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Genetic Polymorphisms SNP (rs5925) of *LDLR* Gene Associated with Familial Hypercholesterolemia in Iraqi Patients

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Abstract: The low density lipoprotein receptor (*LDLR*) allele status is the predominant hypercholesterolemia genetic risk factor. Functional single nucleotide polymorphisms (SNPs) in human *LDLR* gene receptors represent an excellent nominee for association with hypercholesterolemia. So, a common SNP (c.1959T>C ; p.Val653Val , exon 13 , rs5925) in *LDLR* gene was studied using Real-Time PCR and restriction fragment length polymorphism (PCR-RFLP) techniques to show association between *LDLR* SNP with Familial hypercholesterolemia . Seventy of Familial hypercholesterolemia patients who were clinically diagnosed by physician and 30 apparently healthy individuals were conducted within this study. Blood samples were collected from all subjects after 12-14 hour fasting. Genomic DNA was extracted from blood samples and analyzed for rs5925 SNP in *LDLR* gene with specific primers and probes using Real-Time PCR technique. Also, genomic DNA was amplified by conventional PCR with specific primers for detection of this SNP using PCR-RFLP technique. Using two methods in identification of rs5925 SNP for *LDLR* gene in this study come in different performance success percentage of the methods. Where, the Real-Time PCR gave 100% performance success for all subjects, while PCR-RFLP gene gave only 64% performance success for FH patients and 70% for control group. On the other hand, when the two methods were success to be done they gave fairly close results. In Real-Time PCR and PCR-RFLP, FH patients appeared CC homozygous genotype and TC heterozygous genotype significantly higher than in control group while the control group showed significantly increasing in TT homozygous when compared with FH patients. In comparison of the allele frequencies of C and T of *LDLR* gene, FH patients showed that the variable allele C was higher than T allele within this group. This association may be observed between allele polymorphism and risk of FH.

Key words: Familial Hypercholesterolemia, Genetic polymorphisms, *LDLR* gene, SNP (rs5925), Real-Time PCR, and RFLP.

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Introduction:

Familial hypercholesterolemia (FH), is an autosomal dominant inborn error resulting in the loss or damage of low-density lipoproteins receptors (*LDLR*) which leading to an elevated levels of low density lipoprotein cholesterol (LDLc) and total cholesterol (TC) in the circulation as it is dangerous in the incidence of tendon xanthomas and accelerated atherosclerosis, leading to premature cardiovascular events and premature death. The most cause of FH and the loss or damage of LDL's receptor results from the presence of forms of genetic damages in the encoded gene that is located on the

short arm of chromosome 19 (p 13. 3-1. 13) and lead to damage in the transport or metabolism of Low-density lipoprotein (LDL) (1, 2, 5). Familial hypercholesterolemia is one of the most common inherited disorders. There are 10,000,000 people with FH worldwide, mainly heterozygotes. SNP (rs5925) was associated with decreased LDLR splicing efficiency and increased levels of total LDL and cholesterol [11]. Many studies reported that polymorphisms in the *LDLR* gene are associated with FH (3, 4, 8). Early detection of the affected gene can reduce the risk of atherosclerosis and myocardial infraction through the use of available curative methods (6). To our knowledge, the genetic spectrum of familial hypercholesterolemia has not been extensively studied in Iraq. This study aimed to determine the genotype distribution and allele frequency of common polymorphism in *LDLR* gene (rs5925 SNP) in some FH Iraqi patients using two methods Real-Time PCR and PCR-RFLP, and investigate whether this rs5925 SNP was influencing the risk for FH; as well as to estimate the efficiency and accuracy of the best method between the two methods used to identify this SNP.

Materials and method:

Seventy FH Iraqi patients (40 female, 30 male) who were clinically diagnosed , have been included in this study and thirty apparently healthy individuals (17 female, 13 male) as control group were included during this study (pregnant ladies were not enrolled) . All subjects have been informed about the aim of this study and asked to fill in the questionnaire form .Venous blood samples was withdrawn from all subjects after overnight fasting of 12 -14 hours.

