entire system effect is still to be clarified (15). The *MLH1* gene is located on the short arm of the third chromosome with a length of 57375 kb and 19 exons that encodes a protein with 756 amino acids (16).*MLH1* is mostly a target for malignancy studies as its implicated in different type of cancers such as tumors of stomach, ovaries ,ureter, renal pelvis, brain, small bowel, hepatobiliary tract and its proved that most lynch syndrome patients are carries of mutations in *MLH1* and *MSH2*(17). SNP is a point mutation were one base is substituted with another (18). As a Multifactorial disease infertility was studied extensively as a target for SNPs researches yet most researches weren’t replicated properly for confirmation (19). A number of genes have been examined for possible effect of SNPs on male infertility including

eNOS SNPs which was associated with oligoasthenoteratozoospermic and asthenozoospermic cases in different countries(20). MLH3 SNP rs175080 also was linked to oligozoospermia in Caucasian men (21)This study aim to determined genetic polymorphism of MLH1 gene in blood of Iraqi patients with oligozoospermia and fertile patients and specifically two SNPs which arers1800743 and rs4647269.

Material and Methods

Patients and Samples

Samples were collected in Baghdad fertility center for assisted reproduction. Subjects were selected according to diagnosis of a medical expert who would recognize healthy and oligozoospermia patients and send them for Blood and seminal fluid collection. The subjects were divided into two groups (50) apparently healthy and (50) oligozoospermia cases. Seminal fluid was conducted according to WHO standards (22)and blood was collected and into EDTA tubes for DNA extraction.DNA extraction was done by the gSYNC DNA extraction Kit. After genomic DNA extraction, agarose gel electrophoresis has been adopted to underline the presence and integrity of the extracted DNA.

Genotyping (Allelic Discrimination Principle)

The TaqMan technique depends on the exonuclease activity of *taq* polymerase enzyme . The probe contains a reporter dyes (FAM and VIC) at the 5´ end of the probe and a quencher dye (MGB) at the 3´ end of the probe. the quencher suppress the dye when they are close to each on the probe. If the reaction took place then the probe will be attached to a specific amplified sequence leading to cleavage of the probe which would separates the reporter dye and the quencher dye, resulting in increased fluorescence of the reporter. Accumulation of PCR products is detected directly by monitoring the increase in fluorescence of the reporter dye.The increase in fluorescence signal is detected specifically when the target sequence is complementary to the probe and if it is amplified during PCR.

Primer and Probes

TaqMan fluorescent oligonucleotide probes and primers sequences were manufactured by Alpha DNA Ltd (Canada) and stored lyophilized at (-23°C). The sequences of each of the probes and primers used in the experiments are shown in table (1). Primers and probes were spin down prior to opening the tube and dissolved to 100 µM according to instruction of the manufacturer ,later a working solution was prepared by adding 10µl of stock solution to 90 µl of nucleic acid free water.both work and stock solution were stored at –23°C. The wild type detecting probe was labeled with FAM in the 5′ end and Minor groove binder probe (MGB) in the 3′ end. While the probe prepared for the mutant allele (SNP) was labeled with VIC in the 5′ end and MGB in the 3′ end.

Table (1): Primers and probes.

Primer/probe	(Sequence (5′ →3′ direction
***MLH1* gene (rs1800734G>A)**	
Forword	TGTCCAATCAATAGCTGCCG
Reverse	GCCAGAAGAGCCAAGGAAAC
prob -FAM	TACAGCTGAAGGAAGAACGTGAG
probe-VIC	AGCTAAAGGAAGAACGTGAG
***MLH1* gene (rs4647269C>T)**	
Forword	GGTCAGCACTCAGAGGATGT
Reverse	GAGGCTGGTTTGAAGAGGGT
prob -FAM	GATTTGACGTATCCTTGTCTACAGC
probe-VIC	GATGTATCCTTGTCTACAGCA

Real Time PCR Protocols

DNA samples from patients (n=50) and healthy subjects (n=50) were genotyped for SNPs with a Taqman SNP genotyping assay using real time thermacycler,the final volume of the reaction mix was 20 µl. components of the reaction mix is shown in table (2) while the operating program of the thermacycler is shown in Table (3).

Table (2): Components of PCR reaction mix.

