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ORIGINAL ARTICLE

Genetic Variation in Cardiac Troponin T Gene TNNT2 in Iraqi Patients with Cardiomyopathy

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ABSTRACT

Cardiomyopathy is an affliction of the heart muscle that can result in heart failure.It comes in a variety of phenotypes, such as dilated, hypertrophic, and restricted. This study was aimed to investigate the polymorphism in cardiac Troponin T gene TNNT2 in Iraqi patients with cardiomyopathy. Eighty-two diagnosed cardiomyopathy adult patients with age range between (20-70) years, and thirty healthy with same range, were involved in this study during their attendance at Ibn Al- Bitar Center for Cardiac Surgery. The patients were diagnosed by an expert cardiologists based on ECG changes, Echocardiogram, chest x ray. The study was conducted from October 2020 to March 2021, and approved by ethical committees.: CSEC/0920/0052, September 15, 2020 of department of Biology, College of Science, University of Baghdad. Sequencing of the PCR products was performed by the genetic analyzer for forward and reverse primers in macrogen company (South korea), to detect the polymorphism in TNNT2 gene in cardiomyopathy patients. DNA was isolated from both groups (patients and control), and amplification of TNNT2 by using new set of primers to amplify 443 bp from (rs74315380, rs74315379) SNPs and 351bp from (rs3729547) SNP for being use in sequencing portion, and target SNPs will be involved in this region. The genotypes frequency of the rs3729547 SNP showed highly significant ($p<0.001$) different between patients and healthy group. Similarity, there is significant ($p<0.05$) differences among CC, TC and TT genotypes according to patients and healthy individuals. The SNP rs3729547 genotypes frequency in dilated cardiomyopathy (DCM) and hypertrophic cardiomyopathy (HCM) showed significant ($p<0.05$) different between DCM and HCM patients related to TT genotypes, but not for CC and TC genotypes. Similarity, there is significant ($p<0.05$) different among CC, CT and TT genotypes according to DCM and HCM patients.

Keywords: Cardiomyopathy, Troponin T, TNNT2 gene, rs74315380, rs74315379 and rs3729547.

INTRODUCTION

Cardiomyopathies were defined as a diverse category of diseases characterized by structural and functional abnormalities of the myocardium that are not entirely due to coronary artery disease or abnormal loading circumstances (P. Elliott et al., 2008). Cardiomyopathies can be classified into five groups based on morphological and functional phenotypes: dilated cardiomyopathy (DCM), hypertrophic cardiomyopathy (HCM), restrictive cardiomyopathy (RCM), arrhythmogenic right ventricular cardiomyopathy (ARVC), and unclassified cardiomyopathies such as left ventricular non-compaction cardiomyopathy. These types can be further separated into genetic (familial) and non-genetic (non-familial) forms (P. Elliott et al., 2008).

Hypertrophic cardiomyopathy is the most common inherited cardiomyopathy, characterized by abnormal thickening of the ventricular walls in the absence of abnormal loading conditions, (Kramer et al., 2015). Hypertrophic cardiomyopathy is a common cardiomyopathy with maintained left ventricular function (P. M. Elliott et al., 2014) . Affected people have been shown to have a variety of genetic alterations (Lüscher, 2016). Dilated Cardiomyopathy is inherited in around half of cases and is linked to more than 50 genes, most of which code for sarcomere and cytoskeletal proteins (Burke, 2016; McNally, 2017).

Study have found 27 genes linked to HCM and 32 genes linked to DCM, including two X-linked genes (Hershberger & Siegfried, 2011). Sarcomeric contractile protein are encoded by the vast majority of these genes (Landstrom & Ackerman, 2010). Nearly 100 genes whose mutations cause various types of cardiomyopathies have been discovered in recent decades.

Troponin is a protein that is secreted when cardiac cells are injured. It can only be detected in the heart muscle, making it valuable for detecting heart muscle injury (Mehta et al., 2015). The troponin complex, which is responsible for myocardial contractility, contains the majority of troponins (approximately 95%) of the overall number. Troponins are also freely located in the cytoplasm of cardiomyocytes, where they do not participate in the regulation of contraction and relaxation in the heart (Chaulin & Duplyakov, 2020; Duplyakov & Chaulin, 2019).

