Sys Rev Pharm 2020; 11(6): 159 – 168
A multifaceted review journal in the field of pharmacy
E-ISSN 0976-2779 P-ISSN 0975-8453

Detection of CTLA- 4 and PTPN22 Genes Polymorphisms and Relationship with Hyperthyroidism Patients in Thi-Qar Province, Iraq.

Rasha Salih Nuhair[1], Nabeel Mahdi Abid[3], Shaymaa Z.Al-Rumaidh[2]
[1]Department of Biology, College of Sciense, University of Thi-Qar
[2]Department of physiology. College of Vet- Midecine, University of Thi-Qar
[3]Department of Pathology analysis ,College of science,Unvesity of Thi-Qar
E-mail: salihrasha06@gmail.com
nabeelmahdi4@gmail.com
shaymaaalrumaidh@gmail.com

Article History: *Submitted: 02.03.2020* *Revised: 10.04.2020* *Accepted: 31.05.2020*

ABSTRACT
This study was conducted to identify the genetic polymorphisms of the cytotoxic T-lymphocyte-associated protein 4 (CTLA4) gene and protein tyrosine phosphatase non-receptor 22 (PTPN22) gene to investigate the possible association of their polymorphism with the development or progression of hyperthyroidism. Two genetic loci covering two regions within the CTLA4 and PTPN22 genes were amplified by PCR. Next, a direct sequencing strategy was performed for the observed PCR amplicons to assess the pattern of genetic polymorphism in the screened specimens. Subsequently, the observed variants were localized according to their positions within the referring genomic DNA sequences. The results indicated a significant increase ($P<0.05$) in thyroxin(T4) and triiodothyronine (T3) hormones while no significant changes of thyroid stimulating hormone (TSH) compared with health group. In addation , Our results indicated the presence of only one single nucleotide polymorphism (SNP) in the CTLA4 gene that was only localized in only one investigated patient sample compared with the normal referring sequences. This novel SNP, A81G, was observed in one samples, while no other SNP was detected in other samples . However, no variation was detected in all investigated samples concerning the investigated PTPN22 gene. In conclusion, the present study detected a novel A81G SNP within the targeted CTLA4 gene which may be associated with the progression of hyperthyroidism. Further large-scale screening studied is highly recommended to provide a more comprehensive view of this crucial genetic locus in Iraqi populations.

KeyWords:- Hyperthyroidism , CTLA-4 , polymorphism, PTPN22, SNP

Correspondence:
Rasha Salih Nuhair
Department of Biology, College of Science, University of Thi – Qar
E-mail: salihrasha06@gmail.com

DOI: 10.31838/srp.2020.6.25

INTRODUCTION

The thyroid gland is important in the human body because of its ability to produce the hormones triiodothyronine (T3) and tetraiodothyronine (T4), necessaries for appropriate energy levels, and an active life.Thyroid hormones are important in several physiological processes such as metabolism , normal growth development, maintaining the initial level of phospholipids in cell membranes , fatty acids composition of the lipids and any imbalance in their levels could lead to a wide range of clinical conditions(1,2). Autoimmune thyroid disease (AITD) is the most prevalent autoimmune disorder, related to cellular and humoral immune responses targeted at the thyroid gland and affecting many population. Hyperthyroidism is the condition caused by overproduction of thyroid hormone. The prevalence's are varied, depending on different ethnic, geographic area, and the criteria for diagnosis. In the past, this disorder had been found in the young to middle-age group whereas it is now well recognized in the elder group (3,4). Typical symptoms of hyperthyroidism include sudden weight loss , a rapid heart rate ,sweating ,heat intolerance ,nervousness or irritability (5,6).

The major identified AITD susceptibility genes are classified in two functional groups: 1) immune regulatory genes: cytotoxic T lymphocyte-associated antigen 4 (CTLA-4); protein tyrosine phosphatase, nonreceptor type 22 (PTPN22); interleukin 2 receptor (IL-2R); and 2) thyroid-specific genes: the thyroglobulin gene (TG) and the thyrotropin receptor gene (TSHR) (7,8).

CTLA-4 is a member of the immunoglobulin gene superfamily and a negative regulator of T cell responses that is associated with immune tolerance. CTLA-4 is expressed on the surface of T cells mainly in the form of a dimer, and when it interacts with its cognate ligands, this induces inhibitory signals which terminate T cell activation and proliferation. Polymorphisms in CTLA-4 may alter its functionality such that the activation of T cells cannot be inhibited, resulting in a loss of immune tolerance and the occurrence of autoimmunity, making it vital that normal CTLA-4 activity be maintained. CTLA-4 is a major susceptibility gene associated with autoimmune thyroid disease (9).

The protein tyrosine phosphatase non-receptor 22 (PTPN22) gene maps to chromosome 1p13.3-p13.1, and encodes the lymphoid-specific phosphatase known as Lyp, which contains a catalytic N-terminal domain and a non-catalytic C-terminus composed of four proline-rich domains. Lyp is an important down-regulator of T-cell activation through interacting with protein tyrosine kinase (Csk) and inhibiting signaling pathways mediated by the T-cell receptor (TCR) (10, 11).

Many studies show that specific CTLA-4 or PTPN22 genes polymorphisms confer susceptibility to hyperthyroidism as (12,13). Interactions among SNPs at rs231775, rs231779, and rs3087243 significantly increase an individual's susceptibility to Graves' disease (14). But it is still unclear how CTLA-4 and PTPN22 genes polymorphism contributes to the pathogenesis of these diseases. in this study we aimed to investigate of CTLA- 4 and PTPN22 genes polymorphisms of patients with hyperthyroidism in comparison with controls.