No.	Components	(lµ) Volume	
1	GOtaq probe PCR master mix (promega)	10	
2	Forward primer	lµ 10	0.5
3	Reverse primer	lµ 10	
4	probe -FAM	lµ 10	
5	probe-VIC	lµ 10	
6	Template DNA	4	
7	Water , RNase free	5.5	
Final Volume		20	

Table (3) Real time PCR program program

Cycle step		(Temp. (°C	Time	Cycle
Hold1		50	15min.	1
Hold2		95	15min.	
Cycling 1	**Denaturation**	95	5sec.	5
	Annealing	60	20sec.	
	Extension	72	15sec.	
Cycling 2	**Denaturation**	95	5sec.	40
	Annealing*	60	20sec.	
	Extension	72	15sec.	

*** In this step the acquiring Green and Yellow (FAM and VIC) were added**

Hormonal Assay

Hormonal analysis was performed by using fluorescence immunoassay (FIA) on AFIAS autoanalyzer (boditech med incorporated,Korea).the test uses a sandwich immunodetection method. The antibody in buffer binds to the antigen in sample forming antigen antibody complexes which migrate onto nitrocellulose matrix to be captured by another immobilized antibody on the strip. The quantity of antigens in the sample will be represented as a fluorescence signal on detector antibody. The kit consist of cartridge and a pipette tip .the test was done by pippeting 200 µL into the sample pit on

the cartridge and placing the tip into its position ,also the position and the label of the test was set the test finished within 30 minutes the normal range of each test is in table(4).

Statistical Analysis

The Statistical Analysis System-SAS (2012) program was used to affect different factors in study parameters. Chi-square test was used to significant comparison between percentage and Least significant difference –LSD test was used to significant comparison between means in this study (24).

Results and Discussion

This study examined two *MLH1* polymorphism (rs1800734 G>A and rs4647269 C>T) among Iraqi men with oligozoospermia and in apparently healthy controls according to the results achieved by RT-PCR using TaqMan technique in addition to the effect of each SNP on the hormonal profile.Also the seminal parameter of both oligozoospermia and healthy groups were compared.

Seminal Fluid Analysis

Seminal fluid Parameter of control and patients are clarified in table (4), the table include age, volume PH, count, motility, activity and the percentage of normal forms of sperms. Statistically the control and patient group shared a similar age average which would allow eliminating age as a possible variable concerning the semen parameters.The seminal PH showed no significant difference among the two groups which is the same result obtained by both of (25,26) when patients with normal parameters and abnormal ones were compared. This result confirm that PH is not a an influential factor unless in excessive increase or decrease in PH which is what (27) concluded when culturing sperms in growth media with different PH as motility of sperms was affected in PH of 5.2 and 6.2 solutions.The volume of seminal fluid was very convergent in both study groups which contradict what was found by both (28) however volume has no relation to pregnancy outcome and it's not an indicative of infertility unless it's far from average (29 ,30) who studied over 1600 cases in their studies. Spermatozoa count was significantly higher in control group than in oligozoospermic patients group (50.34 *versus* 9.65 million per ml respectively, $p<0.01$).The sperm count of control group are consistent with the normal limits that are specified by(22). There is a global growing concern about decreasing numbers of sperms in semen of healthy fertile men, recent studies showed a drastic drop in numbers since last century fifties from 99 million/ml to 47 million /ml (31),the total count in this study could be an indicator of a similar drop that need to be addressed in the country. The percentages of sperm motility were significantly ($p<0.01$) higher in control than in severe oligozoospermic patients (57.77 *versus* 24.34) respectively. Percentage of active sperms were significantly ($p<0.05$) higher in control than in severe oligozoospermic patients (20.87*versus* 8.06) however, the activity in oligospermic cases are within normal range stated by (22). The percentage of normal sperm were significantly ($p<0.01$) higher in control than in severe oligozoospermic patients (79.73 *versus* 4.6) but the normal forms of oligospermic cases are still within normal range which is 4%. The percentage of abnormal sperm were significantly ($p<0.01$) lower in control

than in server oligozoospermic patients (20.37 *versus* 95.4%) respectively. Low levels of motility and activity and morphology were also cofound with low sperm count (oligospermia) in a number of previous studies (32 ,33).this combined effect on all parameters could be a result in an error in control mechanism of spermatogenesis(34).

