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Association of the growth hormone releasing hormone (rs368475481) polymorphism with Acromegaly disorder in Iraqi patient

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Abstract
Acromegaly (ACM) regarded one of rare endocrine , chronic disorder. its occur because benign tumor in pituitary gland. ACM disorder effect on many hormone as increase growth hormone(GH) secretion that cause increasing of insulin like growth factor-1(IGF-1). also ACM disorder causes increasing of growth hormone releasing hormone(GHRH). biochemical and molecular genetic study is performed from August 2022 to October 2022, including 80 samples Iraqi ACM patients that collected from Diabetic National Center in AL-Mustansiriyah University and 40 samples from healthy . Biochemical study includes 80 samples ACM patients and 40 were as a control. The measuring of fast blood sugar (FBS) by Trinder method , (GH), (IGF-1) were achieved by using sandwich chemiluminescence immunoassay technique and The measuringof GHRH was achieved by using the enzyme-linked immunosorbent assay (ELISA) kit to estimation of variations though these biochemical parameters in healthy and ACM patient. Odds ratio, Chi square and p-value test were used to data analyze for to detect significance of the means. The statistical analysis of hormones measurements has been shown that the level of the GHRH, GH, IGF-1 ,FBS are high significant differences to ACM patients when compared to the control. Sequencing analysis result for amplification of the GHRH gene show rs368475481 mutation where by CC homozygous observe in control 40(100%) , in ACM patients 40(50%) ,heterozygous CT observe in only ACM patients 40(50%) and TT homozygous not observe. In ACM patients there are significant differences ($P<0.05$) between CC, CT (P-value =0.017) with GHRH. .

Key words: GHRH , pituitary adenoma, acromegaly , GH , IGF-1.

Introduction

Acromegaly (ACM) is rare clinical neuroendocrine chronic disorder , ACM arise from increase of growth hormone (GH) after closed of growth plates ,that leads to increasing production of insulin-like growth factor-1 (IGF-1) [1].About 95% from cases the increase production of (GH) because benign tumor called pituitary somatotrophic adenoma [2].The ACM affects about 60 per million people, [3] and the frequency of new cases in year was 3-4 per one million [4]. Neurologist Pierre Marie discovered and diagnosed of ACM in 1886 and he was given first scientifically described the somatic growth tumor, and coined the term ‘acromegaly for description it as distinctive clinical disease [5]. precise time of disease creation is not able to be detected and signs of ACM cannot show and do not improve for long time, caused delay in diagnosis is nearly 10–11 years [6]. Diabetes mellitus (DM), hypertension, sleep apnea, respiratory failure ,Cardiomyopathy leading to heart failure, Hypogonadism are clinical indicators of the

disease [7]. GH is a primarily pathologically affected hormone in ACM [8]. In ACM ,GH and IGF-1 play main role by direct or indirect action in ACM where pituitary secretes amount more than normal of (GH), When GH enters the blood stimulated the liver to increase production of IGF-1 in blood and IGF-1in turn causes bones and body tissue to grow abnormally [9]. Furthermore, the proportion of mortality between patients of ACM with raised (GH , IGF-1) is from 2.6 to 3.5 times more than normal people [10]. As a result to the regulating or decreasing of GH and IGF-1 proportion can lead to similar mortality of ACM patients with the normal people proportion [11]. the drug effectiveness measured in clinical ACM research by using GH and IGF-1. Somatocrinin or growth hormone releasing hormone (GHRH) is a neuropeptide, Consist of singular polypeptide chain involve 44 amino acid , it secreted by the hypothalamus from Arcuate nucleus region , GHRH receptors (GHRH-R) located on somatotrophic cells in pituitary. growth hormone(GH) synthesis and secretion stimulate when GHRH bind to its receptor. the expression of GHRH and its receptor through different extra-hypothalamic locations as cell lines of tumer that derived from human cancers and observed in surgical specimens [12].GHRH gene consist from five exon and located at chromosome 20 in 11.23 position [13].Some cases of ACM was resistant for treated of GH secreting of pituitary adenoma this case called ectopic acromegaly , Growth hormone releasing hormone (GHRH) increasing is main reason of this case, where somatotroph adenoma cells also have ability to secreting GHRH, So it become new useful diagnostic tool for ACM [14].GHRH levels regarded effective diagnostic marker of ectopic acromegaly and marker to the activity of disease after treatment by surgical and a susceptible indicator to detect of disease repetition[15].When tumor engender in pituitary somatotroph cells, irregular production of GH leads to the common signs of ACM ,So the major aim of treatment is decreasing or normalizing of GH and IGF-1level, that leads to inhibition of somatotroph adenoma or at least reducing the tumor [16].

