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Evaluation Level of Gene Expression for PRE gene on Preptin Hormone in Patients with Osteoporosis in Mosul City

Elaf Gh. Nafi **Saba Z. Al-Abachi**
Department of Chemistry /College of Science/ University of Mosul

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corresponding author:
Elaf Gh. Nafi
elaf.23scp165@student.uomosul.edu.iq
Saba Z. Al-Abachi
saba-alabachi@uomosul.edu.iq

ABSTRACT

Osteoporosis is commonly called a "silent condition" due to its tendency to progress without clear symptoms. It is defined by a reduction in bone mass, leading to weakened bones. has become more widespread, leading to sudden fractures in patients without any prior warning. It has been increasingly observed in people under the age of 35, prompting researchers to explore genetic factors. Hormones that regulate nutritional status, including Preptin, they play a critical role in bone metabolism.

Preptin is a recently identified peptide hormone, composed of 34 amino acids, secreted alongside insulin and amylin by pancreatic beta cells. Given its potential link to osteoporosis, the hormone Preptin was selected for further genetic study. Blood samples were collected in EDTA tubes for DNA extraction and in triazole tubes for RNA extraction. Gene expression of Preptin was analyzed in blood samples from osteoporosis and osteopenia patients in Mosul, Iraq, and compared to healthy individuals, due to the high incidence of osteoporosis and its genetic and hereditary associations. The study aimed to assess the genetic variation of Preptin genes in 40 blood samples from patients aged 35 to 75, who were receiving ongoing treatment at Ibn Sina Teaching and Al-Jamhory hospitals. A control group of 30 healthy individuals of similar age was also included.

Additionally, the research focused on detecting genetic mutations in the human DNA sequence, which has been registered in the International Gene Bank under number LC859842.

Keywords: Preptin Hormone, Gene expression, Sequence DNA, Osteoporosis.

INTRODUCTION

Osteoporosis was increasingly become a global epidemic. It's a bone condition marked by changes in bone shape and small-scale damage to bone tissue, leading to higher levels of osteoporosis and a greater risk of fractures (Maffi *et al.,* 2023). Also, osteoporosis is a silent disease (Al-Hariri and Al-Dhafery, 2020) and it was developed without any symptoms until a fracture occurs (Fajar and Azharuddin, 2016).

Bone mass refers to the total mineral content in the bone tissue, while bone mineral contour is a technique used to assess the bone's mineral content (Haseltine *et al.,* 2021). The most common measurement of bone mineral contour is the measurement of X-ray absorption (DEXA) absorptiometry (Dual Energy X-ray absorptiometry) this device works on the high sensitivity of calcium in the absorption of X-rays to measure the relative amounts of bones and other tissues, absolute measurements vary according to different devices significantly so it is necessary to use standardized reference ranges (Diamond and Sheu, 2016).

The value of the reference range of each patient is compared with a database created on the device (Choksi *et al.,* 2018). The diagnosis of osteoporosis and the rate of osteoporosis increase significantly with age (Cech, 2012). Over 200 million people worldwide are currently affected by osteoporosis. Recent figures from the International Osteoporosis Foundation show that globally, 1 in 3 women over 50 and 1 in 5 men will experience osteoporotic fractures during their lifetime (Stephanie *et al.,* 2024).

There are differences in the disease between males and females, with women being more prone than men. In women, it has been hypothesized that menopause is followed by immediate loss of bone mass and contour within a year, this increased rate of bone lead reaches equilibrium about 10 years after menopause (a monthly that merges into a continuous age-related loss after menopause (Kadam *et al.,* 2018). The most recent member of the insulin family is Preptin, which is a 34-amino-acid peptide that is derived of the E-peptide of pro-insulin-like growth factor 2 (pro-IGF2). It is released alongside insulin and helps enhance insulin secretion triggered by glucose (Ungureanu *et al.,* 2023).

I9t was isolated in 2001 by Buchanan and his groups from pancreatic islet granules made up of β mouse cells. It is excreted with insulin and promotes glucose-mediated insulin secretion (Buchanan *et al.,* 2001). In laboratory research Preptin exhibited dose- dependent insulin secretion in high glucose but not normal glucose levels. Its actions are similar to actions of beclamide (Cheng *et al.,* 2012). However, the broader impact of Preptin on glucose metabolism remains unclear.

Since its discovery, Preptin has been found to be secreted not only by beta cells but also by other tissues Including organs like the kidneys, liver, salivary glands, and breast tissue (Aydin, 2014). In female rats with a Preptin knockout, there is a reduction in glucose-enhanced insulin secretion, though this does not occur in male rats. The potential anabolic orthopedic effects of Preptin have sparked interest for use in pharmacokinetic therapies.