Table (4): Comparison between control and patients in seminal parameter

Seminal parameter	Mean ± SE		P-value
	Control No=50	Patients No=50	
Age (year)	36.10 ± 1.03	36.02 ± 0.99	0.761 NS
Volume ( )	2.34 ± 0.09	2.22 ± 0.10	0.187 NS
pH	7.21 ± 1.04	7.10 ± 0.09	0.834 NS
Count ( )	50.34 ± 2.01	9.65 ± 0.89	0.0026 **
Motility (%)	57.77 ± 1.89	24.34 ± 2.072	0.0085 **
Active ( )	20.87 ± 1.41	8.06 ± 1.15	0.0461 *
Nor. Sperm (%)	79.73 ± 1.34	4.60 ± 0.66	0.0001 **
Abo. Sperm (%)	20.37 ± 3.49	95.40 ± 0.61	0.0001 **
*** (P<0.05), ** (P<0.01).**			

Polymorphism

rs4647269 SNP

The SNP rs4647269 is an intron variant as it lies in intron9 (35), In this study the heterozygous form of the SNP (CT) was the most common as 54 individual had these genotype unlike (36) who found that the wild genotype CC was the most common genotype, of course this difference as due to difference in the population in the study . Wild genotype (CC) was found in 24 individuals and 22 had the mutant genotype (TT). Frequencies of C and T alleles were (0.58 and 0.42) in apparently healthy subjects and (0.42 and 0.56) in oligozoospermia patients, respectively.The CC genotype was significantly higher ($p<0.05$) in healthy patient (15(30%) verses 9 (18%) in oligozoospermia patients on the contrary to TT genotype that was significantly higher in oligozoospermia patients .The heterozygous genotype CT had no significance between the two groups. this result suggest a possible connection for the mutant genotype of rs4647269 to oligozoospermia, this result agree with what (37) found, also (36) found that this SNP could be a risk factor for developing azoospermia and oligozoospermia .however due to the low numbers of the samples of mutant and wild genotypes its seems that further research is in need to confirm or deny the results.

Table (5): The frequency of genotypes and alleles at rs4647269 SNP of *MLH1* gene in Iraqi men with oligozoospermia and control.

Genotypes	(%)Frequency, n		$^2\chi$	*OR*	*CI*
	Control	Patients			
CC	15(%30)	9(%18)	4.623 *	0.766	0.86-1.62
CT	28(%56)	26(%52)	1.065 NS	0.183	0.78-1.51
TT	7(%14)	15(%30)	5.439 *	0.902	0.86-1.67
CT+TT	35(%70)	42(%82)	4.428 *	0.715	0.92-1.64
Alleles frequencies					
C	0.58	0.44	--	--	--
T	0.42	0.56	--	--	--
*** (P<0.05), ** (P<0.01).**					

rs1800734 SNP

In this study the heterozygous form of the SNP (GA) was the most common as 89 individual out of 100 had this genotype .while only two individuals had the wild genotype (GG) and 9 had the mutant genotype (AA).The frequency of the A allele was between (52-55)%. Which is close to the percentage found by (38) in turkey patients which was 49.4%. In general the A allele frequency ranges between 54% to 65% in Asian populations studies according to an analysis conducted by (39).There were no significant differences between healthy and oligozoospermia patients in any of the genotypes of the SNP, hence the SNP have no connection to the infertility incidence in this study, this result conflict with what (40.41) stated.

Table (6): The frequency of genotypes and alleles at rs1800734 SNP of *MLH1* gene in Iraqi men with oligozoospermia and control

Genotypes	(%)Frequency, n		$^2\chi$	*OR*	*CI*
	Control	Patients			
GG	1(2%)	1 (2%)	0.00	-	-
GA	46 (92%)	43 (86%)	2.063 NS	0.271	0.72-1.55
AA	3(6%)	6 (12%)	2.063 NS	0.271	0.75-1.59
GA+AA	49 (98%)	49 (98%)	0.00	-	-
Alleles frequencies					
G	0.48	0.45	--	--	--
A	0.52	0.55	--	--	--
*** (P<0.05), ** (P<0.01).**					

Hormonal profile

There was no significant Effect of the SNP 4647269 genotypes on LH levels except for the fact that is in oligozoospermia patients mutant genotype TT showed a significance increase (P<0.05), compared to the wild genotype CC. Some studies do support of the negative effect of chromosomal abnormalities on LH levels by causing an elevation that cause disruption in male infertility(42). Effect of SNP rs4647269 on FSH appear in the wild and heterozygous genotypes as an increase in oligozoospermia patients compared to the healthy ones. Elevated FSH levels could be a sign of reduced spermatogenesis in 60% of infertility cases (43), however; oligozoospermia patients could show normal FSH levels but accompanied by hypospermatogenesis sometimes associated to partial spermatidic arrest (40).In the healthy group the genotype TT showed a significant decrease (P<0.01) compared to CT and CC of the same group .though these differences seems minor compared to results of studies reveling differences in oligozoospermia patients since concentration in control was 4.11 mIU/ml while oligozoospermia patient FSH concentrations were up to 32.81 mIU/ml(44). In the SNP rs4647269 there was a significant increase (P<0.05) in prolactin in both the wild and the mutant genotypes of the oligozoospermia patients compared to the control, only the heterozygous resulted in a significant (P<0.05) decrease in the oligozoospermia patients. It was showed that high level of prolactin is associated with different infertile conditions like oligozoospermia, asthenozoospermia and azoospermia (45) while (21) couldn't find any abnormality in