This study aimed to :
Determine the association of GH,GHRH biochemical parameter as well as *GHRH (rs368475481)* gene polymorphism with the severity of acromegaly disorder in Iraqi patients.

MATERIALS AND METHODS

Samples Collection

The all samples, were taken from Acromegaly patients in Diabetic National Center in AL-Mustansiriyah University. in the period between "August 2022 to October 2022". Demographic information and all of them have history of pituitary adenoma at least more than one year and all are taking monthly long acting octreotide injections for more than one year. Patients were assessed prior and post treatment. Assessment included blood level of GH, IGF1 and by MRI for pituitary adenoma size. All 120 samples were splitted up into two groups. The first group involved 80 samples from Acromegaly patients , the second group include 40 samples from healthy control people.

Genomic DNA Isolation and Genotyping

DNA extraction:
The extraction of DNA from whole blood was performed by utilizing GENEAID Kit (USA). DNA concentration and purity were measured by using Nano-drop.

Primer design:
This primer was designed in primer-Blast at NCBI dependent on (NC_000020.11).

Table(1): Showed primer sequence and size product of the PCR for (rs368475481)C/T of GHRH polymorphism.

GENE	Tm	PRIMER COD	Sequence description (5-3)Sequence	Amplicon size bp
GHRH	62	F1	CTGCAGGGTGTGGGAAGAAA	790
		R1	GCTCCATCACGCCCATTCTA	

PCR master mix preparation:

The primers was purchased as lyophilize from BIONEAR (BIONEAR, KOREA). PROMEGA PCR mastermix was used in the PCR experiment (PROMEGA , USA). Each PCR reaction was performed in a total volume of 50 µl, adding 25 µl of master mix, 2 µl of an isolated genomic DNA solution,2 µl forward primer , 2 µl reverse primer ,and 19 µl nuclease –free water to the PCR tubes. The optimization of the PCR amplifications was done via calculating the (Tm) for each primer of both genes with respect of increasing or decreasing of (Tm) to get the best annealing temperature and detection the DNA bands by electrophoresis. Initial denaturation was began at 94°C for 3 min, and the amplification was began by followed by 35 cycles of denaturation at 94˚C, annealing at 62˚C, and elongation at 72˚C, and was finalized with a final extension at 72°C for 5 min. Amplification was verified by electrophoresis on a Ethidium bromide stain pre-stained 1.5% (w/v) agarose gel in 1× TBE buffer (2 mM of EDTA, 90 mM of Tris–acetate, pH 8.3), using a 100-bp ladder (BIONEER, South Korea) as marker for molecular weight of amplicon. It was made sure that all PCR resolved bands were specific and consisted of only one clean and sharp band in order to be send for sequenciner device successfully.

Table (2):-PCR program for *GHRH* gene amplifications.

NO	Steps	Temperature	Time	No. Of cycles
1	Denaturation 1	94 C°	3 Min	cycle1
2	Denaturation 2	94 C°	30 Min	cycle35
3	Annealing	62 C°	30 Min	
4	Extension 1	72 C°	1 Min	
5	Extension 2	72 C°	5Min	Cycle1
6	Holding	4 C°	-	Cycle 1

Sequencing of PCR amplicons

The resolved PCR amplicons were commercially sequenced from both forward and reverse termini according to the instruction manuals of the sequencing company (Macrogen Inc. Geumchen, Seoul, South Korea). Only clear chromatographs obtained from ABI sequence files were further analyzed, ensuring that the annotation and variations are not because of PCR or sequencing artifacts. By comparing the observed DNA sequences of the investigated samples with the retrieved neighboring DNA sequences of the NCBI Blastn engine, the virtual positions and other details of the retrieved PCR fragments were identified.