However, (Buckels *et al.,* 2022) found that a shorter segment of the N-terminal Preptin specifically, Preptin1-16 preserves its anabolic effects while being more attractive for developing the smaller size means that it is a peptide analogue. The full-length molecule differs in contrast to the full-length molecule, Preptin1-16 has no impact on glucose metabolism. However, its truncated form is enzymatically unstable (Amso *et al.,* 2016). To address this, twenty-eight isotopes of Preptin1-16 were developed and tested on osteoblasts in vitro. The results indicated that Preptin1-8, the shortest of these isotopes, it effectively promotes the formation of bone nodules and the mineralization of the matrix in osteoblasts from mice, making it a promising candidate for the chemical synthesis of new osteoporosis treatments (Ungureanu *et al.,* 2023).

Bone remodeling, a carefully regulated balance between bone resorption by osteoplasty and bone creation by osteoblasts, is essential to preserving the mechanical integrity of the skeleton. the work related to intertextures and osteoblasts. Numerous hormones as well as circulating and local variables control it. Lately, the key control of bone mass by leptin, (Ducy *et al.,* 2000) has been

established. This seems to be driven by a neural mechanism instead of an endocrine one (Amling *et al.,* 2000). Osteoporosis and fragility fractures result from bone loss caused by the separation of components that reshape bone, so that bone resorption surpasses bone production. Although bone resorption is the main target of current osteoporosis preventive treatments, these factors can only partially increase bone mass. Therefore, variables that may increase bone mass by promoting bone development are of great interest, Preptin stimulates osteoblast-like cell to proliferate, differentiate, and survive in vitro. However, Preptin has little effect on the activity of mature bone di aggressors or bone growth. All things considered, Preptin in vitro results suggest that it might have anabolic effect on bone in vivo. According to recent finding, Preptin may have physiological and pathological implications for skeletal metabolism. A large body of evidence suggests that increased body weight and/or fat mass is associated with a rise in bone density and reduced risk of fragility fracture (Buckels *et al.,* 2022).

Of late, most of the nutritional condition regulating or reflecting hormones commentary has been based on such things as insulin, leptin, amylin and more. It was revealed to influence directly on bone metabolism. Overall, the data collected on these studies supports the assumption that the tendency to enhance many diet-related hormones, such as those pancreas-derived adipocytes and cells that is often observed in the peripheral circulation of obese individuals, contributes to the large bone mass and the reduced fracture susceptibility related to obesity. One of the hormones that are a part of these is Preptin that plays a significant role in bone activity. It corresponds with ASP69-Leu102 of IGF-II precursor of 156 amino acids, otherwise known as IGF-IIE. The prohormone to produce the active form of IGF-II in the circulation is composed of the first 67 amino acids (Rotwein et *al*.; 1986).

Very high levels of IGF-II have very potent anabolic activities on the bone and similarly, the biological activities of IGF-II possess a similar pattern (Valenzano *et al.,*1997) where lack of bone mass increment is not rampant in patients who possess tumor secretion of a truncated form of IGF-II lacking the immune response of the amino acids 78-88 but not 89-101. These results show evidence that height peptide levels found in a healthy sequence, Preptin, can make a great difference to status of the bone density in vivo. This was implied that the bone cells of the Preptin effects were observed that could be related to the anabolic steroids in the bone mass within the normal human body functions.

MATERIAL AND METHODS

This study included 40 blood samples (20 osteoporosis patients, 20osteopenia patients, who enrolled Ibn Sina Teaching and Al-Jamhory hospitals for the period from January to June 2024.

Also, there are 30 healthy subjects for comparison), the age of each sample study was between 35-75years. Each of the case studies was supplied with 2 milliliters of venous blood split in two parts, the sample was divided into two parts; the initial one was put in an EDTA tube in order to remove DNA, and the other one was put in a tube with Trizol so to extract RNA.

To obtain the most sensitive and biologically relevant results, the RNA isolation process must incorporate several crucial steps before, during, and after the actual RNA purification. The effectiveness of the RNA extraction is based on the protocol provided by TransGen Biotech (Ritalahti *et al.,* 2010).

In order to conduct a quantitative test on the level of genes expression of PRE gene, there were specific primers of Preptin gene, besides primers of house 2.4. Reverse transcription is the method of converting isolated fragments of mRNA to cDNA

After the isolation of mRNA and microRNA, it is turned into a cDNA molecule using the reverse transcriptase enzyme as per the protocol of Trans Gen Biotech kit (Zhang *et al.,* 2019).