The TNNT2 gene (OMIM number 191045) genes for cardiac troponin T is a 16-exon gene that is found on chromosome 1q32 and covers 25 kb of the genome. Its cytogenetic location in1q32.1, which is the long (q) arm of chromosome 1 at position 32.1. The

molecular location from base pairs 201,359,014 to 201,377,828 on chromosome 1 (Homo sapiens Updated Annotation Release

109.20200228, GRCh38.p13) according to the National Center for

Biotechnology Information (NCBI). This gene has other names like cardiac muscle troponin T, cTnT, LVNC6, RCM3, TNNT2_HUMAN TnTC, troponin T type 2 (cardiac), troponin T, cardiac muscle and troponin T2

The genes TNNT1, TNNT2, and TNNT3 encode three different sets of TnT isoforms for slow, cardiac, and fast skeletal muscle, respectively, alternative splicing produces additional isoforms in each group (Gangadharan et al., 2017) . In about 15% of HCM cases, mutations in the TNNT2 gene have been found (Moore, Abdullah, Tardiff, & biophysics, 2014). Mutations of the gene TNNT2 produce modest cardiac hypertrophy in an autosomal-dominant manner, but they can also result in a high rate of sudden cardiac death (SCD) (Gangadharan et al., 2017).

Over the last few decades, genetic screening has found a number of autosomal inherited mutations as potential 'drivers' or susceptibility factors for cardiomyopathy, with genetic alterations accounting for 40–50% of all instances (Paldino et al., 2018). Genes coding for sarcomere proteins are frequently reported to be defective in both DCM and HCM, with sarcomere genes accounting for 30–40% of DCM-associated mutations and 60% of HCM-associated mutations, respectively (Veselka, Anavekar, & Charron, 2017). Truncating mutations in TTN (TTNtvs) are seen in 25% of end-stage DCM patients and 15% of ambulatory DCM patients (Roberts et al., 2015). Other sarcomere genes with DCM mutations, including as MYH7, MYBPC3, TNNT2, and TPM1, change different residues than HCM mutations and are significantly less prevalent (Marian, van Rooij, & Roberts, 2016).

Most HCM cases are caused by mutations in genes that encode sarcomere proteins, the majority of human TNNT2 mutations are found in the central and C-terminal domains of cardiac troponin T, and they cause both familial and sporadic cardiomyopathy (McConnell et al., 2017). Furthermore, single sarcomere gene mutations, such as cardiac troponin T (TNNT2), are enough to produce cardiomyopathy, and TNNT2 mutations are the most common 'drivers' of thin filament deficit in both DCM and

HCM (Hershberger, Hedges, & Morales, 2013; Veselka et al., 2017). The aim of this study is to detection of TNNT2 gene polymorphism in infected cardiomyopathy Iraqi patients.

MATERIAL AND METHODS

This cohort study was performed on eighty-two adult's cardiomyopathy diagnosed patients, with age range of (20-70) years and the mean age was 47.78 ± 1.35 years during their attendance to Ibn Al-Bitar Center for Cardiac Surgery, Baghdad, also thirty healthy subjects with same age range and mean of 48.53 ±1.64 years were enrolled in this study. The study was conducted from October 2020 to March 2021, written informal consent was obtained from all patients and the study was approved by ethical committees: Ref.: CSEC/0920/0052, September 15, 2020 of department of Biology, College of Science, University of Baghdad.

In this work, 443bp ,351bp were amplified an active binding site of TNNT2 and sequenced using sanger method in order to identify any genetic variability contributing to (DCM and HCM) disease susceptibility among Iraqi patients. DNA was isolated from both groups (patients and control), and amplification of TNNT2 by using by using a DNA extraction kit Norgen's Blood DNA Isolation

Mini Kit. new set of primers to amplify 443 bp from (rs74315380, rs74315379) SNPs and 351bp from (rs3729547) SNP for being use in sequencing portion, and target SNPs will be involved in this region (see Table 1).

Fifty microliter of PCR amplification reaction contained 25 µl from OneTaq (NEB®) master mix, 8 µl of DNA sample, 4 µl 10 pmol/µl from each primer and 9 µl of free-nuclease water. The reaction done under the optimal PCR conditions for this gene. More than 40µl PCR product from each sample have been send to macro gen- Korea for sequencing by Sanger method to identify the single nucleotide polymorphism. Analysis of sequence FASTA files have been done by Geneious Prime software and aligned to Ref Seq of TNNT2 gene with accession number (NG_007556.1: g.17424C>T). The source of all primers used in this study was Macrogen® (Korea). The name, sequence and product size are given in table (2).