Interpretation of sequencing data

The sequencing results of the PCR products were edited, aligned, and analyzed as long as with the respective sequences in the reference database using BioEdit Sequence Alignment Editor Software Version 7.1 (DNASTAR, Madison, WI, USA). The observed variations in each sequenced sample were numbered in PCR amplicons as well as in their corresponding positions within the referring genome. The

identified SNPs were visualized in the dbSNP database to check their details according to their places in the reference genome.

Statistical Analysis:

A statistical study was conducted using the Statistical Package for Social Sciences (SPSS version 26, Inc., Chicago, IL , USA) and the Microsoft Excel Worksheet. The results and examples of the current study were analyzed. P-value significance was determined when the value less than 0.05 (P<0.05), Odd Ratio (OR), and 95% Confidence Interval [17].

Results:

Biochemical results:

Growth hormone releasing hormone (GHRH) was measured in blood serum of all (ACM) patients and healthy controls. Results shown that Growth hormone releasing hormone GHRH is significantly increased in ACM patients in comparison with healthy controls P-value (0.00001) that shows in Table (3).

The mean average of serum GHRH concentration in ACM patients group is (161.2±18.1) compared with (10.63±0.91) in the healthy control group. There was difference is statically significant between the mean value of two groups (control and patients). As the results shows in the Table (3), The study result note that there is increased in the mean average in the group of patient compared to the healthy control.

Table (3):- Comparison between patients and controls in GHRH.

Group	GHRH (ng/l) Mean ± SE
Patients	161.2±18.1
Control	10.63±0.91
P-value	0.00001*
	(P<0.05): *Significant

Growth hormone (GH) was measured in blood serum of all ACM patients and healthy controls. Results shown that GH is significantly increased in ACM patients in comparison with healthy controls P-value (0.0005) that shows in Table (4). The mean average of serum GH concentration in ACM patients group is (8.48±0.74) compared with (3.31±0.34) in the healthy control group. There was difference is statically significant between the mean value of two groups (control and patients). As the results shows in the Table (4), The study result note that there is increased in the mean average in the group of patient compared to the healthy group.

Table (4):-Comparison between patients and controls in GH.

Group	GH (ng/l) Mean ± SE
Patients	8.48±0.74
Control	3.31±0.34
P-value	0.0005*
(P<0.05): *Significant	

Table (5):-Correlation among studied parameters in ACM patients.

parameters	Correlation and P value	GH	GHRH

GH	R	1	
	P-value	0	
GHRH	R	0.551**	1
	P-value	0	0

**** Correlation is significant at P<0.01**

Table (5) illustrates the examined 80 of ACM patients. The results of association between GHRH concentration with parameters that estimated in this study. For GHRH, There are significant differences (P<0.01) between GHRH with GH (P-value=0) ,so GHRH has correlation significant with GH (R=551).

Molecular result

Total of 120 samples were selected from ACM patient and control group that used in Molecular study , these selected depended on disorder in biochemical parameter for patient group, these disorder have confirmed ACM , while the control group selected depended on regular in biochemical parameter. This study was conducted to investigate the possible association of the single nucleic polymorphisms (SNPs) in the Growth hormone releasing hormone GHRH gene with the ACM in a population made of fourty controls (assigned C1 – C40) and eighty - patients (assigned P1 – P80) in IRAQ.

DNA Extraction Result

The isolated genomic DNA from 120 samples including (80 individuals with ACM and 40 apparently healthy control individuals). The quality of DNA samples is also checked by electrophoresis on 0.8% agarose gel and was to be of a high integrity DNA with distinct bands at the top of the gel indicating high quality non-degraded genomic DNA Figure (1).

