Levels of Matrix Metalloproteinase-9 (MMP-9) and its gene polymorphism of Hydatid Cyst Disease in an Infected Patients

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KEYWORDS
Matrix, Hydatid, Patients

ABSTRACT
Hydatid cystic disease is an economic burden in Iraq since it decreases the productivity of sheep, goats, cows, and camels by making the organs unpalpable for human consumption, hence weight-loss and ill health. This is one of the most common zoonosis diseases shared between human beings and animals and appears in man hosts inlcuded some organes, such as liver and lung as hydatid cyst . it causes many complications that may lead to death in repured parasites. Now there are no safe and effective in the fight against this parasite, and the search for such medications is in research. The Matrix Metalloproteinase-9 is a one of the enzymes that belong to the family of matrix metalloproteinases (MMPs), which play a crucial role in the proteolytic degradation of the extracellular matrix (ECM). These enzymes have a zinc ion in the active site that is important for their proteolytic activity. MMP-9, also known as Gelatinase B, catalyzes gelatin and degrades components of the ECM, including abundant type IV collagen in basement membranes. MMP-9 plays an important role in various biological processes, including tissue remodeling, angiogenesis, and tissue inflammation. The objective of the study is to determine the impact of MMP-9 serum levels and specific single nucleotide polymorphisms (SNPs) rs17576 and rs76070157 on patients with hydatid disease, in comparison to a control group of healthy individuals. A total of (62) patients diagnosed with hydatid disease, consisting of 18 males and 44 females, were compared to a control group of(75) individuals, comprising 29 males and 46 females .The study examined the MMP-9 rs17576 and rs76070157 (SNPs) in patients with hydatid disease, comparing them to a control group of healthy individuals. The study revealed no statistically significant difference in the average age between the two groups at a significance level of 0.05. Additionally, the study found higher concentrations of MMP-9 in the sera of the infected group (20.40 ± 1.52 pg/µl) compared to the control group (19.49 ± 1.62 pg/µl). The rs17576 (SNPs) data indicated that the TT genotype and *T* allele exhibited a slightly higher frequency percentage in the group of patients with hydatid disease compared to the healthy group, however this difference was not statistically significant. The values are (98.4% and 99.0%) the value of pc is 0.001. The elevated odds ratio (OR) associated with the TT genotype and *T* allele may serve as a potential risk factor for hydatid disease. The rs17576 SNPs displayed genotypes that were not in accordance with Hardy-Weinberg equilibrium. However, these genotypes were consistent with those observed in a control group. The GG genotype showed a significant increase in frequency compared to the control group (100.0% vs. 100.0%), while the *G* allele exhibited a non-significant increase in frequency. The odds ratio (OR) for the TG genotype was 3.68, and the p-value (pc) was 0.05.Elevated value could potentially increase the risk of hydatid disease. The presence of the TG genotype was shown to considerably increase the frequency percentage in the group of patients with hydatid disease. The odds ratio (OR) value of 1.6 suggests that it may be a risk factor for hydatid disease, with a frequency of 98.4% compared to 0.99 % in the control group. The p-value of 0.001 indicates a statistically significant association. The GG genotype was present in (0%) of the affected group but absent (100.0%) in the healthy group. The odds ratio (OR) of 3.68, with a p-value of 0.001, suggests that the GG genotype is associated with a significantly lower risk of hydatid disease.

1. Introduction

Echinococcosis or hydatidosis caused by the tapeworm *Echinococcus granulosus* larval stage are one of the most important zoonotic diseases for human and domestic animals (Ali *et al*., 2020; Khan *et al*., 2021). This disease leads to many medical, veterinary and economic problems, Iraq is regarded as one of the countries that plagued by endemic sickness of hydatidosis (Deplazes *et al*., 2017; Abdulhameed *et al*., 2019). Around four centuries before Christ, Hippocratus, an ancient Greek physician, linked human hydatid Cysts to the "water-filled tumors" he saw in post-mortem examinations of cattle (Eckert *et al*., 2001). In 1695, Hartmann discovered adult *Echinococcus granulosus* in a dog's small intestine, and in 1782, Goeze identified the larval stage hydatid cysts (Paniker and Ghosh, 2013). In a series of studies published in 1853, Carl Von Siebold revealed that cysts from sheep produced adult tapeworms in dogs, illustrating the life cycle and link between larval

