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Study the effects of STRA 6 gene polymorphism on the incidence of T2DM in a sample of Iraqi patients

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Abstract : This study was aimed to detect STRA6 gene polymorphism in Iraqi type 2 diabetes mellitus patients (T2DM) to found the correlation between the SNP (rs 736118) polymorphism in STRA6 gene and lipid metabolism and impact on the incidence of type 2 diabetes mellitus (T2DM) . Genomic DNA was extracted from the blood samples of T2DM patients and apparently healthy as a control group by using genomic DNA purification kits (Geneaid), the purity of the DNA was between 1.7 -1.9 and the concentration between 30 -100 ng / µl,. total cholesterol TC , high density lipids HDL, low density lipids LDL and Triglycride and fasting blood sugar FBS were measured by kits supplied by (Biolabo, France), while glycosylated hemoglobin HbA1C measured by kit supplied by (Nycocard, Norway). Mean value of triglycerides in diabetic patients was significantly (p<0.001). The result show high Significant difference was observed in FBS level (p<0.001) in patient group (183.47 ± 6.32) and in control (88.61 ± 3.70), in other hand high Significant difference was observed in HBA1C level was (p<0.001) in patient group (8.87 ± 0.21), while in control group was (4.86 ± 0.10). Cholesterol mean level value in diabetic patients was significantly higher than those of control group (p<0.001) , LDL-Cholesterol mean value in diabetic patients was statistically significant (p<0.005) higher than the mean value of control group, (p<0.001), Serum HDL-Cholesterol mean value was significantly (p<0.001), VLDL-Cholesterol mean value in diabetic patients was significantly (p<0.001) increased compared to the mean of control group. Real time PCR (Taq man) RT-PCR were used to detect SNP (rs 736118) C>T in STRA6 gene by using specific probes and primers, As a related with SNP (rs 736118) C>T in STRA6 gene , the percentage of those CT polymorphism genotype in the patients group was significant (p<0.001) compare with control group (58% *versus* 0% respectively) and the individuals with the CT genotype in the patients group is significantly higher than those with CC genotype, the percentage of those with TT polymorphism genotype in the patients group is significantly higher as compared with control group (6% *versus* 0% respectively . The percentage of those with CC polymorphism genotype in the patients group show highly significant differences as a compared with control healthy group (28%, 0% respectively), as a conclusion of this study there was a relationship between polymorphism of STRA 6 gene SNP (rs736118) and the incidence of T2DM in sample of Iraqi patients.

Key word: STRA6 gene , Diabetes mellitus , SNP rs736118 .

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Introduction:

Type 2 diabetes mellitus is illness for polygenic inheritance resulting from genetic factor and environmental factor(1,12). Furthermore the effect of the incidence of candidate genes are different , as well as the effect of gene – environmental interaction can add to different risk of T2DM that different individual suffer form (4,1). As a cell surface receptor ,stimulated by retinoic acid homology 6 (STRA6) ,is also a kind Of cytokines receptors , the gene locates on long arm of the chromosome 15q2 4.1 region and contains 20 exons and 19 introns, which encodes a protein of 667 amino acids, and is a cell surface receptor (3 ,1) the most studied SNPs STRA6 was

rs736118 in exon coding regions, which resulted in changes of protein function and effected the development of T2DM, SNP rs736118, C→T conversion will cause amino acid change of STRA6 from methionine to tyrosine, the location of this SNP is in the c-terminal of STRA6 (1,3), many different studies were conducted on this gene (1,3). The present study focused on Increasing Advanced knowledge about the occurrence of T2DM in over weight Iraqi patients examine the correlation between the SNP(rs736118) polymorphism in the STRA6 gene and lipid metabolism pathway and Detecting STRA6 gene polymorphism in Iraqi T2DM patients by using Real Time PCR (Tag Man).

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Materials and Methods:

This study conducted during the period from November 2016 to May 2017 at University of Baghdad / Institute of Genetic Engineering and Biotechnology for post Graduate Studies. The study consisted of 64 patient with type 2 diabetic were selected from those attended AL – Yarmuok Hospital . Their ages ranged between (25-65) years; patients taking insulin were excluded.