METHODS

This study was carried out on 43 hyperthyroid subjects (women) (mean age 43.3±14.2 years) whom were collected from Endocrine and Diabetic Center in Al-Nasiriya city/Iraq , in the period between 2018-2019 .The T3 and T4 concentrations were assayed by using ELISA kits from Monobind Inc.lake forest CA 92630; USA, Product code : I25-300. TSH also was measured by using ELISA kit from (Calbiotech Inc. a life science company, USA), Product Code: TS227T. The control group includes 26 apparently healthy individuals (women) (mean age 48.7±12.4 years), after having been asked about their health**.**

Hormonal Study

The T3 and T4 concentrations were assayed by using ELISA kits from Monobind Inc.lake forest CA 92630; USA, Product code : I25-300. TSH also was measured by using ELISA kit from (Calbiotech Inc. a life science company, USA), Product Code: TS227T. The control group includes 26 apparently healthy individuals (women) (mean age 48.7±12.4 years), after having been asked about their health.

DNA extraction

The genomic DNA of the investigated samples was extracted using Genaid Kit according to the manufacturer's instructions (Geneaid Biotech, Taiwan). The concentration and purity of DNA were measured by a nanodrop (BioDrop µLITE, BioDrop co., UK), while the DNA integrity was checked by a standard 0.8% (w/v) agarose gel electrophoresis that is pre-stained with a higher concentration of ethidium bromide (0.7 µg/ml) in TAE (40 mM Tris-acetate; 2 mM EDTA, pH 8.3) buffer, using a 1 kb ladder as a molecular weight marker (Cat # D-1040, Bioneer, Daejeon, South Korea). The isolated DNA was used as a template for PCR.

PCR

One PCR fragment was selected for amplification, which partially covered two regions within the *CTLA4* and *PTPN22* genes. The details of these primer's pair were shown in (Table 1). The lyophilized primers were purchased from Bioneer (Bioneer, Daejeon, South Korea). The PCR reaction was performed using *AccuPower* PCR premix (Cat # K-2012, Bioneer, Daejeon, South Korea). Each 20µl of PCR premix was contained 1 U of Top DNA polymerase, 250 µM of dNTPs, 10 mM of Tris-HCl (pH 9.0), 30 mM of KCl, 1.5 mM of $MgCl_2$. The reaction mixture was completed with 10 pmol of each primer and 50 ng of genomic DNA. The following program was applied in PCR thermocycler (MyGenieTM 96/384 Thermal Block, Bioneer, Daejeon, South Korea). The amplification was begun by initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94˚C, annealing (54˚C for 30 s), and elongation at 72˚C, and was finalized with a final extension at 72°C for 30 s. Amplification was verified by electrophoresis on an ethidium bromide (0.5 mg/ml) pre-stained 1.5% (w/v) agarose gel in 1× TBE buffer (2 mM of EDTA, 90 mM of Tris-Borate, pH 8.3), using a 100-bp ladder (Cat # D-1010, Bioneer, Daejeon, South Korea) as a molecular weight marker. It was made sure that all PCR resolved bands are specific and consisted of only one clean and sharp band to be submitted into sequencing successfully.

Table 1: Two specific primers pairs selected to amplify *CLTA4* and *PTPN22* genetic loci within the human genomic DNA sequences. the symbol "Tm" refers to annealing temperature.

Primer	Sequence (5′-3′)	Amplicon size	GenBank Accession Number	Tm	Reference
CLTA4-F	AAATGAATTGGACTGGATGGT	247 bp	NG_011502.1	60° C	(15)
CLTA4-R	TTACGAGAAAGGAAGCCGTG				
PTPN22-F	ACTGATAATGTTGCTTCAACGG	218 bp	NG_011432.1	60° C	(16)
PTPN22-R	TCACCAGCTTCCTCAACCAC				

DNA Sequencing of PCR amplicons

Four samples were selected from patient. The resolved PCR amplicons were commercially sequenced from one direction, forward direction, according to instruction manuals of the sequencing company (Macrogen Inc. Geumchen, Seoul, South Korea). Only clear chromatographs obtained from ABI (Applied Biosystem) 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 local samples with the retrieved DNA sequences, the virtual positions and other details of the retrieved PCR fragments were identified.

Interpretation of sequencing data

The sequencing results of the PCR products of the targeted samples 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 its corresponding position within the referring genome.

Checking the novelty of SNPs

The observed SNP was submitted to the dbSNP database to check their originality. Each particular SNP was highlighted according to its place in the reference genome. Subsequently, the determination of the presence of previous

SNP was performed by viewing its corresponding dbSNP position. Then, the dbSNPs position for the detected SNP was documented.

RESULTS AND DISCUSSION

The results of this study showed there was a significant increase (P<0.05) of concentrations of (T3 and T4) as seen in table (1) as the rate of concentration of T3 in patients (5.302 ± 2.24) compared to the control group (1.807 ± 0.40) with a significant difference (0.00), In addition, T4 concentration(15.20 ± 3.31) for patients compared with the healthy control (8.00 ± 0.69) with a significant difference (0.00) but no significant changes in the serum concentrations of TSH concentration for patients compared with the healthy control. Hyperthyroidism is the condition caused by overproduction of thyroid hormone. Hyperthyroid susceptibility stems from a confluence of genetic, environmental, and immunological factors (9,17). Many studies have observed the incidence of thyroid autoimmune disease in women (4, 18,19) .

Table 2: Comparison of serum (T3 , T4 , and TSH) concentrations of the hyperthyroidism patient groups with healthy controls group.