prolactin in infertile males of his study not to mention that the concentrations of prolactin. The genotype CT showed a significant difference compared to the TT genotype in the healthy group (P<0.05), while in the oligozoospermia patients group the genotype CT resulted in a significant decrease compared to CC and TT. PRL role in male reproduction seems to be modifying and participating rather than a main contributor,it modifies steroidgenesis and gonadotropins by modulating the FSH and LH receptors on Sertoli and Leydig cells,,it may also influence the normal activity of glands like the prostate(46) It remains to be determined whether autocrine PRL plays a significant role in the growth, differentiation, and secretory activity of the prostate gland. Cleaved form of prolactin has been detected in sperm and its supposedly play a role in spermiogenesis and spermatogenesis (47). Only the wild genotype CC showed a significant decrease (P<0.05) in testosterone concentration of oligozoospermia patients. (48) found a significant decrease in testosterone of oligozoospermia patient with chromosomal anomalies compared to healthy males but the decrease was similar to oligozoospermia patient without anomaly while (49) couldn't report differences in testosterone levels between oligozoospermia patients and control or differences among genotypes of rs2274911 of GPRC6A gene.

Table (7): Relationship between genotype of SNP rs4647269 of *MLH1* and hormones in control and oligozoospermia patients.

SNPs	Genotypes	(LH(mIU/ ml		*p- value*
		Control	Patients	
rs4647269 C>T	CC	3.8 ± 0.09 (7)	3.3 ± 0.12 (7)	0.092 NS
	CT	3.8 ± 0.12 (7)	4.0 ± 0.19 (7)	0.654 NS
	TT	4.1 ± 0.17 (7)	4.4 ± 0.21 (7)	0.1552 NS
	p- value	0.702 NS	0.0415 *	---
SNPs	Genotypes	FSH(mIU/ ml)		*p- value*
		Control	Patients	
rs4647269 C>T	CC	5.9 ± 0.24 (7)	5.2 ± 0.16 (7)	0.0841 NS
	CT	4.2 ± 0.19 (7)	5.2 ± 0.24 (7)	0.0362 *
	TT	2.6 ± 0.08 (7)	4.9 ± 0.13 (7)	0.0033 *
	p- value	0.0028 **	0.572 NS	---
SNPs	Genotypes	Prolactin		*p- value*
		Control	Patients	
rs4647269 C>T	CC	12.0 ± 0.53 (7)	15.5 ± 0.24 (7)	0.0463 *
	CT	14.7 ± 0.61 (7)	11.9 ± 0.69 (7)	0.0494 *
	TT	11.6 ± 0.47 (7)	16.5 ± 0.58 (7)	0.0217 *
	p- value	0.0428 *	0.0105 **	---
SNPs	Genotypes	Testosterone		*p- value*
		Control	Patients	
rs4647269 C>T	CC	3.8 ± 0.26 (7)	2.6 ± 0.07 (7)	0.0451 *
	CT	3.3 ± 0.16 (7)	2.7 ± 0.11 (7)	0.0831 NS
	TT	3.3 ± 0.20 (7)	3.2 ± 0.09 (7)	0.712 NS
	p- value	0.1662 NS	0.0793 NS	---
* (P<0.05), NS: Non-Significant.				

LH stimulates Leydig cells in the interstitial of the testes to produce testosterone from cholesterol (50) the only genotype that showed a significant difference between oligozoospermia patients and control is

GA genotype of rs1800734 (P<0.05) (51). Reported an elevation of LH in oligoasthenoteratozoospermia compared to control. GA genotype of control group was accompanied by a significant decrease of LH (P<0.05) compared to GG genotype. FSH results were significantly higher (P<0.01) in oligozoospermia patients of the genotype GG than control of the same genotype.The genotype GG of oligpzoospermia patient also showed a significant increase (P<0.01) compared to GA and AA of the same group. The results show a significant increase (P<0.01) in PRL levels that accompanied the GG genotype in oligozoospermia patient. While the mutant genotype AA resulted in a significant decrease (P<0.05) in oligozoospermia patients. Healthy individual that carry the genotype GG had a significant decrease (P<0.01) compared to the heterozygous and mutant genotype, as for the oligozoospermia patients it was the heterozygous GA genotype that had the significant difference (P<0.01) with concentration of (17.3 ± 0.52). Only GG genotypes (the wild genotypes) showed a significant decrease (P<0.05) in testosterone concentration of oligozoospermia patients. the heterozygous and mutant genotypes of both SNPs didn't show any significant difference between control ant patients .the wild GG genotype was significantly higher compared to the homozygous genotype GA. in the oligozoospermia group the GG genotype decreased(P<0.05) significantly compared to the GA and AA genotypes.

