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RESEARCH PAPER

DNA mismatch repair MLH1 gene polymorphism and infertility in azoospermia males in Iraqi Kurdish population

Muhsin Jamil Abdulwahid[1], Mustafa Saber Al-Attar[2], Samiaa J. Abdulwahid-Kurdi[3], Karzan Abdulmuhsin Mohammad[1]

[1]General Directorate of Scientific Research Center, Salahaddin University-Erbil, Erbil, Iraq.

[2]Department of Biology, College of Science, Salahaddin University-Erbil, Kurdistan Region, Iraq.

[3]Department of General Sciences, Faculty of Education, Soran University. Kurdistan Region, Iraq.

A B S T R A C T:

To ensure the accuracy of DNA replication, to maintain genome stability, and to assure the preservation of a species, the DNA in the cell should be constantly repaired. The mismatch repair (MMR) process is crucial for gametogenesis, meiotic recombination, and the preservation of genomic integrity. Two MutS homologs (MSH4 and MSH5) and three MutL homologs (MLH1, MLH3, and PMS2) are members of the MMR family who actively participate in meiotic recombination and gametogenesis. About 50% of all cases of infertility are caused by male factors, and 15-20% of infertile males have genetic issues that result in azoospermia or severe oligozoospermia. This study aimed to find and discover the nucleotide changes in exons 7, and 8 of *MLH1* gene in seven infertile and three normal fertile males through Polymerase Chain Reaction (PCR) and DNA sequencing. In this study pair of primers was designed according to the NCBI primer designing tool to amplify the exons 7, and 8 of *MLH1* gene by PCR technique then sequencing the amplified product with Sanger sequencing. The sequence results aligned to NCBI database to find the possible nucleotide mismatch. Nine types of nucleotide variations have been detected of which seven of them are considered to be new recording nucleotide variations. These are the 37011581 A>T, 37011685 T>A, 37011767 C>A, 37011695 T> C, 37011579 A>T, 37011595 T>A and 37011717 T> C. However, the other two changes has been reported previously as defined in NCBI database which are 37011835 T> C with rs1553644000 T/C and 37012056 A>C with rs771612764 A/G. this stufy has found that, two nucleotide variants 37011595 T>A in intron 6 and 37012056 A>C rs771612764 A/G in exon 8, are exclusively detected in infertile samples and not in the normal samples. This may indicate a possible link between these variation and infertility.

KEY WORDS: MLH1 gene, Single nucleotide polymorphism, Male infertility, PCR technique, DNA sequencing, Nucleotide change.
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1. INTRODUCTION:

The mutL homolog 1 gene (*MLH1*) is a humans protein-coding gene. The gene consists of 57,329 nucleotides located on chromosome 3 3p22.2 (NC_000003.12:36,987,599 - 37,056,595). It consists of 24 exons that can be transcripted to 756 amino acids making a protein called DNA mismatch repair protein MLH1 or MutL protein homolog 1.

The MLH1 protein can heterodimerize with mismatch repair endonuclease PMS2 to produce MutL alpha, a component of the DNA mismatch repair system. When MutL alpha is bound by MutS beta and some auxiliary proteins, the PMS2 subunit of MutL alpha introduces a single-strand break near DNA mismatches, offering an entrance for exonuclease degradation (https://www.ncbi.nlm.nih.gov/gene/4292). The protein is also associated in DNA damage signaling and can heterodimerize with DNA mismatch repair protein MLH3 to construct MutL

* **Corresponding Author:**
Muhsin Jamil Abdulwahid
E-mail: muhsin.abdulwahid@su.edu.krd
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gamma, which is engaged in meiosis. This gene has been identified as a locus commonly mutated in hereditary nonpolyposis colon cancer(Truninger et al., 2005).

The proteins of the DNA repair pathway play critical roles in gametogenesis. Generally, DNA repair processes in male germline cells include post-replication repair, double strand break repair, DNA mismatch repair, nucleotide excision repair, and base excision repair. Defects in repair pathways can result in aberrant recombination and spermatogenesis arrest. This ultimately may lead to male infertility(Baarends et al., 2001).

Infertility is still a significant clinical issue that affects 15% of couples globally (WHO, 2018). It can be caused by either a female or male factor, with the male-factor accounting for only one-third of cases. Male infertility has many causes, the most significant of which are as follows: Physical reasons, sexually transmitted diseases, environmental and lifestyle variables, hormonal deficiencies, and genetic factors. However, there is no obvious causes for roughly 40% of infertile men (Krausz and Riera-Escamilla, 2018, Ho et al., 2020).