and adult stages. In 1886, the German scientist Leukart described the shape of the parasite and the hydatid cysts resulting from it and called it *Echinococcus granulosus,* This term is derived from the Latin word Echinos, meaning urchin or thorny, while kokakos means bean granulated *Echinococcus*, and the word Granulum means small tubercles. Hydatid is a Greek word, hydatis, meaning a drop of water (Rahman *et al*., 2015). In 1984, Francesco was the first to describe the parasitic nature of the disease (Khalifa *et al*., 2016; Thompson, 2017). The MMP-9 is one of the types of enzymatic proteins that belong to the family of metalloproteinases (MMPs) that contain zinc, which mainly contributes to the disruption and degradation of most components of the extracellular matrix (ECM) and also contributes to chronic inflammation. MMP-9 is also known as collagenase type IV or collagenase IV Gelatinase Gelatinase B (Hasanzadeh *et al*., 2024).Is produced MMP-9 is primarily mediated by many cell types, including macrophages, neutrophils, epithelial cells, mast cells, fibroblasts, and smooth myocytes (Napoli et al., 2020; Tian *et al*., 2021).

He promises MMP-9 is an enzyme that degrades exogenous proteins. In humans, the gene encoding this protein is located on chromosome 20q13.12 and encodes a protein consisting of 707 amino acids, with a molecular weight of 92 kilo Daltons. It is secreted outside the cell and is an inactive pro-enzyme. It is called Pro-MMP-9 because it contains 80 amino acid residues consisting of the N-terminal end, which is cysteine, by forming a complex or linking with the zinc ion in the catalytic domain of the protein. This enzyme is activated, loses 80 amino acids, and transforms from an inactive enzyme into an active enzyme (Tian *et al*., 2021).When infected with hydatid cyst disease, phagocytic cells become active Macrophages, which leads to the release of many factors that kill hydatid cysts, including peptidases, and an increase in their production can cause severe damage to tissues and organs (Hasanzadeh *et al*., 2022). MMP-9 is involved in the degradation and breakdown of type IV collagen, as it is the main component of the basal membrane found in the skin or the main substrate for gelatinase. The enzymatic activity of MMP-9 is inhibited by inhibitor of tissue protein-1 (TIMP-1), and the expression of the Echinococcosis parasite is linked to the degradation of Gp63 compounds present on the surface of the cyst parasite. The appearance of the injury results from an imbalance in the organization of the basement membrane, which leads to an imbalance in the levels of MMP-9 and TIMP-1, in addition to other factors such as the recruitment of edema cells, as TNF-α is a strong stimulator of the production of MMP-9 Excessive TNF-α by monocytes increases the production of MMP-9, leading to an imbalance with its inhibitor TIMP-1 (Jiang *et al*., 2015).

is produced MMPs during infection with the hydatid cysts parasite participate in the innate immune response by decomposing extracellular components, which leads to the migration of white blood cells and increased activity of cytokines and chemicals to the site of infection. As a result, it leads to a lack of control of the activity and leads to tissue damage and the occurrence of pathogenesis, despite the presence of evidence. On the participation of metallopeptidase in liver cell damage during Cystic Echinococcosis (Hasanzadeh *et al*., 2024).It was mentioned that Phagocytic cells macrophages secrete for MMP-9 upon infection with the hydatid cysts parasite and the production of types (MMP1, 7, 8 & 13) increases.

2. Subjects

Materials were collected

Samples were such gathered for further study from those patients having been diagnosed with hydatid disease infection and from control group healthy individuals who visited Baqubah Teaching Hospital/Baqubah City - Diyala Governorate, Al-Muqdadiya Hospital, Khanaqin Hospital. upon personal consent each participant which was taken before sample collection according to science and ethics of scientific research after obtaining Ministry of Health approval the samples were collected from December 2022 to June 2022023. The mean age of onset hydatid disease in this study was 36.76 ± 1.27 years with 18 males and 44 females compared to control group that included 75 individuals 29 males and 46 females their mean age was 34.20 ± 1.20 years. 5 µl of venous blood was provided by each participant: 2 µl in an EDTA tube for genomic analysis of polymorphisms of

the MMP-9 gene (rs17576, rs76070157) and 3 µl in a silicone gel tube for separation of serum for measurement of the level of MMP-9. Those revealing chronic or communicable diseases were excluded after a brief questionnaire looking for any conditions that might already exist.

2.2. Genomic DNA Extraction

DNA was extracted using the traditional method provided by INTRON company, Korea. The extracted DNA had a purity of 1.8-2.0 and a concentration of 50-100ng/µl, as measured by a namedrop spectrophotometer.