Table (8): Relationship between genotype of SNP rs1800734 of *MLH1* and hormones in control and oilgozoospermia patients.

SNPs	Genotypes	(LH(mIU/ ml		*p- value*
		Control	Patients	
rs1800734G > A	GG	5.39 ± 0.00 (1)	4.78 ± 0.00 (1)	0.1083 NS
	GA	3.40 ± 0.18 (7)	4.90 ± 0.22(7)	0.0362 *
	AA	4.11 ± 0.19 (3)	4.30 ± 0.15(6)	0.677 NS
	p- value	0.0288 *	0.0955 NS	---
SNPs	Genotypes	(FSH(mIU/ ml		*p- value*
		Control	Patients	
rs1800734G > A	GG	3.9 ± 0.00 (1)	11.5 ± 0.27 (1)	0.0002 **
	GA	3.8 ± 0.15 (7)	4.0 ± 0.12 (7)	0.673 NS
	AA	4.3 ± 0.10 (3)	4.3 ± 0.15 (6)	0.988 NS
	p- value	0.439 NS	0.0001 **	---
SNPs	Genotypes	Prolactin		p- value
		Control	Patients	
rs1800734G > A	GG	3.69 ± 0.00 (1)	12.95 ± 0.00 (1)	0.0001 **
	GA	16.4 ± 0.73 (7)	17.3 ± 0.52 (7)	0.693 NS
	AA	14.5 ± 0.42 (3)	10.9 ± 0.33 (6)	0.0327 *
	p- value	0.0001 **	0.0003 **	---
SNPs	Genotypes	Testosterone		value -*p*
		Control	Patients	
rs1800734G > A	GG	3.8 ± 0.00 (1)	2.4 ± 0.07 (1)	0.0359 *
	GA	3.1± 0.11 (7)	3.09 ± 0.16 (7)	0.8449 NS
	AA	3.4 ± 0.09 (3)	3.3 ± 0.08 (6)	0.703 NS
	p- value	0.0446 *	0.0352 *	---
*** (P<0.05), ** (P<0.01), NS: Non-Significant.**				

Hablotype

The results of haplotype frequency of rs1800734 and rs4647269 SNPs of *MLH*1 gene in Iraqi men with oligozoospermia and controls are shown in table (9). This study have observed 4 possible haplotypes, Both AC and AT hablotype showed no significant difference between the groups. GC hablotype was the most common in both groups (patients and controls) and it was significantly higher($p<0.05$) in control. Also the GT hablotype was higher($p<0.05$) in oligozoospermia patients than healthy controls.

Table (9): The frequency of hablotypes of rs1800734 and rs4647269 SNPs of *MLH1* gene in Iraqi men with oligozoospermia and controls.

Haplotypes	(%)Frequency, n		$^2\chi$	*OR*	*CI*
	Control	patients			
GC	39 (78%)	31 (62%)	5.421 *	0.918	0.82-1.66
GT	8 (16%)	13 (26%)	4.392 *	0.674	0.88-1.61
AC	3 (6%)	5 (10%)	1.028 NS	0.093	0.59-1.48
AT	0 (0%)	1 (2%)	0.074 NS	0.033	0.60-1.41
Total	50 (100%)	50 (100%)	---	---	---

Hablotype combination

The results of haplotype recombination of rs1800734 and rs4647269 SNPs of *MLH*1 gene in Iraqi men with oligozoospermia and controls are shown in table (10). GC / AC and GC/ AT combination showed a significant difference between oligozoospermic and control groups ($P<0.05$), the combination GT/AT is significantly higher ($P<0.05$) in oligozoospermia patients. Also, as shown in the table (10), 5 of 8 possible haplotype combinations showed no statistically significant differences between oligozoospermia patients and control groups and GC/ AT hablotype combinations was the most common.

Table (10): The frequency of hablotypes combinations of rs1800734 and rs4647269 SNPs of *MLH1* gene in Iraqi men with oligozoospermia and controls.