The DNA samples were extracted using genomic DNA extraction kit (Blood) (WizPure^{TM}, Korea) according to the manufacturer instructions. Specific previously reported primers [7] were used to amplify a specific region of *LDLR* gene that contains rs 5925 SNP(F-**5**´-CTCACAGGTTCCGATG TCAA-3´;R CAAGAGGTAAGGGTG GGTCAG - 3´), and designed probe (Light Cycler® Probe Design Software 2 was used for the design of assay probes) : Dye Fam – BHQ : 5´-TATGGTTCTCTTCCACAACCT-3´ and Dye Vic –BHQ: 5´-GGTCCTCTTCCACAACCTC-3´. Primers and Probes were supplied from the company (Alpha DNA, Canada) as a lyophilized product of different picomols concentrations. They were dissolved in a free nuclease water according to the manufacturer instructions to give a final concentration of (100 pmol/µl), which was used as a stock solution; 10 µl of this solution was added to 90 µl of free nuclease water to obtain 10 pmol/µl final concentration that is utilized in conventional PCR and Real – Time PCR techniques. Genotyping - allelic discrimination of rs5925 SNP in the regulator region of *LDLR* gene was done using PCR-RFLP and Real-Time PCR.Real time PCR was carried out with 20µl reaction mixtures consisting of : 10 µl TaqMan master mix ,1 µl forward primer ,1 µl revers primer,1 µl Vic probe ,1 µl Fam probe , 3 µl DNA templet , 3 µl nuclease free water . Real-time PCR was performed on an ABI Prism 7700 Sequence Detection System (SDS, PE Bio systems) using the following cycling conditions: (The cycling conditions were 95°C for 10 min, 35 cycles of 95°C for 5 s, and

60°C for 20 s.) (7). Each sample was run in duplicate. For quality control, three negative controls were included in each plate, and 5% of the samples were randomly selected for repeated genotyping to verify the results; all of the results were 100% consistent. The statistical analysis system - SAS program was used to show the effect of difference factors in parameters of study. For PCR-RFLP polymerase chain reaction was carried out after several attempts to amplify. PCR was carried out with 25µl reaction mixtures consisting of: 12.5 µl PCR pre Mix; 1 µl Forward primer; 1 µl Reverse primer; 3 µl DNA template and 7.5 µl D.W. PCR amplification was with the following program conditions : 1 cycle for Initial denaturation at 95 ºC for 5 minute ; 35 cycles of (Denaturation at 95 ºC for 30 minute , Annealing at 60 ºC for 30 minute, Extension at 72 ºC for 30 sec) and 1 cycle for Final extension at 72 ºC for 5 minute(7). The PCR products (7µl) had been analyzed by electrophoresis using 2% agarose gel at 100 volt for 45 min. *AvaII* restriction enzyme (Bio Labs, England) which recognizes G^GWCC sites and cuts best at 37°C was used with PCR-RFLP .Genotyping of rs5925 SNP for *LDLR* gene was detected using PCR-RFLP with a mixture component of total volume 20 µl : (10 µl of PCR product , 0.5 µl of *AvaII* Enzyme ,2 µl of Buffer, 7.5 µl of D.W.). The PCR-RFLP mixture components were incubated in water bath for 4 hr. at 37 ºC. The PCR-RFLP product was analyzed on 3.5% agarose gel by electrophoresis with a starting 25 volt for 30 min, then it has been increased to 70 volt for 1hr.The electrophoresed DNA has been visualized under UV light using UV trans illuminator.

Statistical Analysis:

The Statistical Analysis System-SAS (2012) program was used to effect of difference factors in study parameters. Least significant difference –LSD test was used to significant compare between means. Chi-square test was used to significant compare between percentages in this study.

Results and Discussion:

A. Real-Time PCR:

Real-time quantitative PCR is an efficient method for high-throughput genotyping of SNPs. In the present experiment Taq Man Real Time PCR were applied to detect the (rs5925 SNP) (c.1959 T>C; p.Val653Val.Exon 13) in *LDLR* gene. Using Real-Time PCR genetic variation of *LDLR* gene (rs 5925 SNP) was identified in 70 FH Iraqi patients. Remarkable genotypes and allelic frequency of *LDLR* gene polymorphism in FH patients were differ significantly ($p<0.05$) when compared with healthy individual (Table 1). In FH patients group, the homozygous (CC) genotype (44%) was significantly elevated ($p<0.05$) when compared with other genotype with O.R. 1.539 indicating that this genotype represent a risk factor of FH occurrence; followed by mutant heterozygous TC (39%) .In the other hand, homozygous TT genotype was highly significant ($p<0.01$) increase (70%) in control group than other genotypes with O.R.=1.722 indicating that this genotype is a protective factor against FH. This conclusion is supported by that as FH patients with family history were 60 out of total 70 patients , 31 out of 60 (52%) FH patients with family history were with CC genotype and 27 /

60 (45%) of those patients were with TC genotype while only 2 / 60 (3%) were with TT. Comparison of the allele frequencies of C and T of *LDLR* gene between FH patients showed that the variable allele C was higher than T allele, it was (0.64and 0.36, respectively).