The Statistical Analysis System- SAS (2012) program was used to detect the effect of difference groups in study parameters. T-test was used to significant compare between means. Chi-square test was used to significant compare between percentage (0.05 and 0.01) probability in this study.

RESULTS

In this study, 82 patients with cardiomyopathy and 20 healthy individuals were sequenced performed the first primer and analyzed by comparing with the reference sample available in the Gen Bank or National Center for Biotechnology Information (NCBI) By using Basic Local Alignment Search Tool (BLAST) which is available at the NCBI.

The SNP rs3729547 location is in chr1:201365254 and its nucleotide location is 17424 and the highest population MAF is 0.49 (See Figure 6 and 5 respectively). The SNP rs3729547 genotypes frequency in patients describe as follow: CC (5.8%), TC (45.0%) and TT (49.2%). While, in healthy group, the genotypes frequency was: 100.0% for CC, and 0.0% for both TC and TT genotypes. There is highly significant (p<00.001) different between patients and healthy group. Similarity, there is significant (p<0.05) differences among CC, TC and TT genotypes according to patients and healthy individuals.

T allele is in higher frequency in patients (65.05%) than healthy (0.0%), while C allele scored lowest percentage in patients than healthy (34.95% vs, 100.0%) respectively with significant (p<0.001) differences between study groups. Additionally, there was significant (p<0.001) differences between C and T alleles frequency according to patients, but not for healthy (See table 3).

The SNP rs3729547 genotypes frequency in DCM patients describe as follow: CC (4.1%), CT (40.8%) and TT (55.1%). Also, in H.C.M patients was: CC (9.09%), CT (54.54%) and TT (36.36%). There is significant (p<0.05) different between DCM and HCM patients related to TT genotypes, but not for CC and TC genotypes. Similarity, there is significant (p<0.05) different among CC, CT and TT genotypes according to DCM and HCM patients.

T allele is in higher frequency in DCM and HCM patients (68.12% and 58.83%) versus C allele (31.88% and 41.17%) respectively with significant (p<0.05) different for T allele but not for C allele. Conversely, there is significant (p<0.05) different between C and T alleles frequency according to DCM, but not HCM patients (See table 4 and Figure 1 respectively). The rs74315379, rs74315380 SNPs are in chr1:201364336, chr1:201364366 and their nucleotide locations are 18342,18312 respectively (See Figure 7 and 4 respectively).

Their genotypes frequency in patients describe as follow: CC (100%), TC (0.00%) and TT (0.00%). Also, in control group it was: CC (100%), TC (0.00%) and TT (0.00%). There is no significant (p>0.05) different between patients and control group. Similarity, there was no significant (p>0.05) different among CC, TC and TT genotypes according to patients, and control. C allele is in higher frequency (100%) in patients and control while T allele has no frequency in healthy (0.00%) with no significant (p>0.05) different between patients and control group. Conversely, there was significant different between C and T alleles frequency according to patients and control group (See table 5, Figure 2 and 3 respectively).

DISCUSSION

In this study there is significant (p<0.001) different between patients and control group, this result was similar with a study of (Rani et al., 2012), that found the rs3729547 SNP has recently been linked to DCM in the Han Chinese population and hypertrophic cardiomyopathy (HCM) in the Indian population. This SNP was found in the pro band as well as both at-risk sons, and with other previous research has shown that the same genetic mutations can cause HCM as well as DCM (Nanni et al., 2003), though the mechanisms are unknown. Subsequent research has revealed that the same genetic mutations can result in different manifestations of HCM and DCM, which may be due to different functions of specific gene expression in these disorders (Debold et al., 2007; Robinson, Griffiths, Watkins, & Redwood, 2007).

The TNNT2 gene (OMIM number ∗191045) is known to encode the cardiac muscle-specific isoform of troponin T, which binds the troponin complex to tropomyosin in the thin filament of the sarcomere and regulates cardiac muscle contraction (Gao et al., 2020). The TNNT2 gene has 17 exons and is found on chromosome 1q32.1. Other study mentioned that 5% of HCM have been genotyped for TNNT2 mutations (Maron, Bonow, Cannon III, Leon, & Epstein, 1987). The TNNT2 polymorphism are of clinical interest due to their potential role in the development of severe cardiomyopathies. The patients who participated in this study had typical cardiomyopathies.