Twenty eight healthy control with normal fasting blood glucose (80–110 mg/dl). And age range between (25-65) years . Non diabetic control subjects were recruited from the same area as the comprising blood donors, healthy volunteers, or hospital / university . Written informed consent was obtained from all participants.

Genomic DNA extraction:

Total genomic DNA was extracted from the whole frozen blood which was collected in EDTA anticoagulant tubes for molecular studies and it was applied using genomic DNA purification kits (Geneaid ,Taiwan). Figure (1)

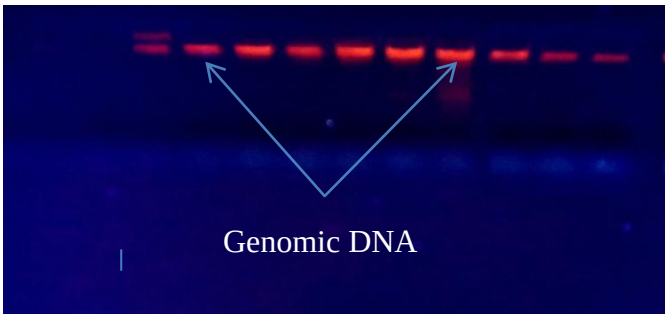

Figure (1): Gel electrophoresis of genomic DNA extracted from blood samples. 1% agarose gel at 5 volt /cm for 30 minutes, then visualized under UV after staining with ethidium bromid.

Total cholesterol TC , high density lipids HDL , low density lipids LDL and Triglycride and fasting blood sugar FBS were measured using kits supplied by (Biolabo, France), while glycosylated hemoglobin HbA1C measured using kit supplied by (Nycocard ,Norway).

Genotyping:

Genotyping was carried out For SNP rs736118 of STRA6 gene polymorphism analysis, DNA was amplified using the forward primer 5'-GTGGATGGCGTTGTAGAGGG -3' and Reverse primer 5'-

TTCTTCTCCCATCCTAGGCG -3 'and VIC – BHQ dye 5'-CCACTATGGCACCCAC-3' and Fam – BHQ 5'-CACCATGGCACCCAC-3' real time Taq man PCR was performed in a 20 µl total volume, Primer forward 1 µl(10 pmol) , Primer reverse 1 µl(10 pmol) ,Template DNA3 µl, (3- 6µg/ml) and 10 µl Taq –man master mix . Fam – BHQ 1 µl(10 pmol) , VIC – BHQ 1 µl (10 pmol), A total of 40 PCR cycles with denaturation at 95 ^{o}C for 15 sec., annealing for 40 Sec at 60 ^{o}C and extension at 72 ^{o}C for 15 Sec. (2).

Results and Discussion:

All serum lipid and lipoproteins were significantly higher in diabetic patients compared to healthy control group. In Figure (2), T. Cholesterol mean level value in diabetic patients was significantly(p<0.01) higher than control group .