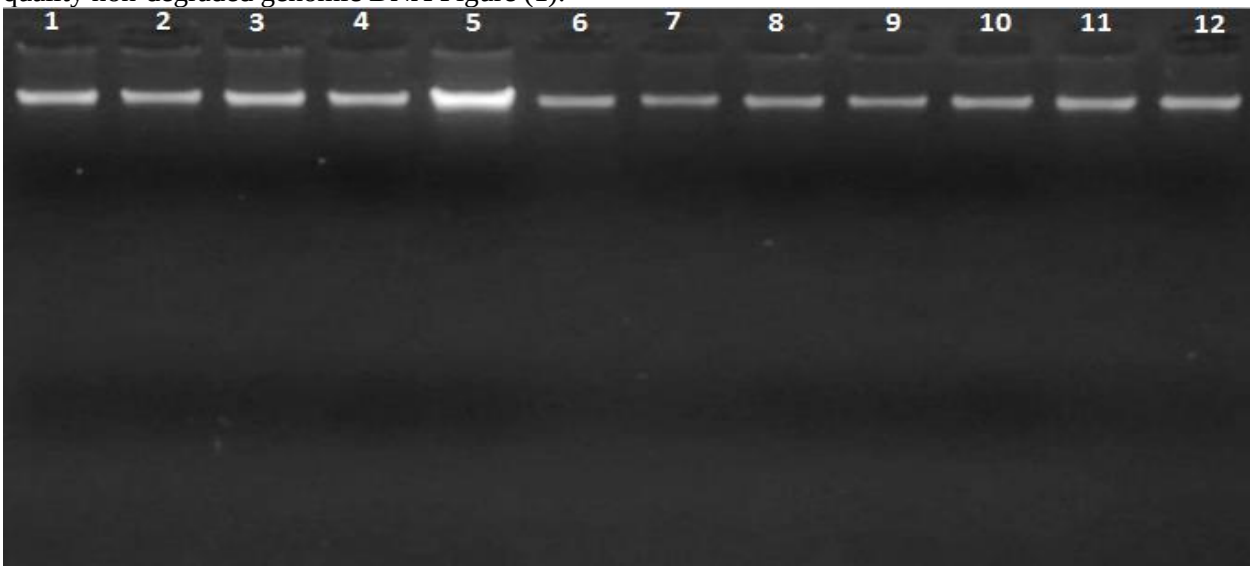


Figure (1):- Integrity of genomic DNA extracted from blood of representative samples. The DNA run on a 0.8% agarose gel at 75 voltages for 60 min, stained in a 500ml of 1X TBE buffer containing 0.6 µ of ethidium bromide, then visualized by a UV transilluminator. Lane order: 1-5, DNA extracted from control individuals and lanes 6-12, DNA from patients sample with acromegaly.

The specific region in GHRH gene that located exon 2,3 and intron 2 was amplified by used conventional PCR technique.The amplification conditions of all the targeting sits were described in table (2) in chapter

three. The annealing temperature for optimization step at first 62 C^{o} was calculated from the primers leaflets sheet and using the Tm formula:-

Tm= 4(C+G) + 2(A+T)

Thus, after preforming the PCR program on three DNA samples extracted from control and six DNA samples extracted from ACM patients. All bands are available and clear and The best optimization degree was 62 C^{o} Figure (2).