Haplotype combinations	(%)Frequency, n		$^2\chi$	*OR*	*CI*
	[1]Control	[2]PCOS			
GC / AC	14 (28%)	8 (16%)	4.743 *	0.792	0.87-1.70
GC/ AT	26 (52%)	22 (44%)	4.277 *	0.635	0.82-1.63
GT / AT	6 (12%)	13 (26%)	5.029 *	0.863	0.87-1.64
AC / AT	3 (6%)	4 (8%)	0.438 NS	0.039	0.59-1.58
GT / GT	1 (2%)	0 (0%)	0.438 NS	0.039	0.57-1.58
GC / GT	0 (0%)	1 (2%)	0.438 NS	0.039	0.57-1.58
AC / AC	0 (0%)	1 (2%)	0.438 NS	0.039	0.57-1.58
AT / AT	0 (0%)	1 (2%)	0.438 NS	0.039	0.57-1.58
Total	50 (100%)	50 (100%)	---	---	---

References

1. Mou, L. and Xie, N. (2016). Male infertility-related molecules involved in sperm-oocyte fusion. *J Reprod Dev*., 63 (1):1-7.

2. Panner Selvam, M.; Agarwal, A.; Pushparaj, P.; Baskaran, S. and Bendou, H. (2019). Sperm Proteome Analysis and Identification of Fertility-Associated Biomarkers in

Unexplained Male Infertility. *Genes*, 10(7): 522.
3. Bieniek, J.; Drabovich, A. and Lo, K. (2016). Seminal biomarkers for the evaluation of male infertility. *Asian J. Androl*, 18(3): 426-433.
4. Anderson, R.; Hanson, H.; Patel, D.; Johnstone, E.; Aston, K.; Carrell, D., *et al*. (2016). Cancer risk in first-and second-degree relatives of men with poor semen quality. *Fertil Steril*., 106(3): 731-738.
5. Nagirnaja, L.; Aston, K. and Conrad, D. (2018). Genetic intersection of male infertility and cancer. *Fertil steril*, 109(1): 20-26.
6. Oud, M.; Volozonoka, L.; Smits, R.; Vissers, L.; Ramos, L. and Veltman, J. (2019). A systematic review and standardized clinical validity assessment of male infertility genes. *Hum Reprod*, 34(5): 932-941.
7. McLachlan, R. (2013). Approach to the patient with oligozoospermia. *JCEM*, 98(3): 873-880.
8. Anaís García-Rodríguez, A.; Gosálvez, J.; Agarwal, A.; Roy, R. and Johnston, S. (2019). DNA damage and repair in human reproductive cells. *Int. J. Mol Sci*., 20(1): 31.
9. Gao, D.; Herman, J. and Guo, M. (2016). The clinical value of aberrant epigenetic changes of DNA damage repair genes in human cancer. *Oncotarget*, 7(24): 37331-37346.
10. Anifandis, G.; Markandona, O.; Dafopoulos, K.; Messini, C.; Tsezou, A.; Dimitraki, M., *et al*. (2017). Embryological results of couples undergoing ICSI-ET treatments with males carrying the single nucleotide polymorphism rs175080 of the *MLH*3 gene. *Int. J. Mol. Sci*., 18(2): 314.
11. Pashaiefar, H., Sheikhha, M., Kalantar, S., Jahaninejad, T., Zaimy, M. & Ghasemi, N. (2013). Analysis of *MLH*3 C2531T polymorphism in Iranian women with unexplained infertility. *Iran J reprod med*, 11(1), 19
12. Feitsma, H.; Leal, M.; Moens, P.; Cuppen, E. and Schulz, R. (2007). *MLH1* deficiency in zebrafish results in male sterility and aneuploid as well as triploid progeny in females. *Genetics*, 175(4): 1561-1569.
13. Edelmann, W.; Cohen, P.; Kane, M.; Lau, K.; Morrow, B.; Bennett, S., *et al*. (1996). Meiotic pachytene arrest in *MLH*1-deficient mice. *Cell*, 85(7): 1125-1134.
14. Eaker, S.; Cobb, J.; Pyle, A. and Handel, M. (2002). Meiotic prophase abnormalities and metaphase cell death in *MLH*1-deficient mouse spermatocytes: insights into regulation of spermatogenic progress. *Dev. biol*., 249(1): 85-95.
15. Mukherjee, S.; Weedin, J.; Addai, J.; Lipshultz, L. and Lamb, D. (2010). Mutations in the MSH5 mismatch repair gene in non-obstructive azoospermic men (NOA). *Fertil Steril*, 94(4): S61.
16. Nassiri, M.; Kooshyar, M.; Roudbar, Z.; Mahdavi, M. and Doosti, M. (2013). Genes and SNPs associated with non-hereditary and hereditary colorectal cancer. *Asian Pac. J. Cancer Prev*., 14(10): 5609-5614.
17. Dowty, J.; Win, A.; Buchanan, D.; Lindor, N.; Macrae, F.; Clendenning, M., *et al*. (2013). Cancer Risks for *MLH* 1 and MSH 2 Mutation Carriers. *Hum. mutat*, 34(3): 490-497.
18. Seal, A.; Gupta, A.; Mahalaxmi, M.; Aykkal, R.; Singh, T. and Arunachalam, V. (2014). Tools, resources and databases for SNPs and indels in sequences: a review. *IJBRA*, 10(3): 264-296.
19. Hotaling, J. and Carrell, D. (2014). Clinical genetic testing for male factor infertility: current applications and future directions. *Andrology*, 2(3): 339-350.
20. Yu, B. and Huang, Z. (2015). Variations in antioxidant genes and male infertility. *Biomed Res. Int.*, 2015:513196.
21. Markandona, O.; Dafopoulos, K.; Anifandis, G.; Messini, C.; Dimitraki, M.; Tsezou, A., *et al*. (2015). Single-nucleotide polymorphism rs 175080 in the *MLH*3 gene and its relation to male infertility. *J. Assist. Reprod. Genet*., 32(12), 1795-1799.
22. WHO. (2010). WHO laboratory manual for the examination and processing of human semen.
23. AL-Baidani, S. (2018). Study of Vitamin D Receptor Gene Polymorphisms and Cytogenetic Changes in a sample of Iraqi infertile women with Polycystic Ovary Syndrome. A thesis Submitted to the Counsel of Genetic Engineering and Biotechnology Institute for Postgraduate Studies University of Baghdad In partial fulfillment of the requirements for the Degree of Master in Genetic Engineering and Biotechnology.
24. SAS. (2012). Statistical Analysis System, User's Guide. Statistical. Version 9.1th ed. SAS. Inst. Inc. Cary. N.C. USA.