About 15% of infertile male cases are due to genetic reasons, which can be split into two groups: chromosomal abnormalities (numerical or structural) and monogenic mutations (Krausz et al., 2011). Standard spermatogenesis is controlled by multiple genes, some of which are not expressed in the testis but regulate multiple physiological processes in the body. Loss-of-function of these genes cause spermatogenesis and sperm functionality imperfections (Linn et al., 2021). To maintain the accuracy of DNA replication during the mitotic, meiotic, and post-meiotic development of both male and female germ cells, DNA repair mechanisms are crucial. Base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), homologous recombination (HR), and non-homologous end joining (NHEJ) are at least five primary DNA repair processes that are active at various periods of the cell cycle and enable the cells to repair DNA damages. Direct chemical reversal and interstrand crosslink (ICL) repair are further methods for treating a small number of particular defects. Cells' ability to preserve genomic stability depends on these repair activities (Chatterjee and Walker, 2017).

The present study aimed to find the association between the nucleotide changes in exons 7, and 8 of *MLH1* gene with infertility among seven infertile and three fertile males through PCR and DNA sequencing in Kurdish population in Erbil city of Kurdistan Region of Iraq.

1. MATERIALS AND METHODS:

2.1 Patients and controls:

In this investigation, an informed consent was gained from each participant. The conset was authorized by the Salahaddin University-Erbil (SUE) College of Science's Research Ethics Committee. This research was performed on 10 Iraqi Kurdish males in the Erbil governorate. Based on their medical histories that were logged at public and private hospitals as well as infertility clinics, patients who agreed to participate in this study were chosen. The participants included seven azoospermia (based on semen fluid analysis results (Organization, 2021) and three normal fertile males as a control group. The participant's ages ranged from 30-48 years old.

2.2 Molecular methods:

2.2.1 Genomic DNA extraction

For Genomic DNA extraction, two ml of venous blood was taken from each participant using an aseptic syringe. The samples wer collected in an anticoagulant EDTA tube utilizing commercial kit (Genomic DNA Mini Kit, Geneaid, Taiwan, Cat. No. GB100). The procedures were conducted according to manufacturing guidelines. The quality and quantity of each genomic DNA sample were measured by NanodropTM 1000 spectrophotometer (Thermo Scientific, USA). The experiments were done in molecular genetics laboratory belong general director of scientific research center/ salahaddin university- Erbil.

2.2.2 Primer design for PCR technique

To investigate the association between the nucleotide changes of exons 7 and 8 of *MLH1* gene with infertility. Utilizing newly designed primers, the exons 7, 8, and intron 7 of *MLH1* gene were amplified simultaneously (689bp) according to the primer-designed tool on the NCBI website (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The foreword primer was F-

5'AGTGGCGTGATATCCTTGATTCT3' with Tm: 59.61 and GC: 43.48% and the reverse primer R-5' CAAGCCTGTGTATTTGACTAAAGC 3' with Tm: 58.61 and GC: 41.67.

2.2.3 PCR sample preparation

For each sample the PCR mixtures were prepared with a final volume 50 µl containing 25 µl of 2X of Master Mix (AMPLIQON), 2.5 µl of each primer (Forward and Reverse primer) with 10 pmol/µl concentration (Macrogen; LIGO), and 5 µl of genomic DNA as a template sample. The mixture completed to 50µl with sterile nuclease-free water. The thermal cycler condition program for amplification consisted of 1 cycle of 95 as Initial denaturation, 35 cycles: 95 °C for 30 sec (denaturation), 56 °C for 30sec (annealing), 72 °C for 45 sec (Extension), and final extension 72°C for10 minutes by utilizing a thermal cycler (Alpha thermal Cycler; code: AC196).

2.2.4 Gel electrophoresis

To visualize the amplified PCR product agarose gel electrophoreses were performed in which five microliters of each amplified PCR product were separated on a 1.5% agarose gel containing 0.5 microliters of ethidium bromide in 100ml of agarose solution throughout the time of 50 minutes of electrophoresis. To compare and confirm the product size of each amplicon, PCR products were tested with a 100bp DNA ladder (GeneDirex). Gel documentation system equipped with UV light (Proxima 2500 Isogene Life science, Netherland) was used to observe the DNA band on the gel (Brown, 2020).

2.2.5 Sanger sequencing

The sequencing was carried out by the bigdye terminator method at Macrogen Company (South Korea, Seoul).