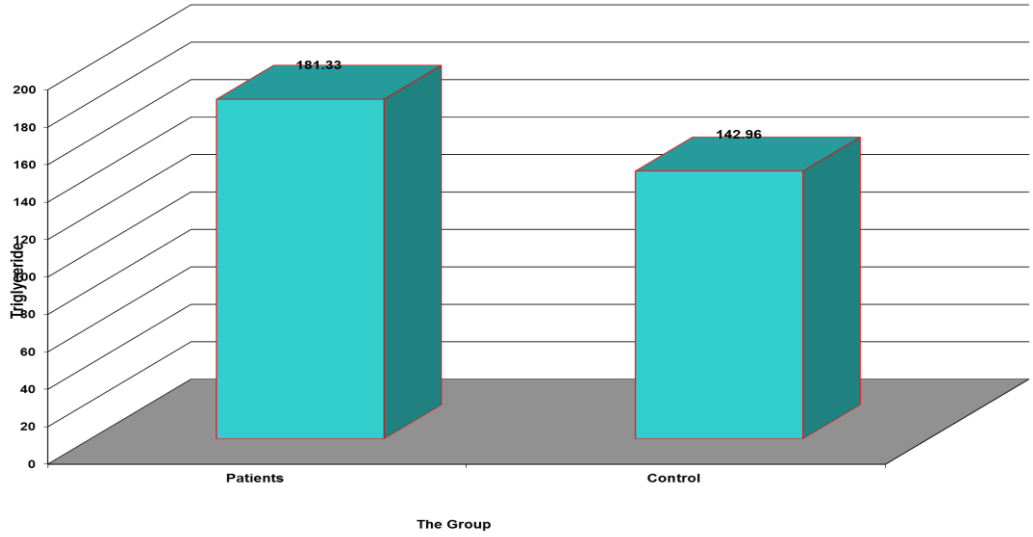

Figure (2): Compare between patients and control In T. cholesterol this increase may be due to an increasing in the plasma concentration of VLDL and LDL, (7).

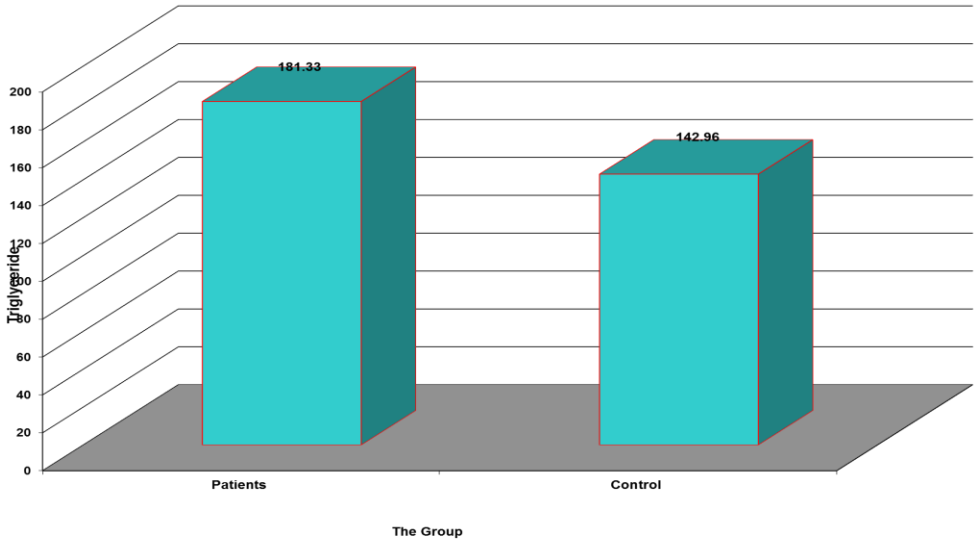

Figure (3): Compare between patients and control In Triglyceride level.

In Figure (3) mean value of triglycerides in diabetic patients was significantly (p=0.001) increased compared to mean of control group in (10) found that Sudanese diabetic patients may be due to overproduction of VLDL lead to increased plasma a levels of triglyceride which, via an exchange process mediated by cholesterol ester transfer protein (CETP), may be due to insulin deficiency which results faulty glucose utilization causes hyperglycemia and mobilization of fatty acids from adipose tissue (10).

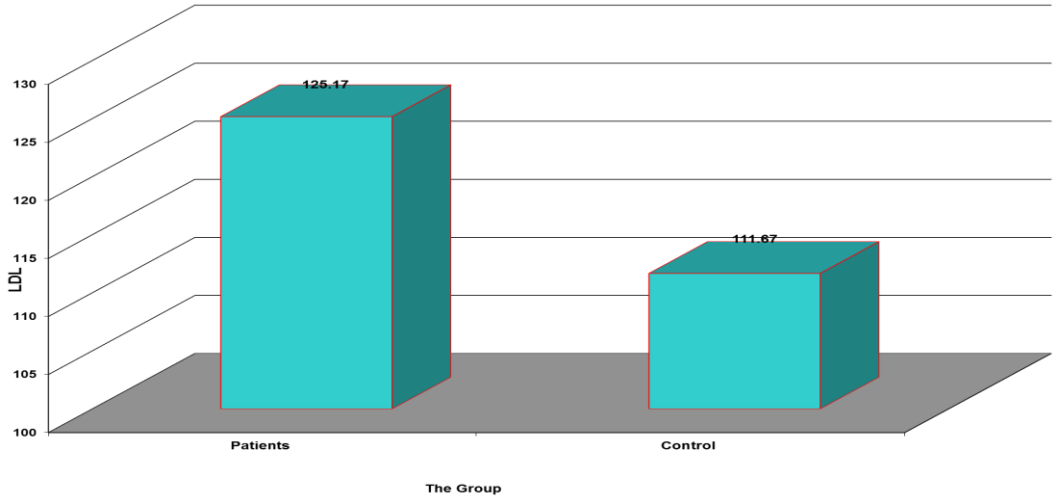

Figure (4): Compare between patients and control In LDL.