25. Harraway, C.; Berger, N. and Dubin, N. H. (2000). Semen pH in patients with normal *versus* abnormal sperm characteristics. *Am J. Obstet. Gynecol.*, 182(5): 1045-1047.
26. Banjoko, S. and Adeseolu, F. (2013). Seminal plasma pH, inorganic phosphate, total and ionized calcium concentrations in the assessment of human spermatozoa function. *JCDR*, 7(11): 2483.
27. Zhou, J.; Chen, L.; Li, J.; Li, H.; Hong, Z.; Xie, M., *et al*. (2015). The semen pH affects sperm motility and capacitation. *PloS one*, 10(7): e0132974.
28. Kadhim, M. (2016). TheAssociation of Androgen Receptor GenePolymorphism with the Incidence of Severe Oligozoospermia in lraqiPatients. A thesis Submitted to the Counsel of Genetic Engineering and Biotechnology Institute for Postgraduate Studies University of Baghdad In partial fulfillment of the requirements for the Degree of Master in Genetic Engineering and Biotechnology:PP31.
29. Gopalkrishnan, K.; Hinduja, I. and Kumar, T. (1992). Volume of semen as a parameter of its quality. *Indian J Med Res*., 96: 361-365.
30. Bostofte, E.; Serup, J. and Rebbe, H. (1982). Relation between sperm count and semen volume, and pregnancies obtained during a twenty-year follow-up period. *Int. J. Androl*., 5(3): 267-275.
31. Gomella, L. (2017). Where have all the sperm gone?. *CJU*, 24, 8972-8972.
32. Hassan, G.M. (2017). Study of GeneticApoptosis According to expression FAS and FASL genes In Severe Oligozoospermic Iraqi Patients. A thesis Submitted to the Counsel of Genetic Engineering and Biotechnology Institute for Postgraduate Studies University of Baghdad In partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Genetic Engineering and Biotechnology. PP47.
33. Thajeel, J. (2016). Study of the Relationship between hsp70 Gene Polymorphisms and the Incidence of Severe Oligozoospermia in Some Iraqi Patients. A thesis Submitted to the Counsel of Genetic Engineering and Biotechnology Institute for Postgraduate Studies University of Baghdad In partial fulfillment of the requirements for the Degree of Master in Genetic Engineering and Biotechnology. PP6.
34. Dua, A. and Vaidya, S. (1996). Sperm motility and morphology as changing parameters linked to sperm count variations. *JPGM*, 42(4): 93.
35. Tulupova, E.; Kumar, R.; Hanova, M.; Slyskova, J.; Pardini, B.; Polakova, V., *et al*. (2008). Do polymorphisms and haplotypes of mismatch repair genes modulate risk of sporadic colorectal cancer?. *MUTAT RES-FUND MOL M*., 648(1-2): 40-45.
36. Ji, G.; Long, Y.; Zhou, Y.; Huang, C.; Gu, A. and Wang, X. (2012). Common variants in mismatch repair genes associated with increased risk of sperm DNA damage and male infertility. *BMC medicine*, 10(1): 49.
37. Zhao, X.; Mu, C.; Ma, J.; Dai, X. and Jiao, H. (2019). The association of four SNPs in DNA mismatch repair genes with idiopathic male infertility in northwest China. *Int J immunogenet*., 6(46): 451-458.
38. Öztaş, E.; Cilingir, U.; Akyüz, A.; Yanar, H. and Özhan, G. (2017). *MLH*1-93G> A and I219V polymorphisms are susceptible to increased risk of sporadic colorectal cancer in a Turkish population. *Istanbul J. Pharm*, 47(2): 63-67.
39. Li, S.; Zheng, Y.; Tian, T.; Wang, M.; Liu, X.; Liu, K., *et al*. (2017). Pooling-analysis on h*MLH*1 polymorphisms and cancer risk: evidence based on 31,484 cancer cases and 45,494 cancer-free controls. *Oncotarget* .93063 ،(54)8.
40. Foresta, C.; Bettella, A.; Merico, M.; Garolla, A.; Plebani, M.; Ferlin, A., *et al*. (2000). FSH in the treatment of oligozoospermia. Mol Cell Endocrinol, 161(1-2): 89-97.
41. Zhang, X.; Ding, M.; Ding, X.; Li, T. and Chen, H. (2015). Six polymorphisms in genes involved in DNA double-strand break repair and chromosome synapsis: association with male infertility. *Syst biol reprod med*, 61(4): 187-193.
42. Arafa, M.; Majzoub, A.; AlSaid, S.; El Ansari, W.; Al Ansari, A.; Elbardisi, Y., *et al*. (2018). Chromosomal abnormalities in infertile men with azoospermia and severe oligozoospermia in Qatar and their association with sperm retrieval intracytoplasmic sperm injection outcomes. *Arab. J. urol*., 16(1): 132-139.
43. Schubert, M.; Lanuza, L. and Gromoll, J. (2019). Pharmacogenetics of FSH action in the male. *Front endocrinol*, 10: 47.
44. Elfateh, F.; Wang, R.; Zhang, Z.; Jiang, Y.; Chen, S. and Liu, R. (2014). Influence of