Detection of the genetic expression levels of Preptin gene by qPCR technique

keeping gene. (Table 1) shows the primer of the Preptin gene combined with respective primer of the housekeeping gene. Primers were used in this study which was designed by primer 3 software and the primers used here are specific to this study.

Table (1): The primer for the Preptin gene is used alongside a specific primer for the housekeeping gene.

Primer	Sequence
PRE-RT-F	AGAGTCACCACCGAGCTTGT
PRE-RT-R	ACCTCAGGACTGGGCTCTCT
H.K-F	GACCCAGATCATGTTTGAG
H.K-R	CGTACAGGGATAGCACAG

Notes: All primers used in this study were designed by primer3 software.

The volume of final reaction was 20 µl as shown in (Table 2), the program which was used in RT-PCR was shown in (Table 3).

Table (2): The volume of final reaction

Component	Volume
Ultra sybrgreen q-PCR master mix	10 µl
RT forward primer	0.5 µl
RT Reversed primer	0.5 µl
cDNAtemplet	4 µl
D.W	5 µl

Table (3): The program uses in RT- PCR reaction

Stage	Temperature	Time
Pre denaturation	95 °C	10 min
Denaturation	95 °C	15 sec
Annealing /Extension	60 °C	1 min
Melting curve analysis	95 °C 60 °C 95 °C 60 °C	15 sec 1 min 15 sec 15 sec

Relative expression rate of genes using the CT value of the target gene was estimated using the control samples that consist of housekeeping gene and patient groups as follows formulation (Haimes *et al*., 2013)

1. test CT = CT target test CT ref test.
Delta CT (control)=CT (target, control) -CT (ref, control).

2. CT (target, test) is the mRNA cycles of the genes Bax and Bcl-2 in the samples of patients.
CT (ref, test) indicates the number of mRNA cycles of the housekeeping gene in sample of the patient.
CT (target, control) implies the mRNA cycles of the Bax and Bcl-2 genes in control samples.
CT (ref, control) is instant of mRNA of the housekeeping gene in control samples.

3. The change in CT of the sample after the treatment as compared to that of the control sample is calculated as follows:
Delta delta CT = Delta CT(test) - Delta CT (control).

4. This formula is used to find out gene expression:
Gene Folding Expression= 2 -Delta -Delta CT.

DNA extraction procedure used was Jones and colleagues (2010) relative to the kit provided by TransGen Biotech. Learn about all the methods and procedures of effective DNA extraction

and purification with maximum quality of the DNA in molecular biology studies. DNA extraction procedure was implemented based on a kit protocol provided by TransGen Biotech, as shown in (Table 4). The peripheral blood was used to extract the DNA in modified procedure (Iranpur and Esmailizadeh, 2009). Plot of DNA extraction Applications DNA may be isolated using many approaches; one example is DNA extraction, which is the process of isolating DNA from the cells of an organism within a sample (typically a biological sample like blood, saliva, or tissue). This involves breaking open the cells, removing proteins and other contaminants, and isolating the DNA, ensuring it's free from other cellular components. The purified DNA can then be used in subsequent applications, such as PCR.

Table (4): Kit components for DNA extraction

Item	Volume	Storage
Proteinase K solution	2ml	At(2-8°C)
Lysis buffer-2	35ml	At(15-25°C)
Washing buffer 1	55ml	At (15-25°C)
Washing buffer 2	55ml	At(15-25°C)
Elution buffer	20ml	At(15-25°C)
Collection tube and spin column	100 pcs	At(15-25°C)

A Bio Drop spectrophotometer was used to determine the level of concentration and purity in the genomic DNA extracted in the samples. They then stored the samples at -20 o C (García *et al.,* 2020).

The nitrogenous base sequences of the PRE gene of the study samples were identified so that the validity of the targeted designed primer in the route of PCR technique application is identified and identification of the presence of other possible variations in the targeted genes is determined. The primers were sent along with PCR result of the PRE gene. Sequences were read at Psomagen Center, in United States of America. This was then led to comparing the gene sequence with that reported at the National Center of Biotechnology Information NCBI and the result was analyzed through the use of the BLAST program. This test required adding 100 nags of template to 10 pmole of each primer to each PCR reaction. The PCR program of Preptin gene shown in (Table 5) and (Table 6) represent the sequences of primers used.