2.3. Preparation of Primers

Designed by entering the GenBank accession number into the NCBI Primer-BLAST online tool, with default settings for primer size, Tm, and GC content. The primers were synthesized according to the specifications of Alpha-DNA Company, Baghdad, Iraq, as detailed in table (1 and 2).

2.4. Polymerase Chain Reaction (PCR) Protocols for Genotyping

It was performed Mutations in the MMP-9 gene using allele-specific primers; the SNPs assessed were rs17576 and rs76070157. Two Eppendorf tubes were prepared per sample, one containing 12.5 µl of master mix, 2 µl DNA, and 2 µl primer (1 µl forward primer, 1 µl reverse primer), the second containing 1 µl of forward primer 2 and common reverse primer, final volume was adjusted to 25 µl with nuclease-free water the thermocycler protocol for both (MMP-9 SNPs) included the following steps Denature at 95°C for 35 seconds; Initial denature at 95°C for 10 minutes Anneal at 60°C for 35 seconds, and extend at 72°C for 35 seconds, repeated for 35 cycles.. Final extension at 72°C for 10 minute.

The PCR amplicons were visualized using electrophoresis on a 1.5% agarose gel, stained with Red Safe stain, and bands were observed under UV trans illumination.

Table :1: MMP-9 primers information and conditions rs17576

rs17576	**Sequence(5'>3')**	**Product length**
Forward primer1	CAATAGGTTTTGAGGGGCATGT	213bp
Forward primer2	CAATAGGTTTTGAGGGGCATGG	
Reverse primer	AACCAGCGGAAAACTTCCTT	

Table 2: MMP-9 primers information and conditions rs76070157

rs76070157	Sequence(5'>3')	Product length
Forward primer1	TAACTTCCAGTCCCCTCCCCC	182 bp
Forward primer2	TGACTTCCAGTCCCCTCCCCC	
Reverse primer	GATCTGTGGCGGTCTCTCTG	

2.5. statistical analysis

uniformity symmetry and symmetrical distribution of boundaries All information checked and confirmed data with calculations using mean and standard deviation Student's t-test was run using IBM SPSS version 26.0 A value of probability less than <0.05 was considered high to pursue the Pearson chi-square value in order to obtain the Probability value for non-parametric data the individual percentage is Fish exact 95% Confidence interval and probability were performed using WinPE version 11.65 (Abramson, 2011) for genotyping and alleles Frequencies such The genotyping frequencies and for alleles calculations of were done The online Hardy-Weinberg calculator was used (Andrews, 2010).

3. Results and Discussion

Results were reported as 62 Hundred patients with hydatid disease (18 males and 44 females) currently compared with one control group included 75 subjects (29 males and 46 females). The distribution of the ages was normal with no significant different in mean ages between the two groups (see Table 3). There was also no significant different at the chose level of significance(0.05) on the average age between the two groups. The current study also reported the concentration rates of MMP-9 in the sera of the study groups' patients; they revealed high concentrations when infected patients were compared to the controls (36.76 ± 1.27, 34.20 ± 1.20pg/µl). There is no significant difference in terms of place of residence, another urban or rural; because it was equal percentage for both areas from table (3). This study found a statistically significant increase at a probability level of (0.05), in the concentration of MMP-9 of serum patients with hydatid cyst disease. Refer to table (4).

Table 3: The demographic data of the studied groups

		Patients group	**Control group**	**Probability**
Sexes No. (%)	Males	18 (29.0)	29 (38.7)	P > 0.05
	Females	44 (71.0)	46 (61.3)	
	Total	62 (100.0)	75 (100.0)	
Living location	Urban	31 (50.0)	50 (66.7)	P < 0.05
	Rural	31 (50.0)	25 (33.3)	
	Total	62 (100.0)	75 (100.0)	
Age mean ± SE (Years)		36.76 ± 1.27	34.20 ± 1.20	P > 0.05

Table4: Serum levels in MMP-9

MMP-9 level mean ± SE (pg/ml)		**Probability**
Patients group	Control group	
20.40 ± 1.52	19.49 ± 1.62	P > 0.05

Single-nucleotide genetic variation rs17576 for MMP-9

Genetic variation was examined for single nucleotide polymorphismsrs17576 MMP-9, there was Allele *T* and *G* They correspond to three genotypes(TT,TG and GG). The genetic patterns were studied by polymerase chain reaction PCR using the allele-specific primer technique (PCR-ASP) (Ye *et al*.,2014).In this technique when the genotype is the same TT a single band will appear upon migration on the agarose gel in the hole of the first primer forward 1. But if a heterogeneous genetic pattern appears TG two bands will appear on the agarose gel, one of which is loaded into the initiator hole forward 1while the second package is in the starter hole forward 2. In addition, the genotype is homozygous GG appeared as a package in the starter's hole forward 2 loaded onto an agarose gel ...as is clear in the figure (1).