In Figure (4), LDL-Cholesterol mean value in diabetic patients was statistically significant (p=0.0199) higher than the mean value of control group , (p=0.001), increased level of LDL in diabetic patients due to insulin increases the number of LDL receptor, so chronic insulin deficiency might be associated with a diminished level of LDL receptor. This causes the increase in LDL particles and result in the increase in LDL-cholesterol value in diabetes mellitus (11).

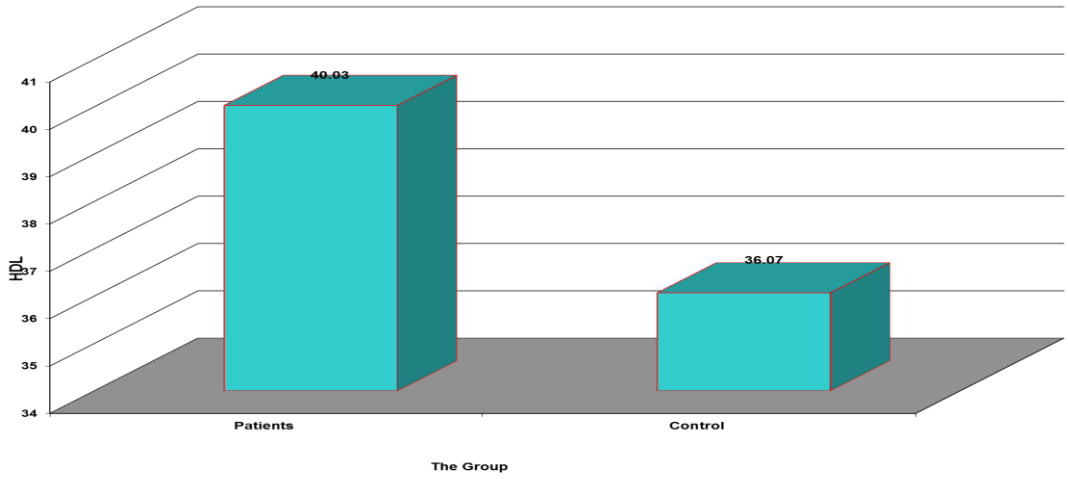

Figure (5): Compare between patients and control In HDL.

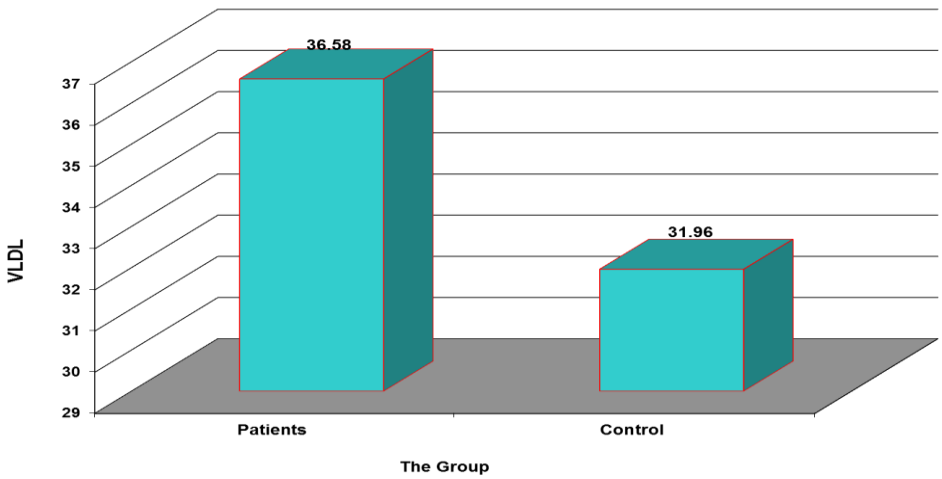

Figure (6): Compare between patients and control In VLDL.