Table (1): Distribution of genotype and allele frequency in FH patients and control group for *LDLR* gene exon 13(rs5925 SNP)by real-time PCR

Genotype	Patients	Control	Chi-square	O.R.
TT	12(17%)	21(70%)	11.24**	1.722
TC	27(39%)	9(30%)	4.294*	0.641
CC	31(44%)	0(0%)	9.831*	1.539
Total	70(100%)	30(100%)	------	------
Allele Frequency				
T	0.36	0.85	------	------
C	0.64	0.15	------	------

O.R.: Odd Ratio. *significance: (P<0.05), **highly significance (P<0.01)

These results which refer to the relationship between the frequency of this SNP and FH are agree with another study that reported the association between the C allele of the rs5925 SNP and higher levels of cholesterol (10). The same finding was reported by (9), who demonstrating that the CC genotype associates to a higher concentration of total and LDL-C and total cholesterol when compared to TC and TT genotypes. In Brazil, (12) also found that even though that there was no amino acid change within this SNP (c.1959 T>C; p.Val653Val.Exon 13.rs5925), it is associated with high levels of cholesterol and LDL cholesterol. Rafiq, *et al*. (11) identified 2 SNPs within *LDLR* gene which were located in exon 12 (rs688 SNP) and exon13 (rs5925) among Pakistan population; an association between these SNPs and increased levels of cholesterol was noted.

B. PCR-RFLP

The DNA fragment (108 bp) including rs5925 SNP in the *LDLR* gene was targeted for amplification by PCR technique. Figure 1 shows the amplified fragment (108 bp) of *LDLR* gene.

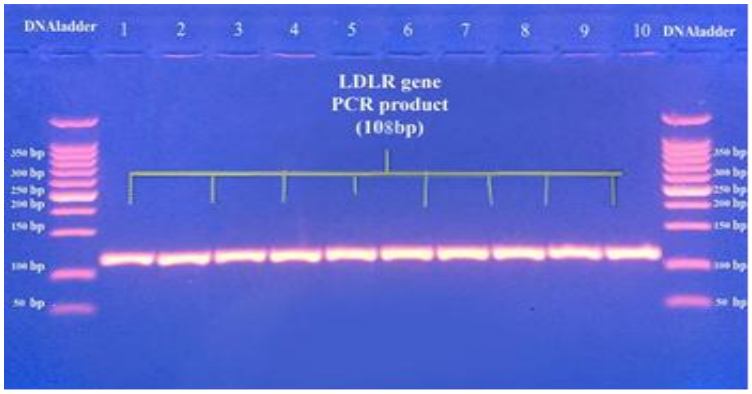

Figure (1): PCR product (108 bp) of targeted fragment (exon 13 of *LDLR* gene) visualized under UV light after staining with ethidium bromide. The electrophoresis was on 2% agarose gel at 70 volt for 90 minutes.

Regarding to rs 5925 SNP in *LDLR* gene, only 45 out of 70 (64%) FH patients and 21 out of 30 (70%) healthy individual gave a result for digesting the targeted fragment (108 bp) with *AvaII* restriction enzyme within PCR-RFLP technique (figure 2). Thus, the conversion of T allele (GGTTC) in to C allele (GGTCC) at c.1959 T>C in mutants created a specific site for *AvaII* enzyme (G^GTTC). Digestion of DNA product with *AvaII* enzyme resulting in three genotype profile :TT (108 bp); TC (108 , 58 and 46 bp) and CC (58 and 46 bp) (figure 2).