2.2.6 Sequence alignment

The Basic Local Alignment Search Tool (BLAST) was used to analyze the results of the *MLH1* DNA sequence which is available on the genome data viewer on the NCBI website (https://www.ncbi.nlm.nih.gov/ genome/gdv/browser/genome/?id=GCF 000001405.40). The BLAST was utilized to evaluate and find the mismatch or similarity of the sample sequence with the database sequence and evaluate the statistical significance of matches.

3. RESULTS

3. 1. PCR testing and sequence analysis

The amplified PCR products for all samples were electrophoresed following PCR amplification **Figure 1.** Every sample has a band size of 689bp, which was equal across all cases. Universally, ten types of nucleotide variations have been detected in this study between all ten samples **Table 1** and **2**.

4. DISCUSSION:

In the current investigation the NCBI primer designing tool used to design a new primer to amplify exons 7, 8, and intron 7 of *MLH1*gene (689bp) **Figure 1.** This is in order to perform DNA sequencing, so that any nucleotide change can be observed. Such changes to be investigated in the infertile azoospermia males and compared with the normal fertile male in the Kurdish population of Iraq. Sequence alignment process using the NCBI-BLAST tool was conducted to identify any nucleotide mismatches. This is in addition to specify the location of any possible mutations in the samples, the alignment were performed separately for each sample of DNA sequence.

This study have revealed ten different types of nucleotide changes among the three fertile and six infertile samples. However no changes were detected in one of the infertile sample. Most changes were detected in intron seven between exon seven and exon eight of *MLH1* gene. Among the ten nucleotide changes only two of them were already described in the NCBI database. These were 37011835 T> C with rs1553644000 T/C and 37012056 A>C with rs771612764 A/G both in exon seven. However the other nucleotide changes as described in **Table 1** and **2** have not been defined in NCBI database. To our knowledge this is the first report indicating these recorded nucleotide changes. The other important finding of this article was the detection of the nucleotide changes 37011581 A>T in intron 7 in four infertile samples (57.1%) and all three normal samples (100%) **Table 2.** This considered finding may indicate the occurrence of this nucleotide polymorphism in Kurdish population since it is present in both normal and infertile samples. Nevertheless, according to the findings of this study, we may conclude an important link between inftertility and two of the nucleotide changes found only in the infertile samples and was absent in the normal fertile males. These variations are 37011595 T>A in intron 6 and 37012056 A>C rs771612764 A/G in exon 8. One limitation to this conclusion could be the sample

size and further studies is recommended with larger sample frequency to certify such claim.

In conclusion, appropriate pair of primers was presented suitable for sequencing the exons 7, and 8 of *MLH1* gene. Generally, seven new types of nucleotide variations have been detected among nine variations that recorded in this study. Two nucleotide variations 37011595 T>A in intron 6 and 37012056 A>C rs771612764 A/G in exon 8 are only found in infertile samples and not in the healthy ones. This might suggest a connection between infertility and these variations.

Table 1. The table shows the nucleotide variation and allele type among three normally fertile and seven infertile Kurdish males in *MLH1* gene's exons 7 and 8 as well as in its introns 6.

Group	Sample No.	Age /Year	Nucleotide change and location on *MLH1* gene	Location on *MLH1* gene	Variation type	Allele type (zygosity)
Normal Control	1	38	37011581 A>T 37011685 T>A 37011767 C>A	intron 6 intron 6 intron 6	Undefined	Homozygous Homozygous Homozygous
	2	48	37011581 A>T 37011695 T> C	intron 6 intron 6		Homozygous Homozygous
	3	46	37011581 A>T 37011579 A>T 37011685 T>A 37011695 T> C 37011705 T> C 37011717 T> C 37011835 T> C	intron 6 intron 6 intron 6 intron 6 intron 6 intron 6 Exon 7	 rs1553644000 T/C	Homozygous Homozygous Homozygous Homozygous Homozygous Homozygous Heterozygous
Infertile patient	1	47	37011581 A>T 37011601 T>A 37011695 T> C	intron 6 intron 6 intron 6		Homozygous Homozygous Homozygous
	2	38	37011581 A>T 37011601 T>A 37011767 C> A	intron 6 intron 6 intron 6		Homozygous Homozygous Homozygous
	3	39	37011579 A>T 37011595 T>A 37011717 T> C 37011835 T> C 37012056 A>C	intron 6 intron 6 intron 6 Exon 7 Exon 8	 rs1553644000 T/C rs771612764 A/G	Homozygous Homozygous Homozygous Heterozygous Homozygous
	4	32	37011581 A>T	intron 6		Homozygous
	5	34	37011581 A>T 37011595 T>A 37011705 T> C	intron 6 intron 6 intron 6		Homozygous Homozygous Homozygous
	6	30	37011595 T>A 37011601 T>A 37011685 T>A 37011695 T>C 370**11**705 T>C 37011717 T>C 37011767 C>A 37012056 A>C	intron 6 intron 6 intron 6 intron 6 intron 6 intron 6 intron 6 Exon 8	 rs771612764 A/G	Homozygous Homozygous Homozygous Homozygous Homozygous Homozygous Homozygous Homozygous
	7	32	non			