Parameter	Subject	No. of cases	Mean ±SD	T-value	Df	P-value
T3	Patients	43	5.302 ± 2.24	7.85	67	0.00
	Control	26	1.807 ± 0.40			
T4	Patients	43	15.20 ± 3.31	10.92	67	0.00
	Control	26	8.00 ± 0.69			
TSH	Patients	43	0.423 ± .13	.006	67	0.617
	Control	26	0.42 ± 0.12			

2-Genetic Study

1. CTLA4 gene

Within this locus, 4 samples were included in the present study. These samples were screened to amplify *CTLA4* gene sequences in the chromosome no. 2. The *CTLA4* is a member of the immunoglobulin superfamily that is expressed by activated T cells and transmits an inhibitory signal to T cells. CTLA4 is homologous to the T-cell co-stimulatory protein, CD28, and both molecules bind to CD80 and CD86, also called B7-1 and B7-2 respectively, on antigen-presenting cells. Emerging studies show that changes in *CTLA4* sequences at the DNA, RNA, and/or protein levels may be associated with the development or progression of hyperthyroidism. Thus, the *CTLA4* gene variant is might be associated with the development of hyperthyroidism (20). The sequencing reactions indicated the exact identity after performing NCBI blastn for these PCR amplicons (21). Concerning the 247 bp amplicons, the NCBI BLASTn engine shown about 99% sequences similarities between the sequenced samples and the intended reference target sequences. By comparing the observed DNA sequences of these investigated samples with the retrieved DNA sequences (GenBank acc. NG_011502.1), the accurate positions and other details of the retrieved PCR fragments were identified (Figure 1).

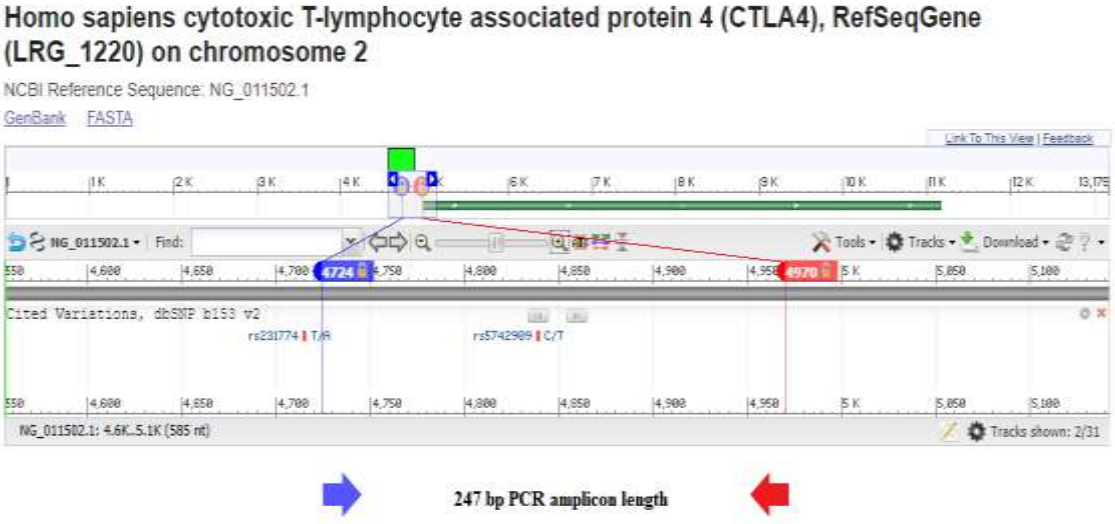

Figure 1-A: The exact position of the retrieved 247 bp amplicon that partially covered a portion of the *CTLA4* gene within chromosome no. 2 (GenBank acc no. NG_011502.1). The cyan arrow refers to the starting point of this amplicon while the red arrow refers to its endpoint.

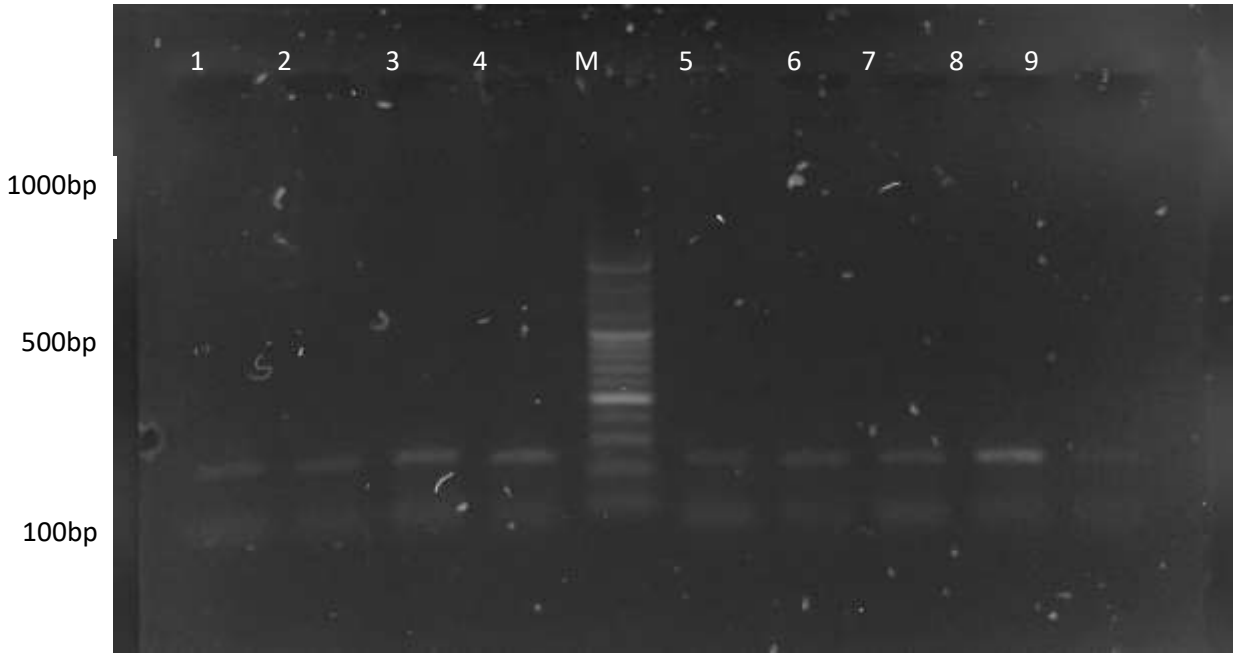

Figure 1-B: Agarose gel electrophoresis of *CTLA4* gene amplification in , where M: ladder, 1-9: positive results

