Original Article

Association between a High Level of Growth Hormone–Releasing Hormone (GHRH) and GHRH Genetic Polymorphism (rs566092278) with Acromegaly Cases

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Abstract

Background: Acromegaly (ACM) is a rare endocrine chronic disorder. It occurs because of a benign tumor in the pituitary gland, which secretes an excess of growth hormone (GH). **Objectives:** The objective of this study is to determine the association between growth hormone-releasing hormone (GHRH) concentration and the GHRH (rs566092278) genetic polymorphism with the severity of ACM cases. **Materials and Methods:** This study, which was carried out from December 2022 to April 2023, involved 80 samples from ACM patients collected from the Diabetic National Center at Al-Mustansiriyah University, Baghdad, Iraq, along with 80 samples from healthy individuals. The measurement of GH and insulin-like growth factor-1 (IGF-1) was performed using the sandwich chemiluminescence immunoassay technique. The measurement of GHRH was performed using the enzyme-linked immunosorbent assay. **Results:** The concentrations of GHRH, GH, and IGF-1 were elevated with significant differences observed in ACM patients compared with controls. Sequencing results for the amplification of the *GHRH* gene show the rs566092278 mutation, whereby CC homozygous was observed in 80 (100%) of controls and 52 (65%) of ACM patients. The presence of CT heterozygous in ACM patients was 28 (35%). There are significant differences ($P < 0.05$) between the control and ACM patients (0% vs. 35%, $P = 0.008$*). There is an association between GHRH concentration and the rs566092278 single nucleotide polymorphism (SNP) in the *GHRH* gene, whereas significant differences ($P < 0.05$) in GHRH concentration between CC and CT genotypes (96.11 ± 21.8 vs. 196.2 ± 24, $P = 0.008$*). **Conclusion:** The elevation of GHRH concentration and the presence of the rs566092278 SNP in the *GHRH* gene were associated with pituitary adenoma that causes ACM in Iraqi patients.

Keywords: Acromegaly, *GHRH* gene, growth hormone, growth hormone-releasing hormone, insulin-like growth factor-1

Introduction

Acromegaly (ACM) is one of the rare chronic neuroendocrine disorders. It arises from increased secretion of growth hormone (GH) after the closure of growth plates, leading to elevated production of insulin-like growth factor-1 (IGF-1).[1] About 95% of cases involve increased production of GH due to a benign tumor called pituitary somatotroph adenoma.[2] ACM affects about 60 people per million, and the annual frequency of new cases is three to four per million.[3,4] Neurologist Pierre Marie discovered and diagnosed ACM in 1886. He was the first to scientifically describe the somatic growth tumor and coined the term "acromegaly" to distinctly describe this clinical disease.[5] The precise time of disease onset in ACM cannot be determined, and signs do not typically improve for a prolonged period. This delay in diagnosis is approximately 10–11 years.[6]

Diabetes mellitus, hypertension, sleep apnea, respiratory failure, cardiomyopathy leading to heart failure, and hypogonadism are clinical indicators of the disease.[7] GH

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is the primary pathologically affected hormone in ACM.[8] In ACM disorder, GH and IGF-1 play a main role through direct or indirect actions. In ACM, the pituitary gland secretes more than the normal amount of GH. When GH enters the bloodstream, it stimulates the liver cells to increase the production of IGF-1 in blood, which in turn causes bones and body tissue to grow abnormally.[9] Furthermore, the mortality rate among ACM patients with elevated GH and IGF-1 is 2.3–3.5 times higher than that of normal individuals.[10] As a result, regulating or decreasing the levels of GH and IGF-1 can lead to a similar mortality rate for ACM patients as that of normal individuals.[11] The effectiveness of the drugs is measured in clinical ACM research, depending on GH and IGF-1 regulation. Somatocrinin or growth hormone-releasing hormone (GHRH) is a neuropeptide consisting of a single polypeptide chain involving 44 amino acids. It is secreted by the hypothalamus from the arcuate nucleus region. GHRH receptors are located on somatotropic cells in the pituitary. GH synthesis and secretion are stimulated when GHRH binds to its receptor. The expression of GHRH and its receptor occurs in different extra-hypothalamic locations, such as cell lines of tumors, derived from human cancers, and is observed in surgical specimens.[12]