Table 2. The table shows the frequency of nucleotide variations in the *MLH1* gene's exons 7 and 8 as well as in its introns 6 in three normally fertile and six infertile Kurdish males.

No.	Nucleotide Changes	Three Fertile Samples	Seven Infertile Samples
1	37011581 A>T Undefined	3(100%)	4 (57.1%)
2	37011685 T>A Undefined	2 (66.6%)	1 (14.3%)
3	37011767 C>A Undefined	1 (33.3%)	2 (28.6%)
4	37011695 T> C Undefined	2 (66.6%)	2 (28.6%)
5	37011579 A>T Undefined	1 (33.3%)	1 (14.3%)
6	37011705 T> C Undefined	1 (33.3%)	2 (28.6%)
7	37011717 T> C Undefined	1 (33.3%)	2 (28.6%)
8	37011835 T> C rs1553644000 T/C	1 (33.3%)	1 (14.3%)
9	37011595 T>A Undefined	Non	3 (42.8%)
10	37012056 A>C rs771612764 A/G	Non	2 (28.6%)

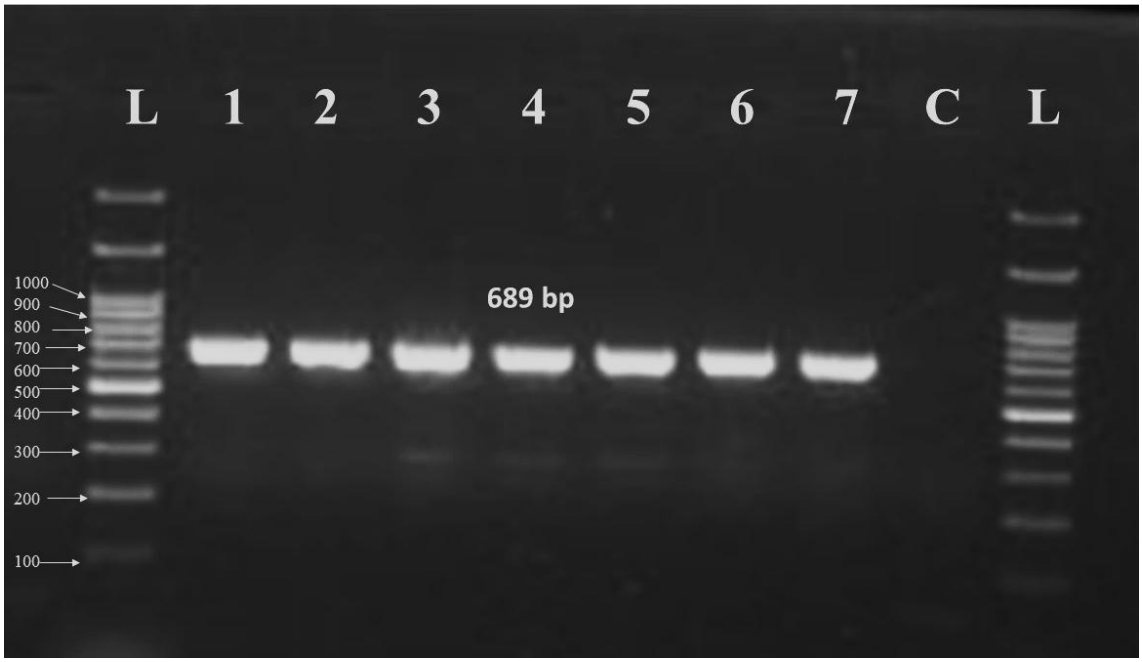

Figure 1. 1X TBE 1.5% agarose gel electrophoresis. Lane L represent 100bp size DNA ladder marker, Lane 1-7 represents samples of PCR product 689bp of the exons 7,8 and intron 7 of *MLH1* gene. Lane C: negative control.

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Conflict of Interest

The authors declare they have no conflicting interests.

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