The most frequent type of genetic variation in the human genome is single-nucleotide polymorphism (SNP), and two large-scale SNP screens in European patients with DCM showed SNPs in multiple genes linked to DCM (Stark et al., 2010; Villard et al., 2011). Because the function of gene expression differs, studies have discovered that the same genes can cause HCM and DCM (Hershberger et al., 2013; Hitomi et al., 2010). Hypertrophic cardiomyopathy (HCM) is the most frequent kind of cardiomyopathy, affecting at least one out of every 500 people, and is the primary cause of sudden cardiac death (SCD) in adolescents and young adults (Lee & Pahl, 2021) .Mutations in sarcomere genes or sarcomere-related proteins have been identified with strong causal evidence of relationship with ventricular hypertrophy typical of HCM (Lipshultz et al., 2019)

Variants in TNNT2 are expected to change the characteristics of myofilaments. TNNT2 variations have been linked to a number of myofilament alterations, which could explain the higher energy (oxygen) consumption and decreased

myocardial efficiency. Higher myofilament Ca+2 sensitivity, which coincides with increased ATPase activity, is a typical trait of myofilaments containing TNNT2 mutations (Witjas-Paalberends et al., 2014). DCM is the third most common cause of heart failure and the most common reason for heart transplantation. Twenty to fifty percent of patients with so-called idiopathic DCM have a genetic cause (Li et al., 2013)

Polymorphisms in the TNNT2 gene have been linked to severe cardiomyopathies, rs3729547 is a single nucleotide polymorphism (SNP) that has been linked to DCM in the Han Chinese population and Hypertrophic cardiomyopathy (HCM) in the Indian population. This SNP was found in the proband as well as both of his at-risk sons (Selvi Rani et al., 2012). The genotype distributions and allele frequencies at the rs3729547 polymorphism loci between patients with DCM and HCM showed statistically significant differences, this was similar to the results of (Li et al., 2013), they showed that rs3729547 (C to T), and another TNNT2 tagging SNP were found to be associated with DCM in a Han Chinese population.

Previous research has shown that the same genetic polymorphism can cause HCM as well as DCM (Nanni et al., 2003), though the mechanisms are unknown. Subsequent research has revealed that the same genetic polymorphism can result in different manifestations of HCM and DCM, which may be due to different functions of specific gene expression in these disorders (Debold et al., 2007; Robinson et al., 2007). DCM with a genetic component account for about half of all cases. Proteins found in the sarcomere, cytoskeleton, nuclear envelope, sarcolemma, ion channels, and intercellular junctions are made by genes involved in this process. Specific mutations in these genes are likely to affect various pathways and alter various structures and mechanisms in the myocardium (Mestroni, Brun, Spezzacatene, Sinagra, & Taylor, 2014)

Pathogenic genetic mutations are the primary cause of DCM. It has been proposed, for example, that mutations in genes encoding contractile proteins cause functional changes and contractile dysfunction in cardiomyocytes (Robinson et al., 2007). The majority of DCM patients have autosomal dominant genetic disease, though there have been reports of recessive, X-linked, and other inheritance patterns (Roh et al., 2014). Compared to other HCM-associated variants, hearts from patients with cardiac troponin T (TNNT2) variants often exhibit much less ventricular hypertrophy than hearts from patients with other HCM-associated variants, because of their limited clinical penetrance, TNNT2 mutations are difficult to identify by echocardiography (Tadros et al., 2020)

Most sarcomeric HCM mutations cause an increase in Ca+2 sensitivity of the contractile apparatus, less energy efficient cross-bridge cycling, a faster cross-bridge turnover rate, and impairment of relaxation, according to in vitro studies (Marston et al., 2012). Hypertrophy cardiomyopathy hearts with TNNT2 polymorphism had less hypertrophy and fibrosis, but more severe myocyte disarray, according to (Varnava et al., 2001). Patients with TNNT2 mutations were also younger and died from sudden cardiac death (SCD) more frequently. Instead of hypertrophy or fibrosis, the increased risk of SCD in TNNT2 patients could be attributed to significant myocyte disarray or myofilament Ca+2 sensitization. SCD and ventricular arrhythmias are linked to troponin alterations that increase myofilament Ca+2 sensitivity (Huke et al., 2013).

To our knowledge, this is the first study in the Iraqi population to show a link between DCM and SNPs in the TNNT2 gene. DCM is regarded as a diverse disease. The current study suggests that rs3729547 in TNNT2 gene may be involved in the pathogenesis at least a subset of DCM patients.