The *GHRH* gene consists of five exons and is located on chromosome 20q11.23 position.[13] Some cases of ACM are resistant to treatment of GH-secreting pituitary adenoma. Clinically, these cases are called ectopic ACM. The main reason for this is the increase in GHRH since somatotroph adenoma cells also can secrete it. GHRH has become a useful diagnostic tool for ACM.[14] GHRH levels serve as both an effective diagnostic marker of ectopic ACM and an indicator of disease activity after surgical treatment, as well as a susceptible indicator to detect disease recurrence.[15] When a tumor develops in pituitary somatotropic cells, irregular production of GH leads to the common signs of ACM. Therefore, the primary aim of treatment is to decrease or normalize GH and IGF-1 levels, which inhibits somatotroph adenoma growth or at least reduces the tumor.[16] The pituitary produces GH under the balance between ghrelin and GHRH.[17] Ghrelin is one of the GH secretagogues that is, secreted by oxyntic cells in the stomach. After its secretion, it is transported toward the hypothalamus, specifically the ventromedial nuclei region, where it stimulates the production of GHRH.[18] ACM disorder is one of the causes of diabetes mellitus and metabolic syndrome.[19]

Materials and Methods

Samples collection

The samples were collected from ACM patients at the Diabetic National Center of Al-Mustansiriyah University, Baghdad, Iraq. The study was conducted from December 2022 to April 2023. A total of 160 samples were divided into two groups. The first group included 80 ACM patients, whereas the second group included 80 control subjects. Demographic information: All patients had a history of pituitary adenoma for at least 1 year and had been receiving monthly long-acting octreotide injections for more than 1 year. Patients were assessed both before and post-treatment. The assessment included measuring blood levels of GH and IGF-1, as well as magnetic resonance imaging to evaluate the size of the pituitary adenoma.

Genomic DNA isolation and genotyping

DNA extraction

Deoxyribonucleic acid (DNA) extraction from whole blood was performed according to the instructions provided in the Geneaid catalog (catalog numbers GS004, GS100, and GS300). The isolated genomic DNA from all samples in this study was analyzed using NanoDrop spectrophotometers. All DNA samples were measured at 260 and 280 nm to assess their purity.

Primer design

Primer-Blast at the National Center for Biotechnology Information (NCBI) based on NC_000020.11 was used to design a primer for detecting the (rs566092278) single nucleotide polymorphism (SNP) in the *GHRH* gene [Table 1].

PCR master mix preparation

The primer was purchased as lyophilized powder from BIONEER (Daejeon, South Korea). PROMEGA PCR Master Mix was used in the PCR experiment (Promega, Madison, WI, USA). Each PCR reaction was performed in a total volume of 50 µL and contained the following components:

Mastermix	25 µL
Genomic DNA	2 µL
F1	2 µL
R1	2 µL
Nuclease free water	19 µL
Total	50 µL

PCR conditions are illustrated in Table 2

Statistical analysis

A statistical analysis was conducted using the Statistical Package for the Social Sciences version 26 (SPSS Inc., Chicago, IL, USA) and Microsoft Excel. The results of the current study were analyzed. Statistical significance was

Table 1: Primer sequence and polymerase chain reaction (PCR) product size for the (rs566092278) C/T SNP in the *GHRH* gene

Gene	T_m	PRIMER COD	Sequence description (5′–3′) sequence	Amplicon size (bp)
GHRH	62	F1	CTGCAGGGTGTGGGAAGAAA	790
		R1	GCTCCATCACGCCCATTCTA	

determined with a P value of less than 0.05 ($P < 0.05$), and the odds ratio (OR) and 95% confidence interval (CI) were calculated.[20]

Ethical approval

The study was conducted following the ethical principles outlined in the Declaration of Helsinki. The study protocol, the subject information, and the consent form were reviewed and approved by a local ethics committee under document number 1742 dated November 24, 2022.