Table (5): The primer sequence that have been used in DNA sequence

Primer	Sequence
PRE–F	GGTTTCTGAAGGAGGGGAAG
PRE –R	CGGCATTTCTCCTCAGTCTC

Table (6): The PCR program for Preptin gene

Stage	Temperature	Time	Number of cycles
Initial denaturation	94 °C	5 min	1
Denaturation	94°C	45min	35
Annealing	57°C	1min	1
Extension	72	1min	1
Final extension	72	7min	1
Stop reaction	4	4min	1

RESULTS AND DISCUSSION

This was then led to comparing the gene sequence with that reported at the National Center of Biotechnology Information NCBI and the result was analyzed through the use of the BLAST program. The results indicated a reduction in the gene expression levels of the PRE gene, with

values of 0.47 in osteoporosis patients and 0.65 in osteopenia patients, in comparison to the control group, which had a gene expression level of 1.0, as shown in Fig. (1).

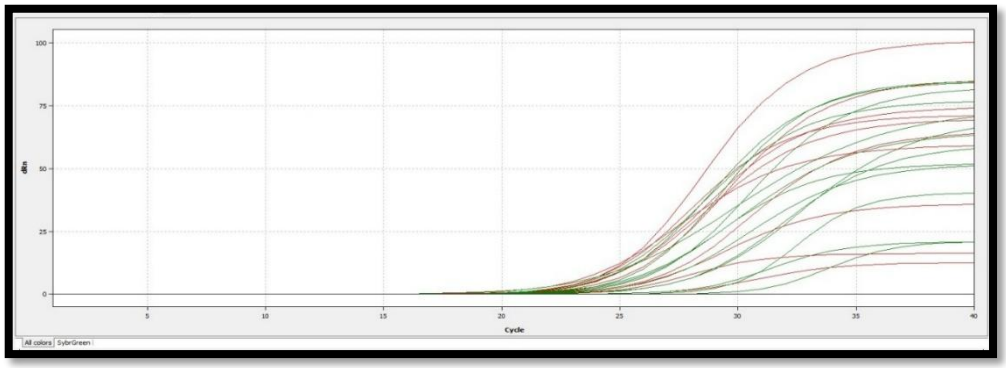


Fig. 1: Values of CT and the level of gene expression for the PRE gene

As shown in (Table 7) and Fig. (2), this suggests that individuals without any health issues may play a crucial role in the progression of the pathological condition. The data highlights the potential influence of a healthy baseline in the development of diseases like osteoporosis and osteopenia.

Table (7): The CT values and gene expression levels for the PRE gene and the housekeeping gene in patients were compared to those of the control group.

Sample	CT target gene	CT housekeeping gene	Δ CT target gene	Δ CT control	Δ Δ CT	Gene Expression folding
C	25.5	24.34	1.16	1.16	0	1
1	33.27	31.99	1.28	1.16	0.08	0.94
2	28.46	24.8	3.66	1.16	3.5	0.1
3	26.07	24.86	1.3	1.16	0.14	0.9
4	28.22	26.13	2.1	1.16	0.94	0.52
5	28.49	26.21	2.28	1.16	1.8	0.28
6	30.74	26.21	4.53	1.16	3.37	0.1
Mean						**0.47**
C	25.32	24.32	1.0	1.0	0	1
1	30.73	24.01	6.7	1.0	5.7	0.1
2	25.36	24.55	0.81	1.0	-0.19	1.14
3	27.29	24.87	2.42	1.0	1.42	0.37
4	26.97	25.56	1.41	1.0	0.41	0.75
5	27.51	26.37	1.14	1.0	0.14	0.9
6	26.74	26.37	0.37	1.0	0.63	0.64
Mean						**0.65**

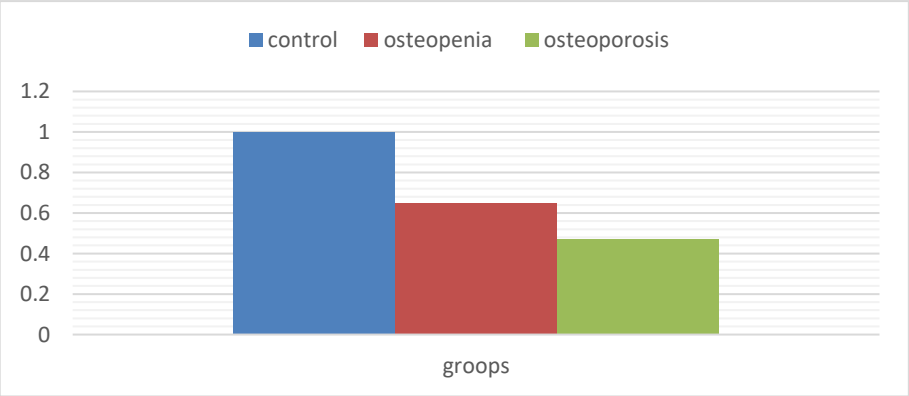


Fig. 2: Level of gene expression of PRE in patients and in the control group

The low level of the genetic expression of a specific hormone means that the quantity that is produced from mRNA of that hormone within cells has decrease in the manufacture of protein or hormone associated with this gene. To produce less hormone itself, access to various factors such as genetic changes or mutations, accessory or medicinal effects, diseases that affect genes regulation (Alberts *et al.,* 2002).