After positioning the 247 bp amplicons' sequences within the chromosome no. 2, the details of its sequences were highlighted, in terms of the positioning of both forward and reverse primers of the 247 bp amplified amplicon (Table 3).

Table 3: The position and length of the 247 bp PCR amplicons used to amplify a portion of the *CTLA4* within chromosome no. 2 (GenBank acc. no. NG_011502.1). The gray-colored sequences referred to the position of the reverse and forward primers, respectively.

Amplicon	Referring locus sequences (5′ - 3′)	length
DNA sequences within the *CTLA4* genetic locus	*AAATGAATTGGACTGGATGGTTAAGGATGCCCAGAAGATTGAATAAAATTG GGATTTAGGAGGACCCTTGTACTCCAGGAAATTCTCCAAGTCTCCACTTAGT TATCCAGATCCTCAAAGTGAACATGAAGCTTCAGTTTCAAATTGAATACATT TTCCATCCATGGATTGGCTTGTTTTGTTCAGTTGAGTGCTTGAGGTTGTCTTT TCGACGTAACAGCTAAACCCACGGCTTCCTTTCTCGTAA**	247 bp

* Refers to the reverse primer sequences (placed in a forward direction)
** Refers to the forward primer sequences (placed in a reverse direction)

The alignment results of the 247 bp samples revealed the presence of only one single nucleotide polymorphism (SNP) in only one of the analyzed samples in comparison with the referring reference DNA sequences (Figure 2). Only one sample showed this SNP, namely S3. Meanwhile, while the other samples have not exerted any detectable SNP.

```
10      20      30      40      50      60      70      80      90      100
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Ref.
AAATGAATTGGACTGGATGGTTAAGGATGCCCAGAAGATTGAATAAAATTGGGATTTAGGAGGACCCTTGTACTCCAGGAAATTCTCCAAGTCTCCACTT
S1  ..........................................................................................
S2  ..........................................................................................
S3  ..........................................................G.....................
S4  ..........................................................................................

110     120     130     140     150     160     170     180     190     200
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Ref.
AGTTATCCAGATCCTCAAAGTGAACATGAAGCTTCAGTTTCAAATTGAATACATTTTCCATCCATGGATTGGCTTGTTTTGTTCAGTTGAGTGCTTGAGG
S1  ..........................................................................................
S2  ..........................................................................................
S3  ..........................................................................................
S4  ..........................................................................................

210     220     230     240
....|....|....|....|....|....|....|....|..
Ref.  TTGTCTTTTCGACGTAACAGCTAAACCCACGGCTTCCTTTCTCGTAA
S1  ...........................................
S2  ...........................................
S3  ...........................................
S4  ...........................................
```

Figure 2: DNA sequences alignment of 4 samples with their corresponding reference sequences of the 247 bp amplicons of the *CTLA4* tic DNA sequences. The symbol "ref" refers to the NCBI referring sequence, letters "S, followed by number" refer to the sample number.

Highly interesting differences were observed in the one nucleic acid substitution as detected in the S3 sample among the other investigated specimens. However, the sequencing chromatogram of the identified variation region, as well as its detailed annotations, were verified and documented, and the chromatograms these sequences were shown according to their positions in the PCR amplicons (Figure 3).

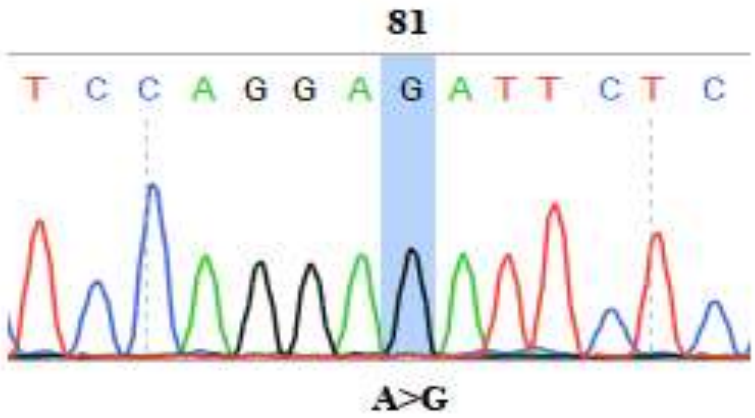

Figure 3: The pattern of the detected SNP within the DNA chromatogram of the targeted 247 bp amplicons of the *CTLA4* gene. The identified substitution SNP is highlighted according to its position in the PCR amplicons. The symbol ">" refers to "substitution" mutation.