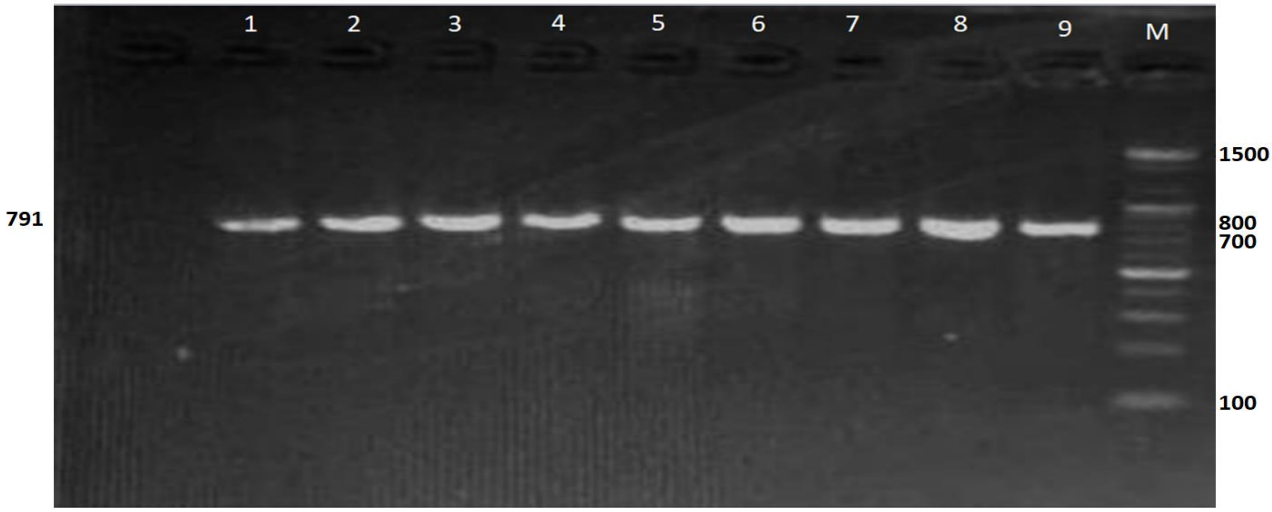
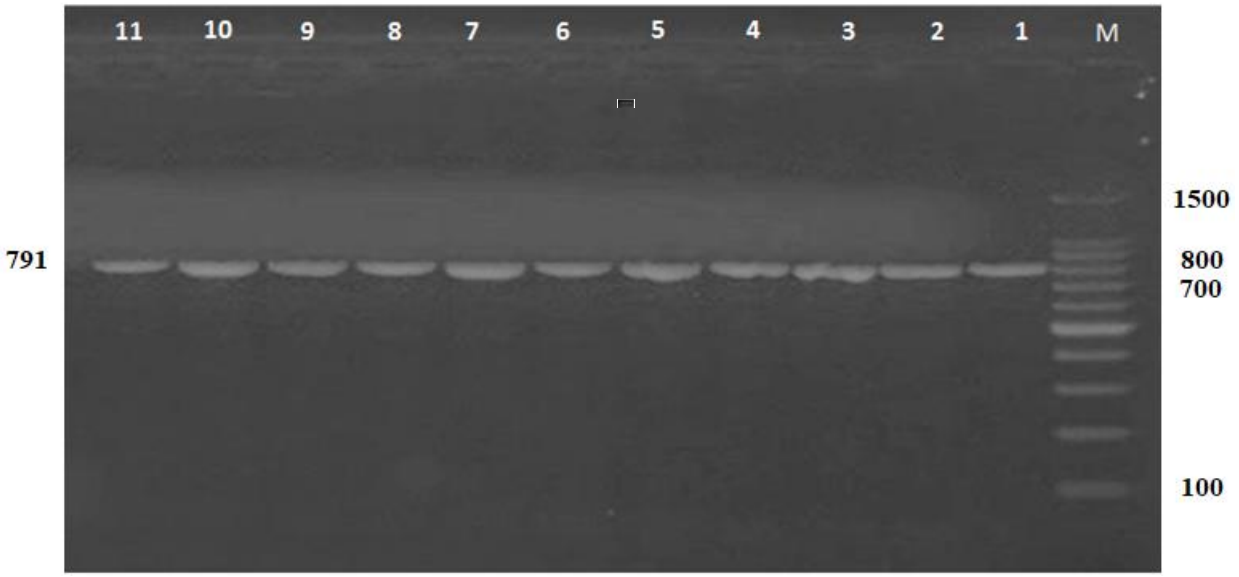


Figure (2):- Optimization of PCR amplification condition of GHRH gene exon 2,3 and intron 2 by using a conventional PCR. PCR product run on a 1.5% agarose gel at 70 voltages for 90 minutes then exposed to a UV transilluminator. Lane order: 1-3, from control individuals and lanes 4-9, patients sample with acromegaly and M is a DNA marker with 100bp ladder.TM begin from 54 C^{o} for lane 1 to 62 C^{o} for lane 9.



Figure(3):- PCR product of amplified 791bp fragment of exon 2,3 and intron 2 of *GHRH* gene. The PCR product obtained with samples from healthy individuals (lanes order 1-5) and PCR product from acromegaly patient samples (lanes 6-11) and (M lane is a 100bp ladder). The PCR product was electrophoresis by a standard 1.5% (w/v) agarose gel that is pre-stained with ethidium bromide (0.6 µg/ml) in TBE buffer, using a 100bp ladder as a molecular weight marker at 75 voltages for 80 min.