Results

Biochemical parameters

GHRH was measured in the blood serum of all ACM patients and healthy controls. The results show that GHRH levels were significantly increased in ACM patients compared with healthy individuals ($P = 0.00001$) as shown in Table 3.

The mean serum GHRH concentration in the ACM patient group was 153.2 ± 18.1 pg/mL, compared with 10.63 ± 0.91 pg/mL in healthy individuals.

GH was measured in the blood serum of all ACM patients and healthy controls. The results show that GH levels were significantly increased in ACM patients compared with healthy individuals ($P = 0.0005$) as shown in Table 4.

The mean serum GH concentration in the ACM patient group was 9.48 ± 0.74 pg/L compared with 3.31 ± 0.34 pg/L in the healthy individuals.

The results of the association between GHRH concentration and the parameters estimated in this study for 80 ACM patients are illustrated in Table 5. For IGF-1, there were statistically significant differences ($P < 0.01$) between IGF-1 and GH ($P = 0$), indicating a significant correlation between IGF-1 and GH ($r = 0.515$). For GHRH, there were statistically significant differences ($P < 0.01$) between GHRH and both GH and IGF-1 ($P = 0$), demonstrating significant correlations between GHRH and GH ($r = 0.551$) and between GHRH and IGF-1 ($r = 0.382$).

Table 2: PCR program for *GHRH* gene amplification

No	Steps	T_m (°C)	Time (min)	No. of cycles
1	Denaturation 1	94	3	One cycle
2	Denaturation 1	94	30	Cycles 35
3	Annealing	62	30	
4	Extension 1	72	1	
5	Extension 2	72	5	One cycle
6	Holding	4	—	One cycle

Table 3: Difference in GHRH levels between ACM patients and the control group

Group	GHRH (pg/L) Mean ± SE
Patients	153.2 ± 18.1
Control	10.63 ± 0.91
P value	0.00001*

*Significant difference $P < 0.05$

Table 4: Difference in GH levels between ACM patients and the control group

Group	GH (ng/L) Mean ± SE
Patients	9.48 ± 0.74
Control	3.91 ± 0.34
P value	0.0005*

*Significant difference at $P < 0.05$

Molecular study

A total of 160 samples were selected from ACM patients and control groups for use in a molecular study. Samples were selected based on disorders in biochemical parameters for the patient group, as these disorders have confirmed ACM. This study was conducted to investigate the possible association of SNPs in the *GHRH* gene with ACM in a population consisting of 80 controls (assigned C1–C80) and 80 patients (assigned P1–P80) in Iraq.

Optimization of PCR amplification conditions for the *GHRH* gene

The annealing temperature for the optimization step was calculated based on the primer datasheet and using the T_m formula:

$$T_m = 4(\mathrm{C+G}) + 2(\mathrm{A+T})$$

The optimal annealing temperature was found to be 62°C [Figure 1]. The results of the PCR products amplified from the 791 bp fragments of exon 2, exon 3, and intron 2 of the GHRH gene are shown in [Figure 2].

Sequencing analysis

The sequencing reaction indicated the exact identity of this genetic fragment after performing NCBI BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi). For the currently investigated 791 bp amplicons of the *GHRH* gene, the NCBI BLASTn engine showed up to 99% sequence similarity between the sequenced samples and the intended reference target sequences, which completely covered exons 2 and 3, and some of their upstream and downstream portions. By comparing the observed DNA sequences of these samples with the retrieved DNA sequences (GenBank acc. NC_000020.11), the accurate positions and other details of the retrieved PCR fragments were identified [see Figure 3].