To elucidate the position of the detected substitution SNP concerning its deposited SNP database of the sequenced 247 bp fragment, the corresponding positions of the *CTLA4* gene was retrieved from the dbSNP server (https://www.ncbi.nlm.nih.gov/projects/SNP/). So, to find out the nature of this nucleic acid substitution SNP, a graphical representation was performed concerning the *CTLA4* dbSNP database within chromosome no. 2 (GenBank Acc. No. NG_011502.1). By reviewing the genomic position of this SNP, it was found that this SNP is a novel one and not deposited SNP was observed in its genomic position within the *CTLA4* gene known (Figure 4). However, this novel SNP is located in the 5′-untranslated region (5′-UTR) sequences of the studied *CTLA4* gene. Interestingly, there was a tendency for this substitution SNP to be positioned within the patient samples. This is due to its specific localization in S3 samples, which was belonged to the patient specimen. Therefore, it's highly recommended to

study the frequency of such SNP using the statistical analyses. Though the small number of samples represents the main limitation of this study, the high tendency of this novel SNP to be present in the patients needs to be highlighted to see if there is a possible association between this substitution and the progression or development of hyperthyroidism. Though no previous study was reported to associate this substitution SNP with the development of hyperthyroidism, it has been known that the region in which this SNP positioned has belonged to 5′-UTR region. This region is highly linked with a variety of hyperthyroidism diseases.

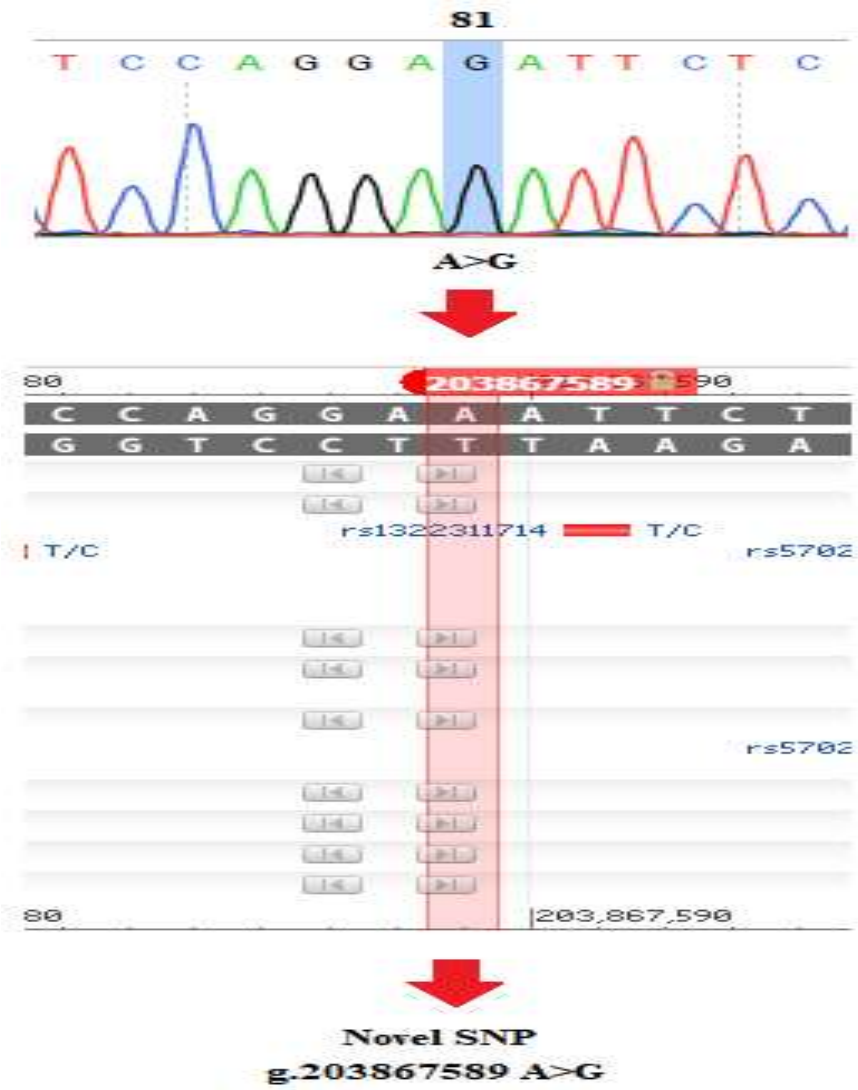

Figure 4: The SNP's novelty checking of *CTLA4* genetic single nucleotides polymorphisms using the dbSNP server. The identified SNP is marked with variable colors respectively. The GenBank acc. no. NC_000001.11 is used in the positioning of the highlighted substitution SNP. The position of the targeted sequences is found in the positive strand. The red arrow refers to the genomic position of the observed novel SNP, while yellow lines refer to the position at which this SNP was inserted.

To summarize all the results obtained from the sequenced 247 bp fragments, the exact position and annotations of the observed nucleic acid substitution SNP is described in the NCBI reference sequences as shown in (Table 3).

Table 4: The pattern of the observed SNPs in the 247 bp amplicons of the *CTLA4* gene in comparison with the NCBI referring sequences (GenBank acc. no. NC_000002.12). The symbol "A followed by number" refers to the investigated sample numbers.

Sample No.	Native	Allele	Position in the PCR fragment	Position in the reference genome	SNP type	Variant summary
S3	A	G	81	203867589	Substitution SNP in 3′-	Novel SNP NC_000002.12;g.203867589