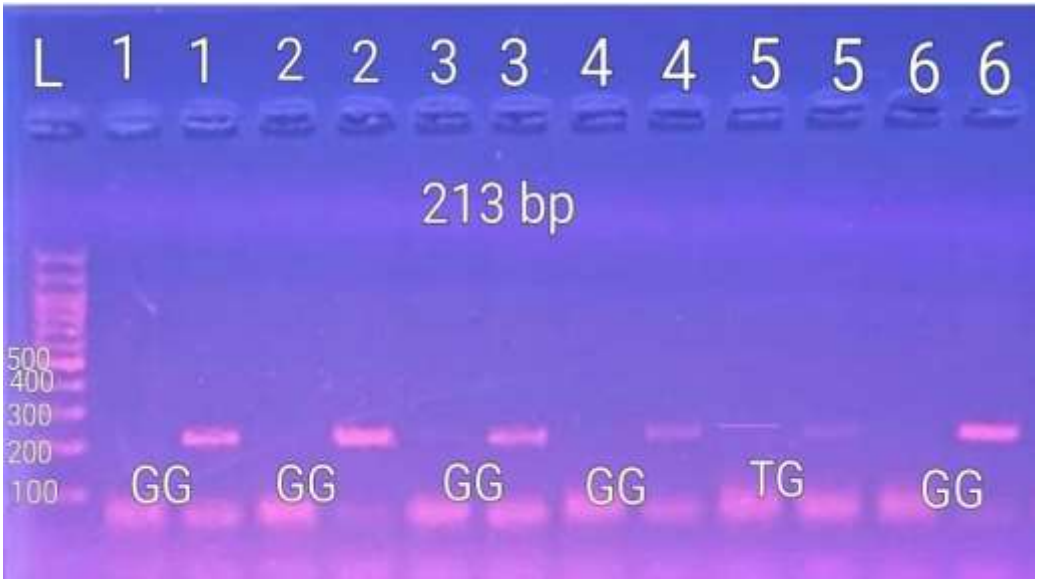

Figure 1. Gel electrophoresis of MMP-9 SNP rs17576 PCR product was performed on

1.5% agarose gel stained with Red Safe stain (Intron company, South Korea).

1-6: electrophoresed samples' numbers, the single band for the TT and GG genotypes

(Wild and mutant homozygote, respectively) the double bands for the TG genotype

(mutant homozygote).

The results of the current study showed that the frequency ratio of genotypes and alleles forMMP-9 rs17576For the injured group Hydatid cysts the control group is consistent with the Hardy-Weinberg equation because there are no significant differences, as shown in the table (5).

The results of the current study showed a non-significant decrease in the frequency of these genotypes in the group of patients with hydatid cysts and the control group.

Table 5: The genotyping and alleles frequencies of MMP-9 rs17576 SNPs

Genotypes	Patients group (n=62)		Control group (n=75)	
	Observed	Expected	Observed	Expected
TT	61 (98.4)	61.0 (98.39)	0 (0.0)	0 (0.0)
TG	1 (1.6)	0.99 (1.60)	0 (0.0)	0 (0.0)
GG	0 (0.0)	0.0 (0.001)	75 (100.0)	75 (100.0)
Total	62 (100.0)		75 (100.0)	75 (100.0)
P-HWE	P > 0.05		-	

The current results also showed a significant increase in the percentage frequency of the allele *T* in the group of patients with hydatid cysts compared with the control group, while the allele *G* show presence decrease not significant in percentage frequency in the group of patients with hydatid cysts compared with the control group as shown in table (6), as genetic patterns are(TT, TG, GG)and the night from the factors the infectious hydatid cysts the value of the likelihood ratio for this is a for pattern and the night is (6191.0, 3.68 ,24782.33,0.00004) as shown in the table (6).