As can be observed in Fig. (3 and 4), and (Table 8), the result of the DNA extraction and the PCR reaction served as an indication that a correlation existed between the patients and genetic variation of PRE gene.

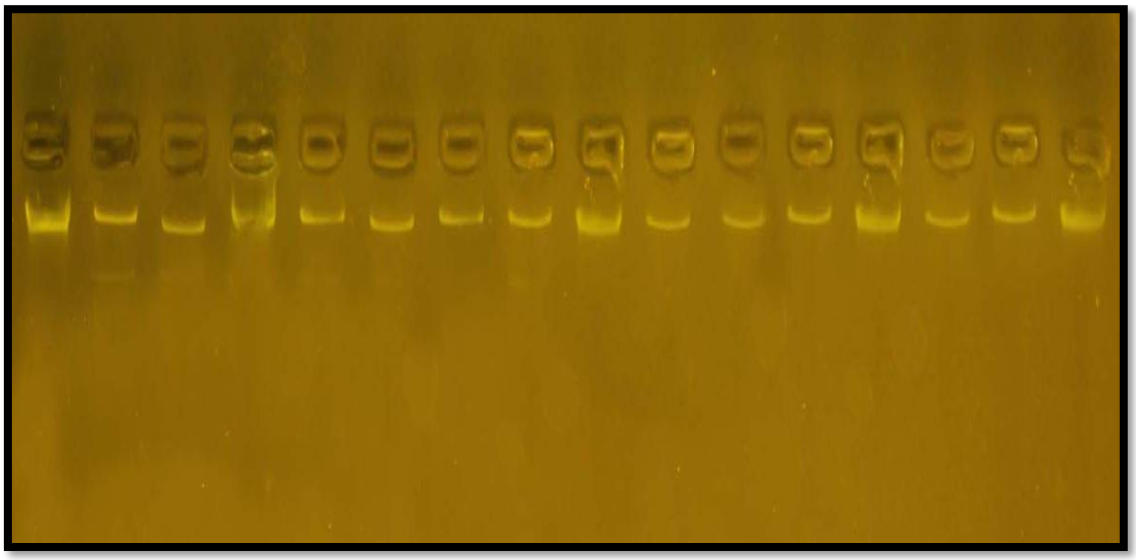


Fig. 3: DNA extraction from blood that separated by 1% agarose gel electrophoresis