	UTR region	A>G

In this study, it was found that *CTLA4* genetic polymorphism was only detected in patients. For this reason, an interesting outcome could be associated with hyperthyroidism progression. However, the currently analyzed fragment of the *CTLA4* gene was located in a non-coding portion within the same targeted gene, which was found to be localized within the 5′-UTR. Within these sequences, the currently detected novel SNP was not described elsewhere in the literature. Nevertheless, this SNP has a high prevalence in the analyzed specimens of patients and did not exert the same positioning in their control counterparts. This entails that such SNP may be implicated with one of the mechanisms involved in the multifactorial hyperthyroidism. However, it is highly important to describe the current finding using a large-scale investigation to get a more comprehensive perspective on the possible role of A81G SNP in the development of hyperthyroidism in the Iraqi population. Moreover, it might be possible to involve the 5′-UTR flanking regions of A81G SNP in this suggested investigation to describe such a relation between the *CTLA4* gene and hyperthyroidism. This finding comes in agreement with other findings that confirmed the importance of this upstream region in the development of hyperthyroidism (22). Though our detected A81G SNP, was not detected previously, it was found that this SNP was positioned within a highly polymorphic region within the 5′-UTR sequences of the *CTLA4* gene. This, in turn, increases the importance of our detection in terms of the critical position this SNP that characterized. However, the 5-UTR region is not the only locus that could be associated with the development of hyperthyroidism, since several other regions were also reported to be associated with this disease (20). Thus, it can be stated from the above-mentioned results that the *CTLA4* gene has a strong association with the progression of hyperthyroidism. This finding may suggest the A81G SNP as a potential candidate within the *CTLA4* gene which could be associated with the development of hyperthyroidism.

2. PTPN22 gene

Within this locus, 4 samples were included as well. These samples were screened to amplify *PTPN22* genetic sequences in the chromosome no. 1. The *PTPN22* gene encodes a protein called protein tyrosine phosphatase non-receptor 22 (*PTPN22*) gene helps to prevent hyper-responsiveness of T cells, which causes negative T cell regulation (23). Accumulated pieces of literature have been shown that *PTPN22* polymorphism could be associated with the development of hyperthyroidism (24,25). Thus, the *PTPN22* gene variant is potentially correlated with the progression of this disease (26). The sequencing reactions indicated the exact identity after performing NCBI blastn for these PCR amplicons (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Concerning the 218 bp amplicons, the NCBI BLASTn engine shown about 100% sequences similarities between the sequenced samples and the intended reference target sequences. By comparing the observed DNA sequences of these investigated samples with the retrieved DNA sequences (GenBank acc. NG_011432.1), the accurate positions and other details of the retrieved PCR fragments were identified (Figure 5).

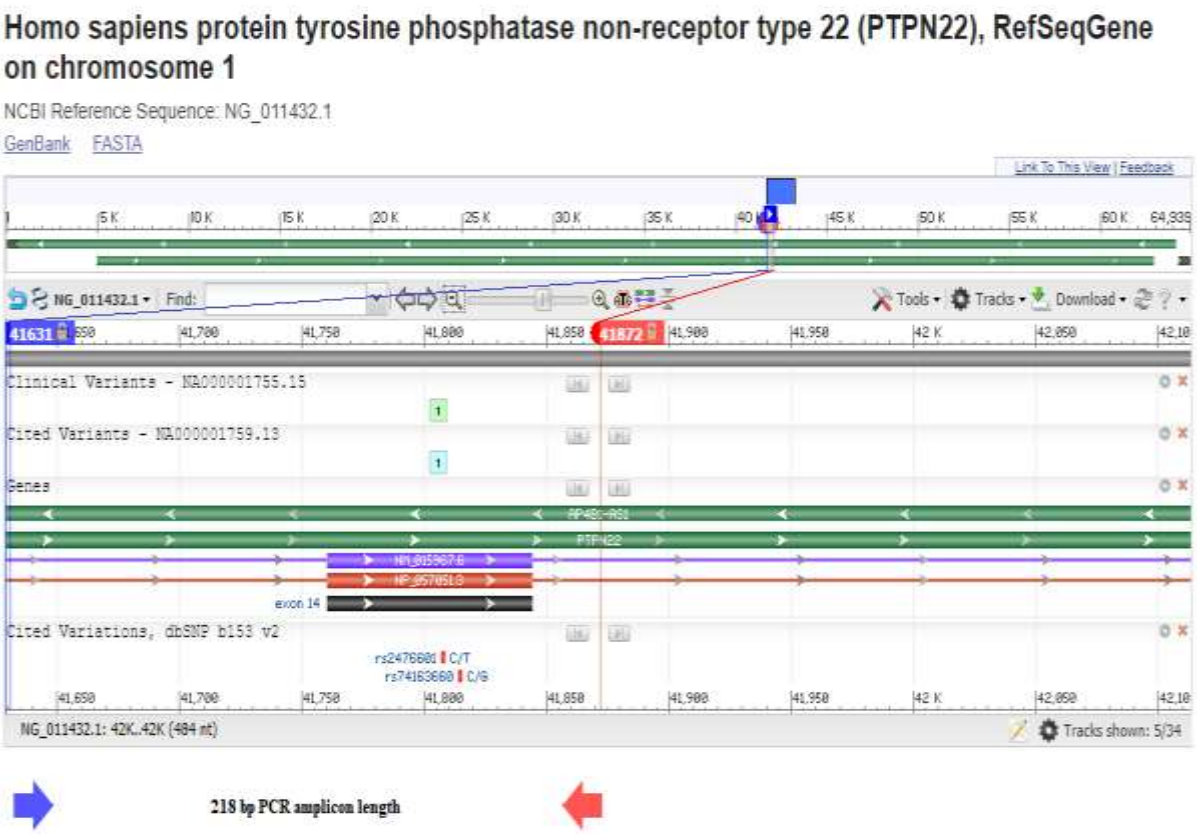

Figure 5-A: The exact position of the retrieved 218 bp amplicon that partially covered a portion of the *PTPN22* gene within chromosome no. 1 (GenBank acc no. NG_011432.1). The cyan arrow refers to the starting point of this amplicon while the red arrow refers to its endpoint.