Table 6: MMP-9 genotyping and allele frequencies of rs17576 between the groups

Genotypes	**Patients group (n=62)**	**Control group (n=75)**	***X²***	**OR (95% CI)**	**Fisher's exact Probability**
T	123 (99.0)	0 (0.0)	269.99	24782.33 (1012.0-606903.8)	P < 0.001
G	1 (0.01)	150 (100.0)	269.99	0.00004 (0.000002-0.0010)	P < 0.001
TT	61 (98.4)	0 (0.0)	133.02	6191.0 (253.4-151260.8)	P < 0.001
TG	1 (1.6)	0 (0.0)	1.219	3.68 (0.15-89.98)	P > 0.05
GG	0 (0.0)	75 (100.0)	137.0	0.00005 (0.000001-0.0026)	P < 0.001
Total	62 (100.0)	75 (100.0)			

The results of the current study showed the presence of decrease level MMP-9 between the group of patients with cystic fibrosis and the control group in genotype TT comparison (20.51 ± 1.54 , 19.49 ± 1.62) as shown in the table (7).

The results of the current study indicate that this may be the first study on the level of MMP-9 and the genetic pattern of rs17576 as mentioned for the first time the relationship with the increase level MMP-9 in patients with hydatid disease compared with the control group.

Table 7: MMP-9 levels among rs17576 single nucleotide polymorphism (SNP) between the groups

Genotypes	MMP-9 level mean ± SE (pg/ml)		Probability
	Patients group (n=62)	Control group (n=75)	
TT	20.51 ± 1.54	19.49 ± 1.62	P > 0.05
TG	13.65	-	-
GG	-	-	-
Probability	P > 0.05	-	

Single-nucleotide genetic variation rs7606707157 for MMP-9

Genetic variation was examined for single nucleotide polymorphismsrs7606707157 MMP-9 there were two alleles *A* and *G* they correspond to three genotypes (AA,AG and GG). The genetic patterns were studied by polymerase chain reaction PCR using allele-specific primers technology (PCR-ASP) (Ye *et al*.,2014).In this technique when the genotype is the same AA a single band will appear upon migration on the agarose gel in the hole of the first primer forward 1. But if a heterogeneous genetic pattern appears AG two bands will appear on the agarose gel, one of which is loaded into the initiator hole forward 1while the second package is in the starter hole forward 2. In addition, the genotype is homozygous GG he appeared as a package in the starter's hole forward 2 loaded onto an agarose gel as is clear in the figure (2).