genetic abnormalities on semen quality and male fertility: A four-year prospective study. *Iran J reprod med*, 12(2): 95.

45. Raut, S.; Deshpande, S. and Balasinor, N. (2019). Unveiling the Role of Prolactin and its Receptor in Male Reproduction. *Horm. Metab. Res*., 51(04): 215-219.
46. Tenorio, F.; Simões, M.; Teixeira, V. and Teixeira, Á. (2015). Effects of melatonin and prolactin in reproduction: review of literature. *Rev. Assoc. Méd. Bras*., 61(3): 269-274.
47. Marano, R. and Ben-Jonathan, N. (2014). Minireview: extrapituitary prolactin: an update on the distribution, regulation, and functions. *Mol endocrinol*, 28(5): 622-633.
48. Zhang, Z.; Jiang, Y.; Yun, X.; Yang, X.; Wang, R.; Dai, R. and Liu, R. (2012). Male infertility in Northeast China: a cytogenetic study of 135 patients with non-obstructive azoospermia and severe oligozoospermia. *J assist reprod genet*, 29(1): 83-87.
49. De Toni, L.; Di Nisio, A.; Speltra, E.; Rocca, M.; Ghezzi, M.; Zuccarello, D., *et al*. (2016). Polymorphism rs2274911 of GPRC6A as a novel risk factor for testis failure.*JCEM*, 101(3): 953-961.
50. Gurung, P. and Jialal, I. (2019). Physiology, Male Reproductive System. In StatPearls. .StatPearls Publishing.
51. Wei, T.; Huang, W.; Lin, A. and Chen, K. (2013). The role of hormones on semen parameters in patients with idiopathic or varicocele-related oligoasthenoteratozoospermia (OAT) syndrome. *J CHIN MED ASSOC* , 76(11), 624-628.