Table 5: Correlation among studied parameters in ACM patients

Parameters	Correlation and *P* value	GH	IGF-1	GHRH
GH	R	1		
	P value	0		
IGF	R	0.515**	1	
	P value	0	0	
GHRH	R	0.551**	0.382**	1
	P value	0	0	0

**Correlation is significant at $P < 0.01$

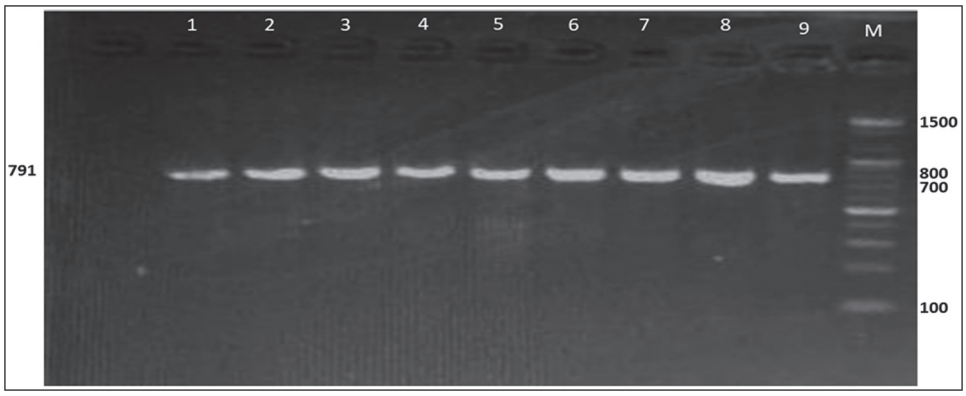

Figure 1: Optimization of PCR amplification conditions for *GHRH* gene exons 2 and 3, and intron 2 using conventional PCR. PCR products were run on a 1.5% agarose gel at 70 V for 90 min, then exposed to an ultraviolet transilluminator. Lane order: Lanes 1–3 were from control individuals, lanes 4–9 were from patient samples with ACM, and M was a DNA marker with a 100 bp ladder. The annealing temperature (T_m) ranged from 54°C for lane 1 to 62°C for lane 9

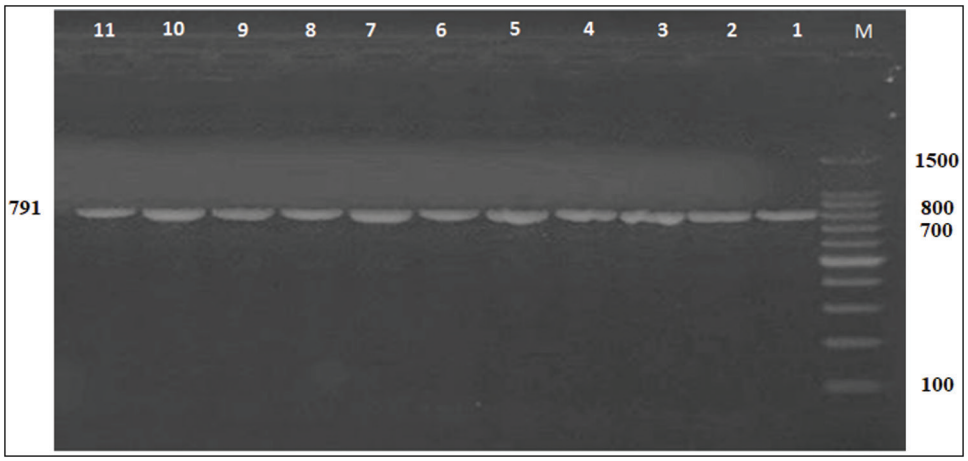

Figure 2: PCR products of amplified 791 bp fragments of exon 2, 3, and intron 2 of the GHRH gene. Lanes 1–5 showed PCR products from healthy individuals, lanes 6–11 showed PCR products from ACM patient samples, and lane M represented the DNA ladder (100 bp). The electrophoresis was performed using a 1.5% (w/v) agarose gel stained with ethidium bromide (0.6 μg/mL) and Tris/borate/ethylenediaminetetraacetic acid buffer. The electrophoresis was conducted at 70 V for 80 min