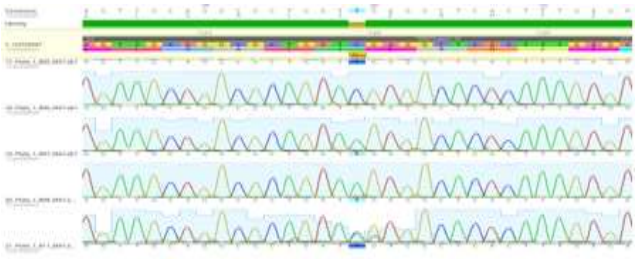

Figure 1: TNNT2 sequence alignment showing rs3729547 SNP in patents with DCM and HCM by using genius software with Gen Bank database for SNP.

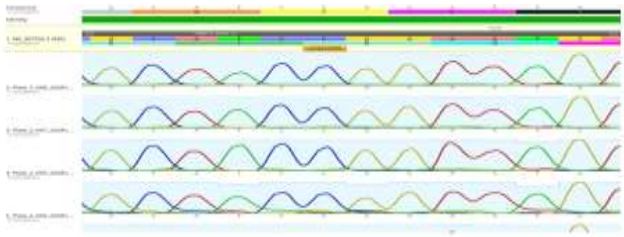

Figure 2: TNNT2 sequence alignment showing rs74315379 SNP.

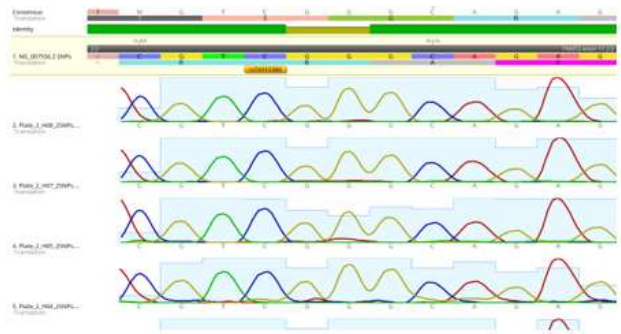

Figure 3: TNNT2 sequence alignment showing rs74315380 SNP.

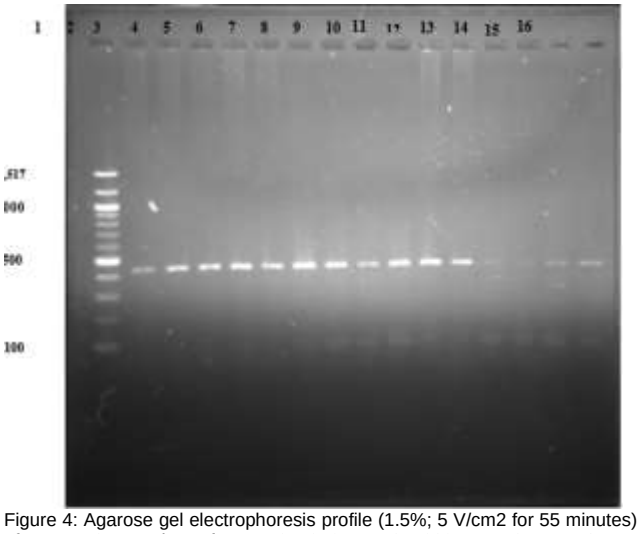

Figure 4: Agarose gel electrophoresis profile (1.5%; 5 V/cm2 for 55 minutes) of DNA–PCR products for rs74315379, rs74315380 TNNT2 gene SNPs. These SNPs showed two alleles (443bp); C allele and T allele. Genotypes of each SNP is indicted for each sample. M: Lane (M) refers to DNA ladder (100bp).

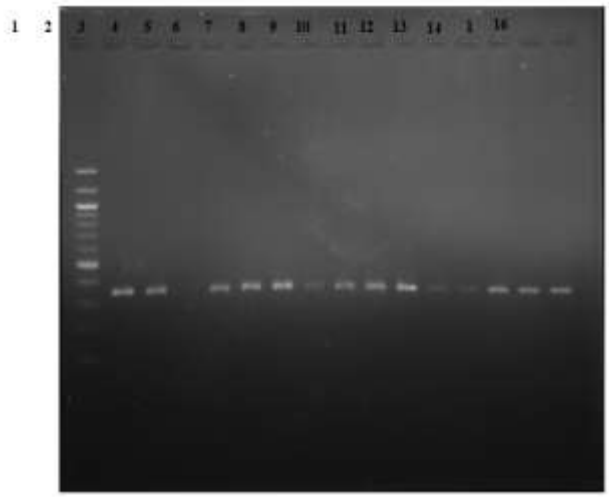

Figure 5: Agarose gel electrophoresis profile (1.5%; 5 V/cm2 for 55 minutes) of DNA–PCR products for rs3729547 TNNT2 gene SNPs. This SNP showed two alleles (351bp); C allele and T allele. Genotypes of each SNP is indicted for each sample. M: Lane (M) refers to DNA ladder (100bp).