In Figure (5) Serum HDL-Cholesterol mean value was significantly (p= 0.0036).

In Figure (6) VLDL-Cholesterol mean value in diabetic patients was significantly (P=0.00385) increased compared to mean of control group an increasein VLDL occurred in diabetes mellitus due to increase availability of glucose for VLDL synthesis and decrease in lipoprotein lipase activity leading to decrease of VLDL from peripheral circulation (8). This result agree with (7,9). The same results ($p<0.05$) were found between subgroups patients they compared to control. Elevated lipid and lipoprotein level in diabetic patients may be due to insulin resistance because impaired insulin action increase free fatty acid release from intra-abdominal adipose tissue promoting lipoprotein lipase activity results in reduced triglyceride clearance(7).

Distribution of genotype and allele frequency in patients and control:

Diabetic patients ,genotype frequencies of sixty four 64 diabetic patients were studied using Real-Time PCR to detected the presence of single nucleotide polymorphism of STRA6 gene (rs 736118), rs 736118 single nucleotide polymorphism is characterized by C>T , the genotype frequency of heterozygous type (CT) had the highest frequency at (90.63%) and genotype , and mutant type (TT)had the frequency at (9.37%), while the homozygous wild type had the lowest frequency at (0.00%) Table(1).

The allelic frequencies were calculated using genotype frequencies which indicate that the mutant alleles (CT) had a higher frequency (0.55) than the wildtype (C) (0.45) in diabetic patients Table (1).

Genotype frequency of twenty eight healthy control were studied using Real –Time PCR , the genotype frequency of homozygous wild type (CC) had the highest frequency at (100%) and genotype frequency of heterozygote (CT) and mutant type (TT) have the lowest frequency at (0.00%,0.00%) respectively. Table (1).

Allelic frequencies were calculated which indicate that wild allele (C) (1) had a higher frequency than mutant allele (T) (0.00) in healthy control Table (1).

For association studies , Chi-Square indicated significant association between the diabetic and control group (P <0.0001) for both genotype and allele frequencies , the odd ratio for genotype frequency was calculated as (CC) wild type O.R (2.00), and the odd ratio for heterozygous type (CT) was (1.82) and the mutant (TT)was (0.695).

However genotype frequency analyzed data revealed that as diabetics are more 1.82 times more likely to have a (CT) allele as compared to (CC) allele and about (0.695) times more likely to have a (TT) allele, that indicates that people with (CT) allele have (1.82) times higher risk of developing diabetes and those with (TT) allele have (0.695) times higher risk of developing diabetes as a compered to people with (CC) allele Table (1).

Table (1): Distribution of genotype and allele frequency in patients and control

Genotype	Patients	Control	Chi-square	P-value	O.R.
CC	**0 (0.00%)**	**28 (100%)**	**15.00****	**0.0001**	**2.00**
CT	**58 (90.63%)**	**0 (0.00%)**	**14.17 ****	**0.0001**	**1.82**
TT	**6 (9.37%)**	**0 (0.00%)**	**4.372 ***	**0.0442**	**0.695**
Allele frequency					
C	**0.45**	**1**	--	--	--
T	**0.55**	**0.00**	--	--	--

Table (2): Effect of genotype of gene in study parameters of patients

Parameters	Genotype		LSD value
	CT	TT	
FBS	186.89 ± 6.57	150.33 ± 19.17	42.742 NS
HbAIC	9.02 ± 0.22	7.43 ± 0.34	1.408 *
BMI	27.50 ± 0.44	25.26 ± 1.40	2.921 NS
Total cholesterol	203.71 ± 4.1sz8	191.50 ± 16.97	28.097 NS
Triglyceride	181.15 ± 5.91	183.00 ± 19.39	38.846 NS
LDL	126.39 ± 3.77	113.33 ± 14.25	25.119 NS
HDL	39.91 ± 0.79	41.17 ± 2.84	5.243 NS
VLDL	36.56 ± 1.12	36.67 ± 3.97	7.423 NS
*** (P<0.05), NS: Non-significant.**			