Sequencing Result

In the present study, the investigated sequences of the *GHRH* geneare suited to chromosome 20. The *GHRH* gene encodes for the growth hormone-releasing hormone, which stimulates the secretion of growth hormone (https://www.uniprot.org/uniprotkb/P01286/entry). The sequence reaction indicated the exact identity of this genetic fragment after performing NCBI blastn(https://blastn.ncbi.gov.Blast.cgi). Concerning the currently investigated 791 bp amplicons of the GHRH gene, the NCBI BLASTn engine showed up to 99% sequence similarities between the sequenced samples and the intended reference target sequences, which completely cover all the exon 2 and exon 3 and some of their upstream and downstream portions. By comparing the observed DNA sequences of these investigated samples with the retrieved DNA sequences (GenBank acc. NC_000020.11), the accurate positions and other details of the retrieved PCR fragments were identified (Fig 4).

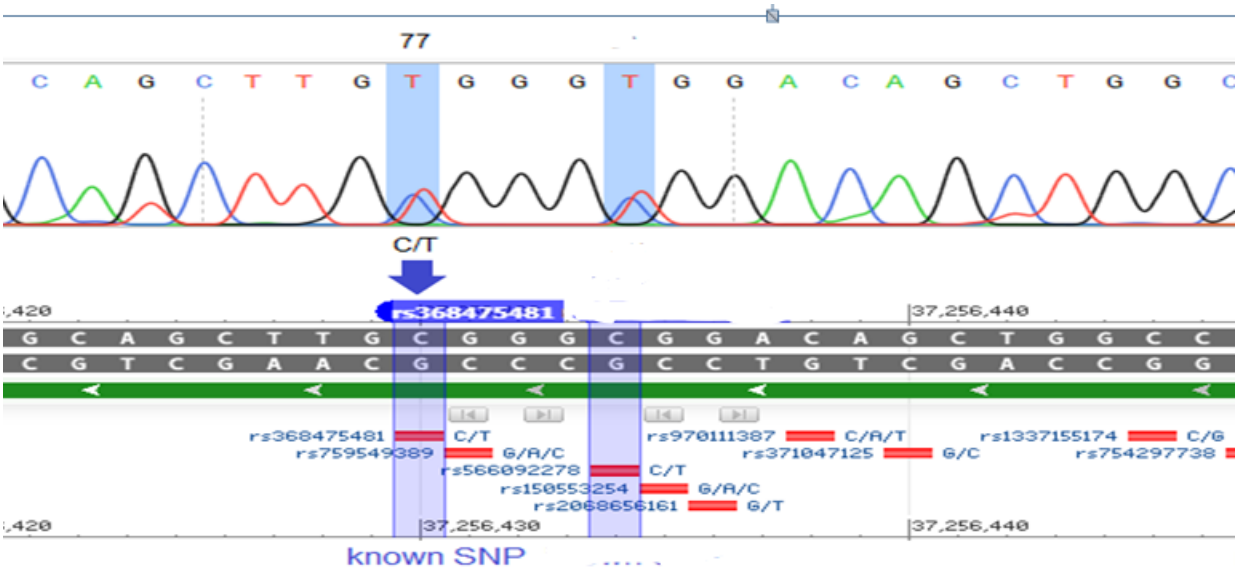


Figure (4):-The SNP's annotations checking of *GHRH* genetic single nucleotide polymorphisms using the dbSNP server. The identified known SNPs were marked with a blue color. GenBank acc. no. NC_000020.11 was used in the positioning of the identified SNPs in the *GHRH* gene. The positions of targeted sequences are positioned in the positive strand.

The result of Sequencing analysis for amplification of the GHRH (rs368475481) gene polymorphism Allele C showed in control was 80(100%) , in ACM patients group was 120(75%), while Allele T only in ACM patients group was 40(25%) and not observe in control group .In control group CC was 40(100%), CT 0(0%) and TT 0(0%). In ACM patients CC was 40(50%), CT 40(50%) and TT 0(0%). There is significant difference (P<0.05) between control and ACM group (100 versus 50%, OR= 0.012, 95%CI= 0.007 - 0.2077, X2=30, Etiological fraction = 0.31, P-value = 0.012). There is significant difference (P<0.05) between control and ACM group (0 versus 50%, OR= 81, 95%CI= 4.8150 - 1362.62, X2=30, Etiological fraction = 0.18, P-value = 0.012), so that may be use CT as indication for ACM this result showed in table (6).