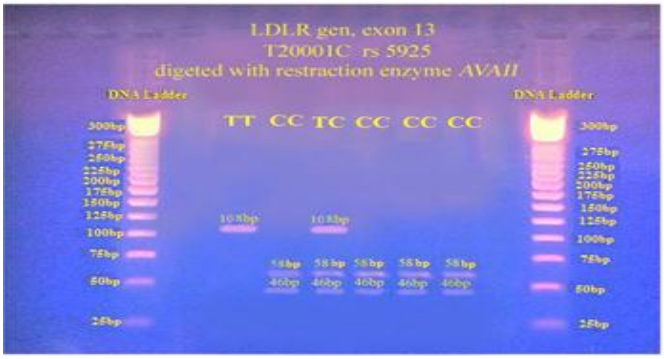

Figure (2): PCR product (108 bp fragment) of *LDLR* gene digested with *AvaII* restriction enzyme and electrophoresed on 3.5% agarose. The genotypes at exon 13 are: TT (108 bp), TC (46, 58 and 108 bp) and CC (46 and 58 bp).

With regard to *LDLR* gene polymorphism, the distribution of genotype and allele frequency of rs5925 SNP was presented in Table (2). The CC genotype frequency was significantly higher (O.R. =1.526; X2 =9.67), ($p<0.01$) in FH patients when compared with apparently healthy subjects (43 % *versus* 0%, respectively) ,while TC genotype frequency was statistically significant higher ($p<0.05$) in FH patients (37%) with O.R.=0.073 ; X^2 =0.893 .The frequency of TT genotype was highly significant ($p<0.01$) with O.R=1.481; X^2 =9.52 in apparently healthy subjects when compared with FH patients (73 % *versus* 20 %, respectively). Comparison of the allele frequencies of C and T of *LDLR* gene within FH patients showed that the variable allele C was higher than T allele, it was (0.61and 0.39, respectively). As a conclusion, there was a significant difference in the genotype and allele frequencies of *LDLR* gene between FH patients and apparently healthy subject group, referring to the present a relationship between these variants and the risk for FH.

Table (2): Distribution of genotype and allele frequency in FH patients and control group by using PCR-RFLP

Genotype	Patients	Control	Chi-square	O.R.
TT	9(20%)	15(73%)	10.527**	1.657
TC	17(37%)	6(27%)	4.263*	0.703
CC	19(43%)	0(0%)	9.841*	1.368
Total	45(100%)	21(100%)	------	------
Allele Frequency				
T	0.39	0.87	------	------
C	0.61	0.13	------	------

O.R.: Odd Ratio. *significance: (P<0.05), **highly significance (P<0.01)

C. Comparison between Real-Time PCR and PCR-RFLP Results:

Using two methods in identification of rs5925 SNP for *LDLR* gene in this study come in different performance success percentage of the methods. Thus, the real-time PCR gave 100%, performance success, while PCR-RFLP gene only 64% performance success for FH patients and 70% for control group. On the other hand, when the two methods were success to be done gave fairly close results (Table 3).

Table (3): Percentage of performance success for Real-Time PCR and PCR-RFLP in rs 5925 SNP identification

Genotype	FH Patients		Control group	
	Real-Time PCR	PCR-RFLP	Real-Time PCR	PCR-RFLP
TT	17%	20 %	70%	73%
TC	39 %	37%	30%	27%
CC	44 %	43%	0%	0%

This difference in the percentage of the performance success for the two methods related to the high accuracy of Real-time PCR which come from the possibility of recognition the amplified of targeted specific product at each cycle, also for the using sequence-specific probes containing a fluorescent reporter dye on the 5´ end and a quencher dye on the 3´ end to monitor the real-time PCR reaction.

Conclusion:

As conclusion this study shows many scientific aspects : results here pointed for CC genotype and C allele as a risk factor for FH , while TT genotype and T allele as a protective factor against FH ; Real-Time PCR more accurate in genotyping than PCR-RFLP in addition to that it is high-throughput and more clinical laboratory that it could be useful to monitor the inheritance of FH in Iraqi families rather than PCR-RFLP ;and the present study are helpful to provide information to other researchers about the gene variation analyzed in Iraqi FH patients which may be helpful for tailoring therapy in clinical practice .

In the light of the findings here, the present study recommend for further studies to evaluate the clinical significance of this rs5925 SNP in the *LDLR* gene, and recommend include large scale of Iraqi FH patients within such genetic study of *LDLR* gene variation. Study other SNPs in the *LDLR* gene and other genes (APOB, PCSK9) related with FH in Iraqi population. This study offers recommendation on DNA sequencing of different regions of LDL receptor to cover the complete targete *LDLR* gene region in Iraqi FH patients seeking for a new variants SNPs related with FH.

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