In the present study the association of SNP rs736118 polymorphisms of STRA6 gene in the incidence of type two diabetes mellitus was examined. The results of this study support the association between genetic variation of STRA6 gene SNP rs736118 polymorphisms and the increased risk of T2DM in Iraqi population. showed that the STRA6 ,SNP (rs 736118) was C/T dimorphism and was significantly associated . as their wild alleles are (C), and risk variants alleles are (T). their genotypes were wild homozygous (CC), heterozygote risk variant (CT), and homozygous risk variant (TT) ,as to the relation between SNP (rs 736118)of STRA6 and T2DM, result showed that frequencies distributions of genotype of SNP (rs 736118) was statically significant between patients and healthy control, Table(2).

Moreover the present study showed that parameters (FBS ,HbA1C, BMI, Total cholesterol, triglyceride, HDL, LDL and VLDL was not statically significant between genotype (TT) and (CT) in T2DM patients which firstly found and has not been reported previously showed in table (2), result of the resent study agreement with (1), In southern Han Chinese who found that genotype (TT) of SNP (rs 736118) on STRA6 was significantly associated

with T2DM and protected human against T2DM, and disagree with (3) who found that G/A on its complementary strand was associated with T2DM .

STRA6 act as a cell surface receptor of RBP 4 , which activated Jaknus kinase and signal transducer and activeter of transcription JAK2 – STAT5 signal pathway , induced production of suppressor of cytokine signaling (SOC-3) by combining with retinol –RBP complex ,and led to insulin resistance to promoter the occurrence of T2DM. For SNP of rs736118, C→T conversion will cause change of amino acid STRA6 from methionine to tyrosine, The location of this SNP is in the c-terminal of STRA6, which has been implicated in signal transduction via phosphorylation(1)

Tyrosine conversion could potentially have an impact on the signal transduction pathway (6). had been found that STRA6 in adipocyte may be important in the maintenance of normal body fat and physiological insulin action , it has been hypothesized that increased signaling through STRA6 could cause insulin resistance , while the reduction of STRA6 in adipocyte result in leanness and improved insulin sensitivity (3),as in Table (2).

That represent serum level of total cholesterol and high density lipoprotein and low density lipoprotein and very low density lipoprotein all has no difference between the T2DM patients in both genotype (TT) (CT) .

Furthermore this study proved an evidence that STRA6gene may be play an important role in increasing the susceptibility of type 2 DM , but this increase in number of risk allele had no effect on BMI, FBS, HbA1C on (Table 2).

Indicating that the increasing in the susceptibility of the T2DM is modulated through some other pathway most probably by effecting the measure of insulin resistance (3), This study involves a smaller sample size and the population is restricted to the Baghdad province of Iraqi , thus population based study with larger sample size needs to be carried out.

Allelic quantitative by real time PCR:

Real-time quantitative PCR is an efficient method for high-throughput genotyping of single nucleotide polymorphisms (SNPs).in the present experiment utilized the Taq Man Primers and probes for the TaqMan assays were designed by Alpha DNA Ltd (Canada), The probes for the two alleles were labelled with the reporter dyes FAM and VIC respectively. The TaqMan probe contains a reporter dye (6FAM, VIC) at the 5´ end of the probe and a quencher dye (TAMRA) at the 3´ end of the probe. During the reaction, cleavage of the probe 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. When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence. During PCR, if the target of interest is present, the probe specifically anneals to the target. The 5´ to 3´ nucleolytic activity of the AmpliTaq Gold, UP enzyme cleaves the probe between the reporter and the quencher only if the probe hybridizes to the target. In Figure (7) As amplification proceeds, the Taq polymerase enzyme cleaves the bound probe, and a fluorescent signal is generated.