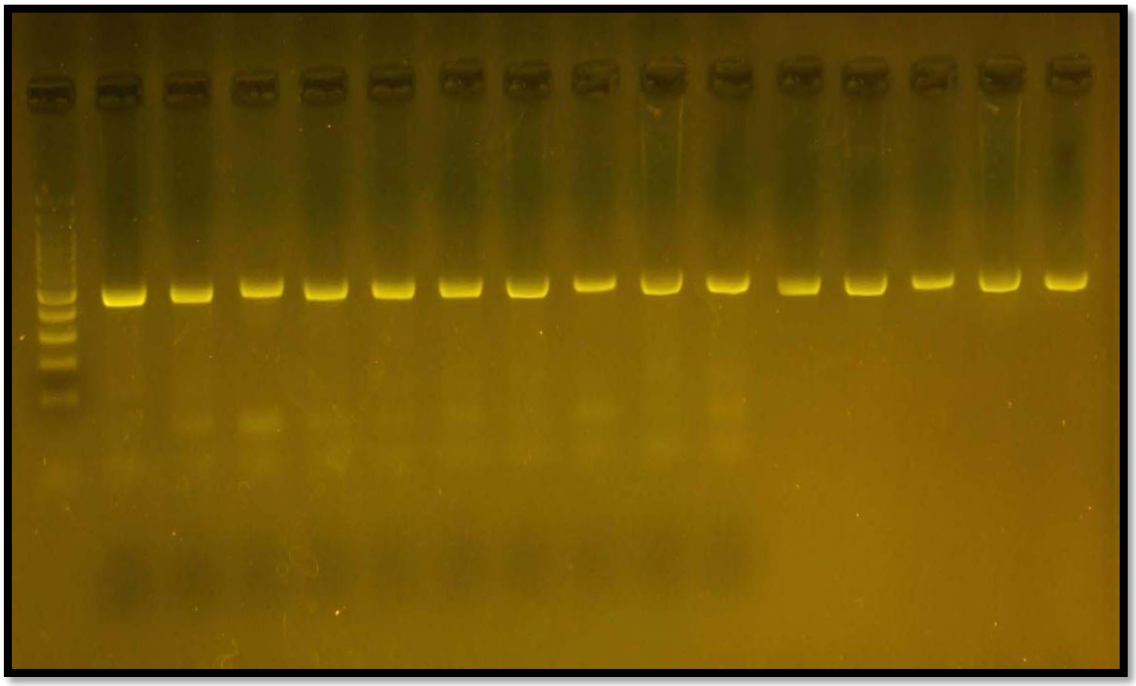
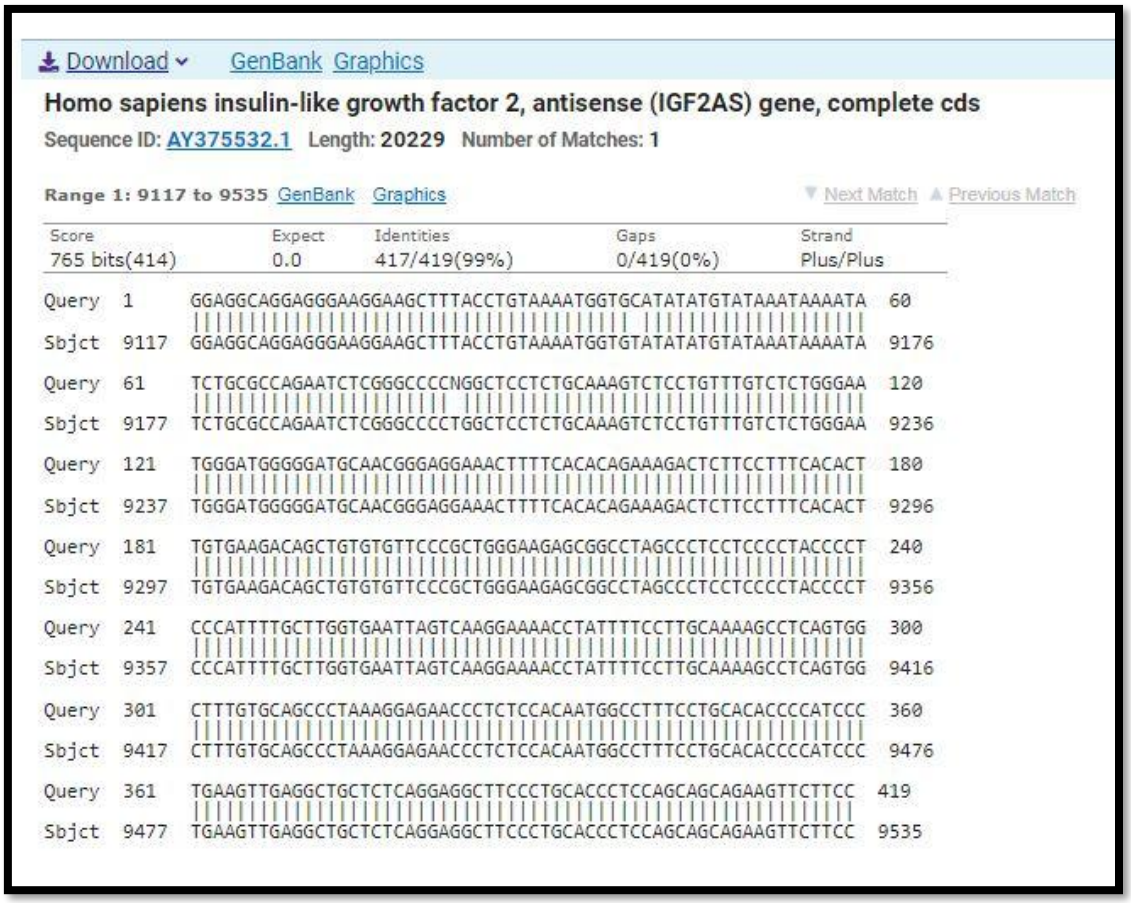


Fig. 4: The PCR product for the PRE gene, which has a size of 490 bp, was separated using 2% agarose gel electrophoresis.