The results of the sequencing analysis for the amplification of the rs566092278 SNP in the GHRH gene showed the presence of allele C in all 80 control samples (100%). In the ACM patient group, allele C was present in 132 samples (82.5%), whereas allele T was present in 28 samples (17.5%). Allele T was not observed in the control group. The genotypic distribution in the control group was as follows: CC in 80 samples (100%), CT in 0 samples (0%), and TT in 0 samples (0%). For ACM patients, the genotypic distribution was CC in 52 samples (65%), CT in 28 samples (35%), and TT in 0 samples (0%). Statistically significant differences ($P < 0.05$) were found between the control and ACM groups for the CT genotype (0% vs. 35%, OR = 43.97, 95% CI = 2.6054–42.1, $\chi^2 = 18.26$, Etiological fraction = 0.34, $P = 0.008$). The CT heterozygous genotype was observed exclusively in ACM patients, indicating its association with ACM. This result is illustrated in Table 6.

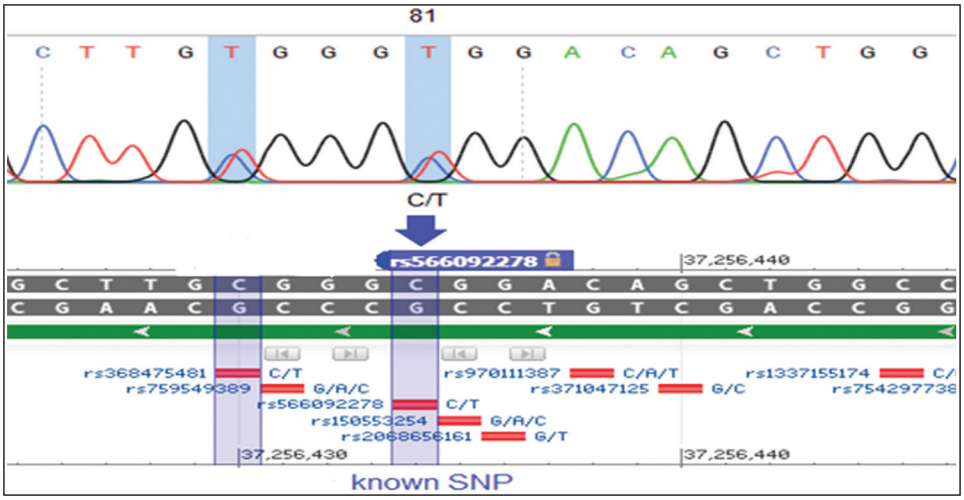

Figure 3: SNP annotation for GHRH gene single nucleotide polymorphisms was performed using the dbSNP server. The identified known SNPs were marked in blue. GenBank accession number NC_000020.11 was used to position the identified SNPs in the *GHRH* gene. The positions of the targeted sequences were on the positive strand

Table 6: Genotype and allele frequency for the rs566092278 SNP in the *GHRH* gene

Genotype	Patients *N*.= 80		Control *N* = 80		χ^2	*P* value	OR	Etiological fraction	95% CI
	N	%	*N*	%					
CC	52	65	80	100	18.26	0.008*	0.022	0.34	0.0013–0.3838
CT	28	35	0	0	18.26	0.008*	43.97	0.34	2.6054–742.1
TT	0	0	0	0	0	1	0.51	0	0.0098–25.82
Alleles									
C	132	82.5	160	100	15.85	0.01*	0.028	0.37	0.0017–0.4795
T	28	17.5	0	0	15.85	0.01*	34.63	0.07	2.0854–575.06

*Significant difference at $P < 0.05$

Table 7: Association of GHRH genetic polymorphism (rs566092278) with GHRH concentration in patients (Mean ± SE)

Gene SNP	Parameters	Groups	Genotypes (mean± SE)			*P* value
			CC	CT	TT	
rs368475481	GHRH	GHRH	96.11 ± 21.8	196.2 ± 24	—	0.008*

*Significant difference at $P < 0.05$

The results of the association between GHRH concentration and the genotype for GHRH in 80 ACM patients are illustrated in Table 7. For the rs566092278 SNP, there were statistically significant differences ($P < 0.05$) in GHRH concentrations between the CC and CT genotypes (96.11 ± 21.8 vs. 196.2 ± 24, $P = 0.008$).