Figure 6: Gene graph showing the location of the rs3729547 synapse on the TNNT2 gene in the chromosome 1, located in the exon 10.

Figure 7: Gene graph showing the location of the rs74315379, rs74315380 synapse on the TNNT2 gene in the chromosome 1, located in the exon 11.

Table 1: The name, sequence and product size of primers used in this study.

Name of Primer	Sequence	Product size (bp)	Reference
TNNT2 (2-SNPs)	F-GTTTCTGTACCTGCGATGTCA R-TCCTGAGTAGCTAGGATGACC	443	Newly Designed
TNNT2 rs3729547	F-GAGGTCTTTTGCACTGCGTT R-CCTCTGAAGGAGACCTCACA	351	Newly Designed

Table 2: The name, sequence and product size of primers used in this study.

Name of Primer	Sequence	Product size (bp)	Reference
TNNT2 (2-SNPs)	F-GTTTCTGTACCTGCGATGTCA R-TCCTGAGTAGCTAGGATGAC C	443	Newly Designed
TNNT2 rs3729547	F-GAGGTCTTTTGCACTGCGTT R-CCTCTGAAGGAGACCTCACA	351	Newly Designed

Table 3: Comparative genotypes and alleles frequency of SNP rs3729547 between patients and control group diagnosis by chi-square test and based on Hardy-Weinberg Equilibrium.

Genotypes			Groups		Total	P value	OR (C.I.)
			Patients	Healthy			
SNP rs3729547 C>T	CC	N	4	20	24	Reference value	
		%	5.8%	100.0%	26.37%		
	TC	N	32	0	32	P<00.001***	0.003 (0.001-0.06)
		%	45.0%	0.00%	35.16%		
	TT	N	35	0	35	P<00.001***	0.003 (0.0015-0.06)
		%	49.2%	0.00%	38.46%		
	Total	N	71	20	91	OR= Odd Ratio C.I.= Confidence intervals	
		%	100.0%	100.0%	100.0%		
	P value		P<0.01**	1.00	p>0.05		
	C	N	36	20	56	p<00.001***	0.013 (0.007-0.22)
		%	34.95%	100.0%	45.52%		
	T	N	67	0	67		
		%	65.05%	0.00%	54.48%		
	Total	N	103	20	123	OR= Odd Ratio C.I.= Confidence intervals	
		%	100.0%	100.0%	100%		
	P value		P<0.01**	1.00	p>0.05		

Table 4: Comparative genotypes and alleles frequency of SNP rs3729547 between DCM and HCM patients' diagnosis by chi-square test and based on Hardy-Weinberg Equilibrium.

Genotypes			Diagnosis		Total	P value
			DCM	HCM		
SNP rs3729547 C>T	CC	N	2	2	4	1.00
		%	4.1%	9.09%	5.8%	
	TC	N	20	12	32	p>0.05

		%	40.8%	54.54%	45.0%	
	TT	N	27	8	35	P<0.01**
		%	55.1%	36.36%	49.2%	
	Total	N	49	22	71	P<0.01**
		%	100.0%	100.0%	100.0%	
	P value		P<0.001***	P<0.05*	p>0.05	
	C	N	22	14	56	
		%	31.88%	41.17%	45.52%	p>0.05
	T	N	47	20	67	P<0.01**
		%	68.12%	58.83%	54.48%	
	Total	N	69	34	123	
		%	100.0%	100.0%	100%	
	P value		P<0.01**	p>0.05	P>0.05	P<0.01**

Table 5: Comparative genotypes and alleles frequency of rs74315379and rs74315380 SNPs between patients and control groups diagnosis by chi-square test and based on Hardy-Weinberg Equilibrium.

		Patients		Control		Chi- Square	
		No	%	No	%	(χ2)	P-value
SNP rs74315379	Genotypes						
	CC	60	100	18	100	0.00 NS	1.00
C>T	CT	0	0.00	0	0.00	0.00 NS	1.00
	TT	0	0.00	0	0.00	0.00 NS	1.00
	Total	60	100%	18	100%		
	C	1		1			
Allele frequency	T	0.00		0.00			
	NS: Non-Significant.						

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