Table (6):- Genotype and Allele Frequency for *(rs368475481)* in *GHRH* gene.

Genotype	Patients No.=80		Control No.=40		X^2	p-value	OR	Etiological fraction	95 % CI
	No.	%	No.	%					
CC	40	50	40	100	30	0.012*	0.012	0.31	0.007 to 0.2077
CT	40	50	0	0	30	0.012*	81	0.18	4.8150 to 1362.62
TT	0	0	0	0	0	1	0.503	0	0.009 to 25.82

Alleles									
C	120	75	80	100	24	0.005*	0.01	0.4	0.0011 to 0.3049
T	40	25	0	0	24	0.005*	54.11	0	3.2802 to 892.66

*** Significant difference at P<0.05**

Table (7):- The association of *GHRH* gene polymorphism with GHRH concentrations for patients (Mean ± SE).

Gene SNP	Parameters	Groups	Genotypes (mean± SE)			P-value
			CC	CT	TT	
rs368475481	GHRH	Patients	118.2±22.7	204.2±26.7	-	0.017*

*** Significant difference at P<0.05**

Table (7) illustrates the examined 80 ACM. The results of association between GHRH concentration and genotype for GHRH. For rs368475481,There are significant differences (P<0.05) between CC, CT (118.2±22.7 versus 204.2±26.7, P-value =0.017) with GHRH.

Discussion

Previous study by Dineen and colleagues (2017). Showed that the ACM arise because pituitary adenoma which causes hypersecretion of GH that lead to soft tissue enlargement with frontal bossing, skeletal overgrowth, mandibular prognathism, jaw malocclusion and overbite and skin thickening.

In addition, these results are agreement with Minuto and colleagues (2012), they showed that 13% of acromegalic patients going on LAR treatment were normal level of GH concentration and 87% were with abnormal concentration.

Previous study by Melmed (2017) provided that hypothalamus gland produce the GHRH in to the portal system, impinges upon the somatotroph cells, binds to specific surface receptors, and elicits intracellular signals that modulate pituitary GH synthesis and/or secretion. Hypothalamic tumors,including hamartomas, choristomas, gliomas, and gangliocytomas may produce excessive GHRH with subsequent GH hypersecretion and resultant ACM. These patients may suffered from somatotroph hyperplesia, or very rarely a pituitary GH-cell adenoma,supporting the notion that excess hypothalamic GHRH leads to pituitary hyperplasia and subsequent adenoma formation.

These results are agreement with Borsonand and colleagues (2012), they investigated GHRH expression in different tumor types and showe GHRH expression was present in 25% of tumors of endocrine , particularly in pheochromocytomas, gastroenteropancreatic tumors and small cells lung carcinomas and rarely, in non-endocrine tumors where by reported one case of GHRH secreting tumor associated with both, pituitary hyperplasia and somatotropic adenoma.

With regard to the identified 77C>T SNP, it was found that the detected SNP was found to be deposited in this sever under the name rs368475481 (Fig 4.5A). This SNP was detected with an extremely low frequency within the coding sequences of the GHRH gene. The deposited frequency of the allele T was accounted to be 0.00007 (https://www.ncbi.nlm.nih.gov/snp/rs368475481). In contrast with frequency of this research found this allele with a frequency of 33.3%. This observation indicated the presence of remarkable differences between study populations and the reference data. However, this SNP leads to missense variations in the encoded protein, namely NP_001171660.1:p.Arg51His. the C77T variant it will change the amino acid arginine which has positive charged guanidino group to histidine which has an imidazole group. His regarded only common amino acid own the ionizable side chain that having pKa near from neutrality, in many enzyme-catalyzed reactions, a His residue facilitates the reaction by serving as a proton donor/acceptor. There are no publications have mentioned this SNP in the PubMed indices, this study is first investigation about mentioned this SNP in all world.

Homozygous CC of rs368475481 have no any related with ACM disorder because its the wild type and present in control and patients, Heterozygote CT was observed only in 40(50%) of ACM patients and not observed in control ,so may highly associated with the development of ACM in the investigated population and can used as marker for diagnosis disorder ACM.

Conclusion: Association of the GHRH 77C>T*(rs368475481)*polymorphism with pituitary adenoma that cause acromegaly disorder in Iraqi patients.

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