Discussion

A previous study by Akirov *et al.*[21] demonstrated that ACM is a slowly progressive disease caused by persistent excess of GH and IGF-1. While most cases of ACM are due to a GH-secreting pituitary adenoma, ACM may, though rarely, be secondary to the hypothalamic secretion of GHRH, ectopic GHRH, or GH secretion.[21]

A previous study by Campana *et al.*[22] proved that hypersecretion of GH causes ACM. Excess GH stimulates the liver to produce IGF-1, which is responsible for most of the clinical manifestations of ACM.[22]

A previous study by Melmed and co-workers[23] demonstrated that the hypothalamus produces GHRH, which is released into the portal system and acts on somatotroph cells in the pituitary gland. GHRH binds to specific surface receptors on these cells, eliciting intracellular signals that modulate pituitary GH synthesis and/or secretion. Hypothalamic tumors, such as hamartomas, choristomas, gliomas, and gangliocytomas, may produce excessive GHRH, leading to GH hypersecretion and resultant ACM. These patients may exhibit somatotroph hyperplasia or, very rarely, a pituitary GH-cell adenoma, supporting the notion that excess hypothalamic GHRH can lead to pituitary hyperplasia and the subsequent formation of an adenoma.[23]

A previous study by Lamback *et al.*[24] included the measurement of *GHRH* gene expression in different tumor types. GHRH

gene expression was found in 25% of endocrine tumors, particularly in pheochromocytomas, gastroenteropancreatic neuroendocrine tumors (NETs), and small-cell lung carcinomas. *GHRH* gene expression is rare in non-endocrine tumors, with only one reported case of a GHRH-secreting tumor associated with somatotropinoma and pulmonary NETs.[24]

In the present study, the investigated sequences of the *GHRH* gene are located on chromosome 20. The *GHRH* gene encodes GHRH, which stimulates the secretion of GH (https://www.uniprot.org/uniprotkb/P01286/entry).

About the identified 81C>T SNP, it was found that this SNP is also registered under the name rs566092278 [see Figure 3]. This variant was detected at an extremely low frequency within the coding sequences of the *GHRH* gene. The reported frequency of the T allele is 0.00012 (https://www.ncbi.nlm.nih.gov/snp/rs566092278), but in the present study, the frequency of the T allele was 23.3%. This observation indicates notable differences between the study populations and the reference data. Moreover, this SNP led to missense variations in the encoded protein, specifically NP_001171660.1:p.Ala50Thr. For this variant, the mutation changed the amino acid alanine, which is nonpolar, aliphatic, and hydrophobic, to threonine, which is polar, uncharged, and hydrophilic. No publications were mentioning this SNP in PubMed. This study represents the first investigation of this SNP across all databases.

Homozygous CC of rs566092278 has no association with ACM disorder, as it is the wild-type genotype and is present in both control and patient groups. The heterozygous CT genotype was observed only in 28 (35%) of ACM patients and was not observed in controls. Therefore, it is highly associated with the development of ACM in the investigated population and can be used as a marker for diagnosing ACM.

Conclusion

The GHRH concentration was elevated in patients with ACM, indicating an association between elevated GHRH levels and the disorder. Statistically significant differences were observed between patients with ACM and the control group. The heterozygous (CT) form of the rs566092278 SNP in the *GHRH* gene was associated with the development of ACM in the studied population.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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