The alignment between the nucleotide sequence of the PRE gene from the studied blood samples and the reference gene sequence at the National Center for Biotechnology Information (NCBI) was analyzed. The detailed matching results are presented in Fig. (5).



Download GenBank Graphics

Homo sapiens insulin-like growth factor 2, antisense (IGF2AS) gene, complete cds

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Range 1: 9117 to 9535 GenBank Graphics Next Match Previous Match

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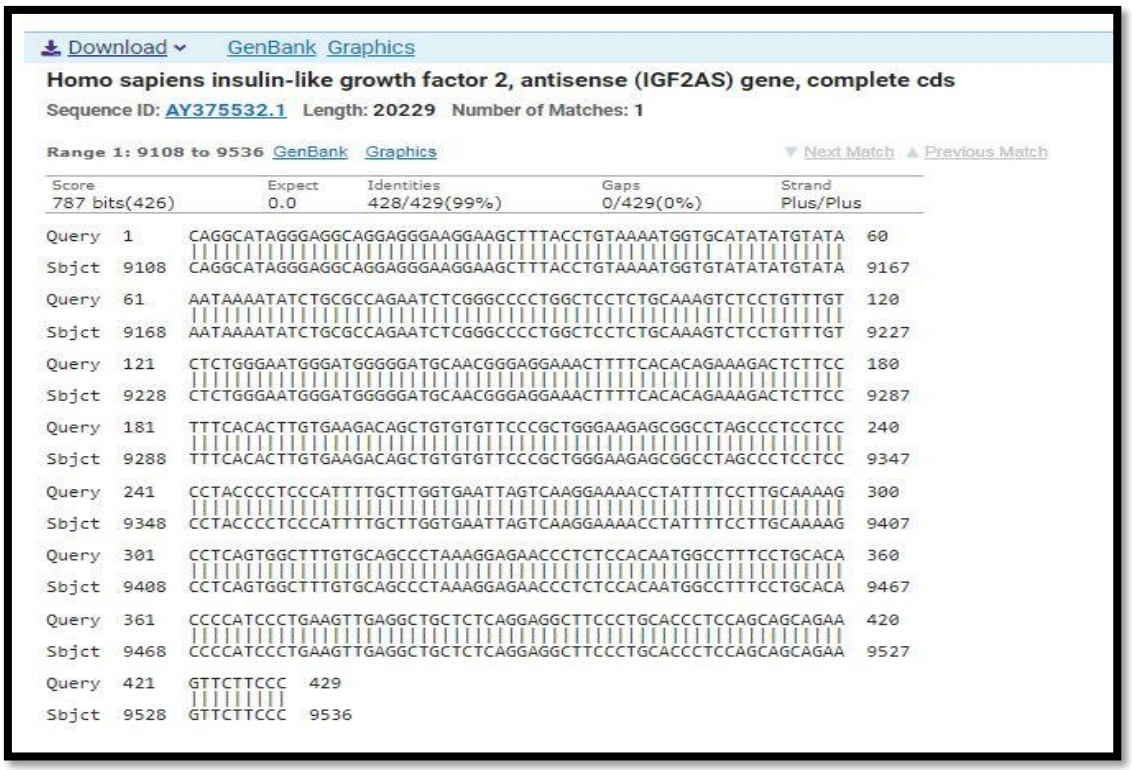

Download GenBank Graphics

Homo sapiens insulin-like growth factor 2, antisense (IGF2AS) gene, complete cds

Sequence ID: AY375532.1 Length: 20229 Number of Matches: 1

Range 1: 9108 to 9536 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
787 bits(426)	0.0	428/429(99%)	0/429(0%)	Plus/Plus

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Sbjct  9528  GTTCTTCCC  9536
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Download GenBank Graphics

Homo sapiens insulin-like growth factor 2, antisense (IGF2AS) gene, complete cds

Sequence ID: AY375532.1 Length: 20229 Number of Matches: 1

Range 1: 9108 to 9536 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
787 bits(426)	0.0	428/429(99%)	0/429(0%)	Plus/Plus

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Fig. 5: The matching results of nucleotide sequence of the *PRE* gene in the studied blood samples with the nucleotide sequence of main gene at National Centre for Biotechnology Information (NCBI)

The results in (Table 8) showed the presence of types of variances that were identified through the technique of DNA sequence and its location in the gene, which are mutation and deletions.

Table (8): Nucleotide sequences of PRE gene by DNA sequence, this is a presentation of the results where they are discussed.