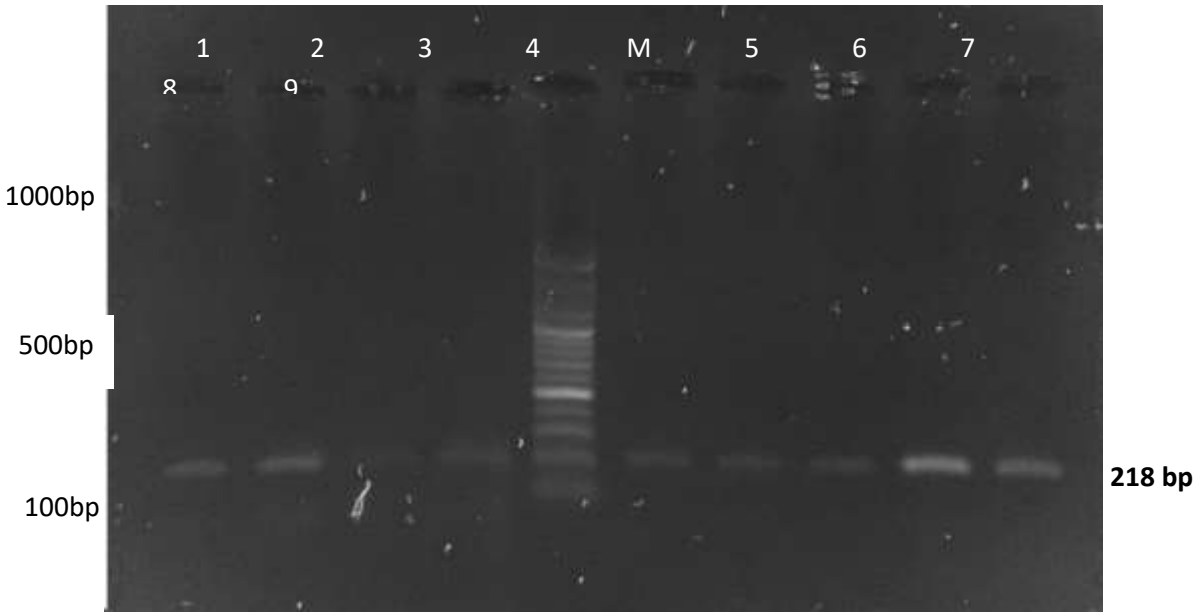

Figure 5-B: Agarose gel electrophoresis of PTPN22 gene amplification in , where M: ladder, 1-9: positive results

After positioning the 218 bp amplicons' sequences within the chromosome no. 1, the details of its sequences were highlighted, in terms of the positioning of both forward and reverse primers of the 218 bp amplified amplicon (Table 5).

Table 5: The position and length of the 218 bp PCR amplicons used to amplify a portion of the *PTPN22* gene within chromosome no. 1 (GenBank acc. no. NG_011432.1). The gray-colored sequences referred to the position of the reverse and forward primers, respectively.

Amplicon	Referring locus sequences (5′ - 3′)	length
DNA sequences within the *PTPN22* genetic locus	*ACTGATAATGTTGCTTCAACGGAATTTAAATATAAATTATGGTAAATTTATA TTTAATATTAGAATATAAGAATTTCCTTTGGATTGTTCTAATTAACAATTGTT ACAATATTTTTGACATTTTGGATAGCAACTGCTCCAAGGATAGATGATGAAA TCCCCCCTCCACTTCCTGTACGGACACCTGAATCATTTATTGTGGTTGAGGA AGCTGGTGA**	218 bp

* Refers to the reverse primer sequences (placed in a forward direction)
** Refers to the forward primer sequences (placed in a reverse direction)

The alignment results of the 218 bp samples revealed the absence of any mutation throughout some of the analyzed samples in comparison with the referring reference DNA sequences (Figure 6).

Figure 6: DNA sequences alignment of 4 samples with their corresponding reference sequences of the 218 bp amplicons of the *PTPN22* genetic DNA sequences. The symbol "ref" refers to the NCBI referring sequence, letters "S, followed by number" refer to the sample number.

Our finding of the absence of any detectable correlation between PTPN22 and hyperthyroidism is not the only one that entailed this observation. Following our finding, recently studied indicated the absence of any association between the *PTPN22* gene and the progression of hyperthyroidism in some Asian populations (27).

CONCLUSION

Concerning the *CTLA4* gene, one substitution SNP was only detected in the patient's specimen. This novel detection of this SNP was confirmed in this study as no previous SNP was detected in the same genomic position. Concerning *PTPN22* genetic polymorphism, no detectable variations were observed in this study, which signifies the non-remarkable association between polymorphism of this gene with hyperthyroidism. Unfortunately, the low number of samples may be considered as the only limiting factor in this study. Therefore, further studies are required to incorporate a larger number of samples to assess the actual distribution of this novel SNP in a variety of hyperthyroidism patients.

REFERENCES

1. Prasad R, and Kumar, V. (2005). Thyroid hormones increase Na+-Pi co-transport activity in intestinal brush border membrane: role of membrane lipid composition and fluidity. Molecular and Cellular Biochemistry, 278 (1-2): 195-202.