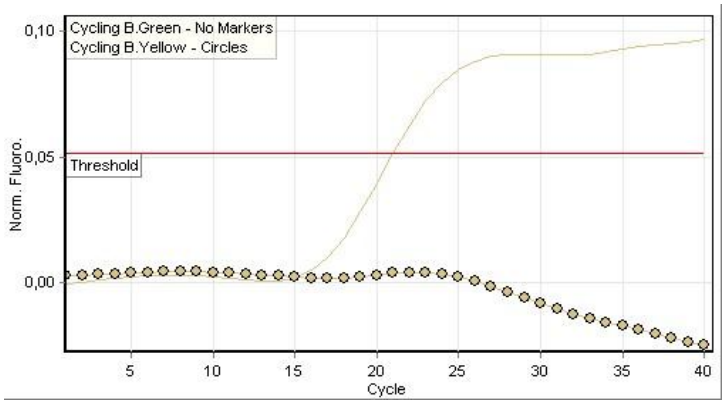

Figure (7 -A): Wild character of STRA6 gene , only Fam – BHQ is hybridyzed with DNA sample (sample is healthy NO. 13).

A - Fluorescent signals are interpreted automatically using sequence detection software dedicated to real-time PCR instrumentation A fluorescent signal from only the Fam dye indicates homozygosity for Allele "B" (wild type).

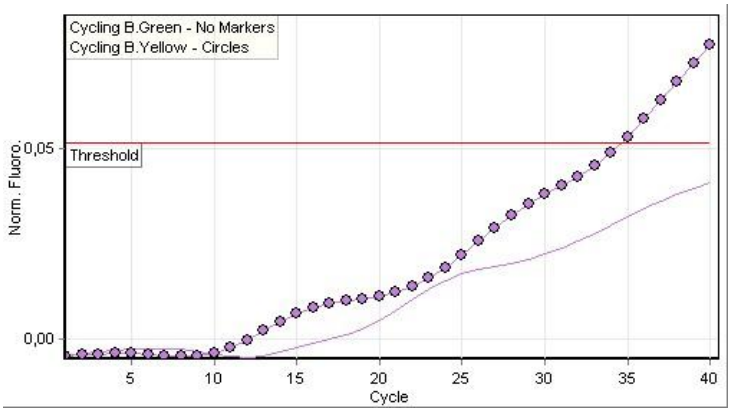

Figure (7-B): Mutant character of STRA6 gene , only VIC – BHQ is hybridized with DNA sample (sample is infected NO. 18).

B - A fluorescent signal from only the VIC dye indicates homozygosity for Allele "A" figure (7) here only curve for VIC dye only raising (Mutant).

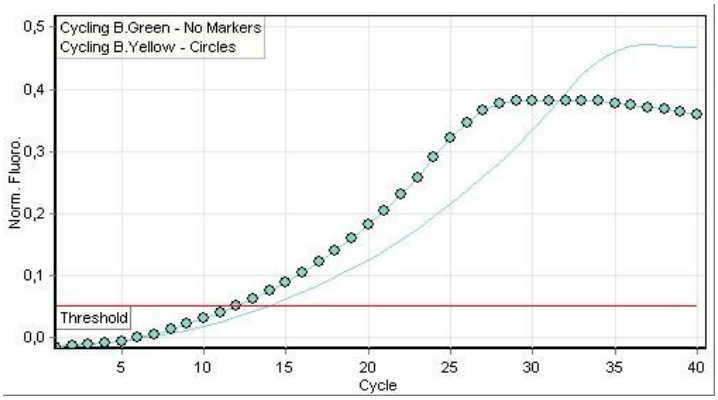

Figure (7-C): Heterozygote character both VIC and FAM dye hybridized with DNA sample (sample NO .15).

C - polymerase enzyme cleaves the bound probe, and a fluorescent signal is generated. Fluorescent signals are interpreted the presence of both fluorescent signals indicates Allele "A"/Allele "B" heterozygosity. fig(7-C).

The probe fragments are then displaced from the target, and polymerization of the strand continues. The 3´ end of the probe is blocked to prevent extension of the probe during PCR. This process occurs in every cycle, and it does not interfere with the exponential accumulation of product. The increase in fluorescence signal is detected only if the target sequence is complementary to the probe and if it is amplified during PCR. Because of these requirements, nonspecific amplification is not detected (2).

Statistical analysis:

The analysis of variance was used at $P<0.05$ and the test of least significant difference (LSD) was used to compare averages of some parameters. The test of Chi – square was used to compare averages of some other parameters . the standard error for all parameters was calculated . the SAS sowftware (product ver .2012) was used.

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