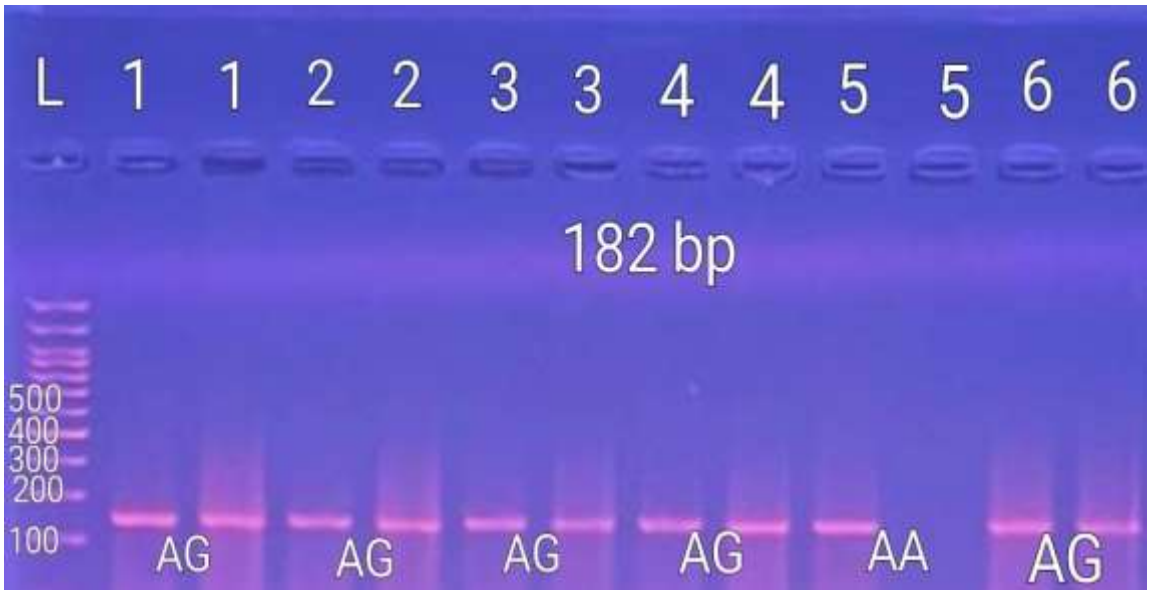

Figure 2. MMP-9 SNP rs7607157 PCR product was electrophoresed on 1.5% agarose gel. Staining was done using Red Safe dye (Intron Biotechnology, South Korea). The figure shows lanes 1 to 6, which correspond to the different electrophoresed samples, where 1 is the marker. The single band observed indicates the AA and GG genotypes (wild and mutant homozygote, respectively), while the double band represents the AG genotype (mutant heterozygote). The results of the present study showed that genotype and allele frequencies of MMP-9 rs7606707157 for hydatid cyst disease the injured group did not differ from those in the control group and were in equilibrium with Hardy-Weinberg because shown in the table (8). The results of the present study showed the presence of to rise Noteworthy in frequency of the ratio percentage results of these genetic patterns in the population with hydatid cyst disease and the control population. The results of the present study showed the presence of to rise moral in frequency ratio percentage these genotypes were observed in patients with hydatid disease and the control group.

Table 8: The genotyping and alleles frequencies of MMP-9 rs76067057 SNPs

Genotypes	Patients group (n=62)		Control group (n=75)	
	Observed	Expected	Observed	Expected
AA	2 (3.2)	3.23 (16.0)	10 (13.33)	14.08 (18.78)
AG	59 (95.2)	95.16 (30.99)	45 (60.0)	36.83 (49.11)
GG	1 (1.6)	1.61 (15.0)	20 (26.67)	24.08 (32.11)
Total	62 (100.0)		75 (100.0)	75 (100.0)
P-HWE	P < 0.001		P > 0.05	

As the present results showed the presence of low non moral in the percentage to repeat for the alleles *G* in the group of patients with hydatid disease compared with the control group ,also fortune alleles *A* show presence decrease there was no significant difference in the frequency of the percentage in the group of patients with hydatid cysts compared to the control group as shown in Table (9), as it is considered embroider genetic(AA, AG, GG)And the alleles *G* of the factors danger for the incidence of hydatid disease, the odds ratio for this pattern and allele was(0.22, 13.11, 0.05, 0.74) as shown in table (9).

Table 9: MMP-9genotyping and allele frequencies of rs76067057 between the groups.

Genotypes	Patients group (n=62)	Control group (n=75)	X^2	OR (95% CI)	Fisher's exact Probability
A	63 (51.0)	65 (43.0)	1.523	1.35 (0.84-2.17)	P > 0.05
G	61 (49.0)	85 (57.0)	1.523	0.74 (0.46-1.19)	P > 0.05
AA	2 (3.2)	10 (13.33)	4.339	0.22 (0.05-1.02)	P > 0.05
AG	59 (95.2)	45 (60.0)	22.949	13.11 (3.80-45.25)	P < 0.001
GG	1 (1.6)	20 (26.67)	16.415	0.05 (0.01-0.34)	P < 0.001
Total	62 (100.0)	75 (100.0)			

The results of the current study showed that down moral at level MMP-9 between the group of patients with cystic fibrosis and the control group in genotype AA(16.93 ± 5.67 comparison 25.22 ± 6.27), as well as for both genotypes (AG, GG) (20.66 ± 1.58,16.80 ± 1.57comparison11.67,22.14 ± 3.56) respectively As shown in the table (10).The results of the current study indicate that this may be the first study on the level of MMP-9 and the pattern genetic of rs7606707157It was first mentioned that the relationship with the increase in the level of MMP-9 in patients with hydatid disease compared with the control group.

Table 10: MMP-9levels among rs76067057 single nucleotide polymorphism (SNP) between the groups.

Genotypes	MMP-9 level mean ± SE (pg/ml)		Probability
	Patients group (n=62)	Control group (n=75)	
AA	16.93 ± 5.67 ^{A}	25.22 ± 6.27 ^{A}	P > 0.05
AG	20.66 ± 1.58 ^{A}	16.80 ± 1.57 ^{A}	P > 0.05
GG	11.67 ^{A}	22.14 ± 3.56	P > 0.05

Conclusion

Present findings, MMP-9 serum levels were , it was found that there is significant increase with a in hydatid cyst disease .The current results also showed a significant increase in the percentage frequency of the allele *T* in the group of patients with hydatid cysts of rs 17576 , the allele *T* might be potential risk factors for hydatid disease .while alleles *A* in rs 76067057 show presence decrease there was no significant difference in the frequency of the percentage in the group of patients with hydatid cysts and risk factor of hydatid cyst patients.

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