2. Fliers E, Bianco AC, Langouche L,and Boelen A (2015). Thyroid function in critically ill patients. Lancet Diabetes Endocrinol 3: 816-825.
3. Panita, L.M.D.;Kittisak ,S. M. D*, Ajanee, M.M.D; Chaiyasit ,C M.D. (2006). Clinical Manifestations of Primary Hyperthyroidism in the Elderly Patients at the Out-Patient Clinic of Srinagarind Hospital. J Med Assoc Thai Vol. 89 No. 2 :178-181.
4. Magtooph , M.G.(2015).Study of Thyroid and Reproductive hormones levels in fertile and infertile women. J.Thi-Qar Sci. Vol.5 (2):31-35.
5. Jonathan, H .Li,Safford ,Aduen J F,Heckman M G,Crook JE and Burger CD.(2007). Pulmonary hypertension and thyroid diseases .Chest; , 132:793-797.
6. Alkinany, A.S.J. (2013). Relationship between hyperthyroidism and pulmonary function tests in female patients . J.Thi-Qar Sci. Vol.4(1) : 1991 – 8690.
7. Hemminki, K.; Li, X.; Sundquist, J.; Sundquist, K.(2010). The epidemiology of Graves' disease: evidence of a genetic and an environmental contribution. J Autoimmun; 34: J307-13.
8. Hodge SE, Ban Y, Strug LJ, et al.(2006). Possible interaction between HLA-DRbeta1 and thyroglobulin variants in Graves' disease. Thyroid; 16: 351-5.
9. Sun,, W .; Zhang ,X.; Wu ,J.; Zhao,W.; Zhao,S. and Li ,M.(2019). Correlation of TSHR and CTLA-4 Single Nucleotide Polymorphisms with Graves Disease. https://doi.org/10.1155/2019/6982623
10. Behrens, T.W. (2011). Lyp breakdown and autoimmunity. Nature genetics 43: 821–822.
11. Wawrusiewicz-Kurylonek, N.; Koper-Lenkiewicz, **O.M.; Gościk, J.; Myśliwiec , J.; Pawłowski, P. and Krętowski.(2019).** Association of *PTPN22* polymorphism and its correlation with Graves' disease susceptibility in Polish adult population—Apreliminary study. Mol Genet Genomic Med.;7:e661. https://doi.org/10.1002/mgg3.661
12. Zhao, S. X. ; Pan, C. M. ; Cao, H. M. et al.,(2010). "Association of the CTLA-4 gene with Graves disease in the Chinese Han population," PLoS One, vol. 5(3) : e9821.
13. Ting, W. H. ; Chien, M. N. ; Lo, F. S. et al.(2016). "Association of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) gene polymorphisms with autoimmune thyroid disease in children and adults: case-control study," PLoS One, vol. 11(4): article e0154394.
14. Chen, x; Hu, Z. ; Liu, M. et al.,(2018). "Correlation between CTLA-4 and CD40 gene polymorphisms and their interaction in Graves disease in a Chinese Han population," BMC Medical Genetics, vol. 19 (1) :171.
15. Astermark J, Wang X, Oldenburg J, et al. MIBS Study Group.(2007). Polymorphisms in the CTLA-4 gene and inhibitor development in patients with severe hemophilia A. J Thromb Haemost 2007; 5: 263–265.
16. Aliparasti MR, Almasi S, Majidi J, Zamani F, Khoramifar AR, Azari ARF.(2013) Protein tyrosine phosphatase non-receptor type 22 gene polymorphism C1858T is not associated with leprosy in Azerbaijan, Northwest Iran. Indian journal of human genetics. 19(4):403.
17. Shukla,S.K.; Singh, G. ; Ahmad, S. and Pant, P.(2018). "Infections, genetic and environmental factors in pathogenesis of autoimmune thyroid diseases," Microbial Pathogenesis, vol. 116, : 279–288.
18. Petta C. A., Arruda M. S., Zantut-Wittmann D. E., Benetti-Pinto C. L. (2007). Thyroid autoimmunity and thyroid dysfunction in women with endometriosis. Hum. Reprod. 22, 2693–269710.1093.
19. Krassas G. E., Poppe K., Glinoer D. (2010). Thyroid function and human reproductive health. Endocr. Rev. 31, 702–755.
20. Pastuszak-**Lewandoska D, Sewerynek E, Domańska D, Gładyś A, Skrzypczak R, Brzeziańska E.(201).** CTLA-4 gene polymorphisms and their influence on predisposition to autoimmune thyroid diseases (Graves' disease and Hashimoto's thyroiditis). Arch Med Sci. 2012;8(3):415–421. doi:10.5114/aoms. 28593
21. Zhang Z, Schwartz S, Wagner L, and Miller W. A greedy algorithm for aligning DNA sequences(2019). J Comput Biol 2000, 7(1-2):203-14. doi: 10.1089/10665270050081478
22. Rubattu S, Forte M, Marchitti S, Volpe M. (2019). Molecular Implications of Natriuretic Peptides in the Protection from Hypertension and Target Organ Damage Development. Int J Mol Sci. 13;20(4):798. doi: 10.3390/ijms20040798.
23. Burn GL, Svensson L, Sanchez-Blanco C, Saini M, Cope AP.(2011). Why is PTPN22 a good candidate susceptibility gene for autoimmune disease? FEBS Lett. 1; 585(23):3689-98.
24. Skorka A, Bednarczuk T, Bar-Andziak E, Nauman J, Ploski R.(2005). Lymphoid tyrosine phosphatase (PTPN22/LYP) variant and Graves' disease in a Polish population: association and gene dose-dependent correlation with age of onset. Clin Endocrinol (Oxf) 62:679–82.
25. Bottini N, Musumeci L, Alonso A, Rahmouni S, Nika K. A.(2004). functional variant of lymphoid tyrosine phosphatase is associated with type I diabetes. Nat Genet 36: 337–338.
26. Ichimura , M.Kaku, H, Fukutani, T, Koga, H, Mukai, I et al., (2008). Association of protien tyrosine phosphate nonreceptore 22(PTPN22) gene polymorphism with susceptability to Graves diseases in Japanese population .Thyroid, 18: 625-630.
27. Shehjar F, Dil-Afroze, Misgar RA, Malik SA,(2018). Laway BA. PTPN22 1858 C/T Exon Polymorphism is not Associated with Graves' Disease in Kashmiri population. Indian J Endocrinol Metab. 22(4):457–460. doi:10.4103/ijem.IJEM_105_18.