ID Sequence	Nucleotide	Location	Mutation type	Identify	Gaps
AY35532.1	T ——— C	9157	Transformation	%99	0 %
AY35532.1	T ——— C	9157	Transformation	%99	0 %
AY35532.1	T——— C	9157	Transformation	%99	0 %
AY35532.1	A——— C	9177	Transition	%99	0 %
AY35532.1	G ———T	9482	Transition	%99	0 %
AY35532.1	G——— A	9520	Transformation	%99	0 %
AY35532.1	T——— C	9157	Transformation	%99	0 %

(Table 8) presents the nucleotide sequences of the PRE gene obtained through DNA sequencing. The data show the presence of mutations at specific locations in the gene sequence, with mutation types identified as either transformation or transition. All samples showed a high identity percentage (99%) with 0% gaps, indicating reliable sequence alignment. A recurrent mutation at position 9157 (T to C) was observed multiple times, suggesting a possible hotspot for genetic variation in this region. Other notable transitions occurred at positions 9177, 9482, and 9520, involving changes from A to C, G to T, and G to A respectively. These variations may influence gene expression or the functional structure of the PRE gene product. Overall, the findings highlight key mutation points that could be significant for further genetic or functional analysis.

CONCLUSIONS

Most of the mutation found are transformation mutations (T to C), (G to A), while transition mutations (G to T), (A to C). The presence of many genetic mutations from transition and transformation, especially the shift from (T to C), and although they are frequent, this mutation can affect the function of the protein depending on their location within the gene. Transition mutation (T to C) can lead to nonsense or silent mutations, depending on the affected codon. These mutations are able to alter the form of the protein or its performance, which harm and may be entertaining with the relation that the gene performs. Alterations of active sites or regulatory regions may have grave effects. This sort of mutation can have benign results to significant alterations in the course of action of the protein, which can be involved in the disease process, not to mention the reduction of genetic expression of osteoporosis and osteopenia.

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تقييم مستوى تعبير جين PRE في هرمون بريبتين لدى المرضى المصابين بهشاشة العظام في مدينة الموصل

ايلاف غزوان نافع **صبا زكي العبايجي**

جامعة الموصل/ كلية العلوم/ قسم الكيمياء/ الموصل/ العراق

الملخص

الهشاشة العظمية تُعرف عادة بالمرض الصامت لأنها تتطور غالبًا دون ظهور أعراض ملحوظة. يتمثل هذا المرض في انخفاض كثافة العظام، وقد أصبح أكثر انتشارًا في الآونة الأخيرة، مما يؤدي إلى حدوث كسور مفاجئة في المرضى دون أي تحذير مسبق. وقد لوحظ بشكل متزايد في الأشخاص الذين تقل أعمارهم عن 35 عامًا، مما دفع الباحثين إلى دراسة العوامل الجينية المرتبطة به. تؤثر العديد من الهرمونات التي تنظم الحالة الغذائية على التمثيل الغذائي للعظام، ومن بينها هرمون بريبتين.

يعتبر بريبتين هرمونًا ببتيديًا مكونًا من 34 حمضًا أمينيًا، يتم إفرازه مع الإنسولين والأميلين من خلايا البنكرياس بيتا. نظرًا للارتباط المحتمل بينه وبين الهشاشة العظمية، تم اختيار هرمون بريبتين لدراسة تأثيره الجيني. تم جمع عينات دم في أنابيبEDTA لاستخراج الحمض النووي، وفي أنابيب تريازول لاستخراج RNA. تم تحليل تعبير الجينات لهرمون بريبتين في عينات دم مرضى هشاشة العظام ومرضى نقص كثافة العظام في مدينة الموصل بالعراق، وقارنتها مع الأشخاص الأصحاء، نظرًا للانتشار العالي للهشاشة العظمية ووجود ارتباط جيني ووراثي في سبب الإصابة. هدفت الدراسة إلى تقييم التباين الجيني لجينات بريبتين في 40 عينة دم لمرضى هشاشة العظام ونقص كثافة العظام من كلا الجنسين، تراوحت أعمارهم بين 35 و75 عامًا، والذين كانوا تحت علاج مستمر في مستشفيات ابن سينا التعليمية والمستشفى الجمهوري في مدينة الموصل. كما شملت الدراسة 30 شخصًا سليمًا من كلا الجنسين في نفس الفئة العمرية كمجموعة تحكم. بالإضافة إلى ذلك، تم التركيز على الكشف عن الطفرات الجينية في تسلسل الحمض النووي البشري الذي تم تسجيله في بنك الجينات الدولي تحت الرقم LC859842.

الكلمات الدالة :هرمون بريبتين، تعبير الجينات، تسلسل الحمض النووي، هشاشة العظام.