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Circulating CTLA-4 Level and CTLA-4 rs733618 Gene Polymorphism Role in Immunological Response to Pfizer BioNTech (BNT162b2) COVID-19 Vaccine in Iraq

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ABSTRACT

Background and Aim: The immune response to the BNT162b2 COVID-19 vaccine among people is not the same. Genetic polymorphism in inhibitory immune checkpoints may have an important role in immune response after vaccination; therefore, we aimed to demonstrate the role of CTLA-4 rs733618 gene polymorphism and the amount of circulating CTLA-4 in individuals vaccinated with the BNT162b2 vaccine following the booster dose.

Materials and Methods: This research is a cross-sectional study performed on 180 healthy adults (above 18 years old) vaccinated with the BNT162b2 COVID-19 vaccine 21-30 days after the second dose at the community-dwelling from December 2021 to April 2022. After DNA extraction from the sample's blood, Allele-specific polymerase chain reaction (ASPCR) was developed to detect single nucleotide polymorphisms of CTLA-4 rs733618. The levels of IgG in the serum, which are directed towards the spike protein-1 and soluble CTLA-4, were quantified using an Enzyme-linked Immunosorbent Assay (ELISA).

Results: The current study showed no significant association in CTLA-4 rs733618 genotype distribution and immune response to the BNT162b2 vaccine, Furthermore, there was a highly significant difference between CTLA-4 serum levels and CTLA-4 rs733618 genotype frequency.

Conclusion: CTLA-4 -1722 T/C rs733618 is not significantly related to immunological response to the BNT162b2 vaccine. The level of s-CTLA-4 production may be affected by CTLA-4rs733618 polymorphism. rs733618 (T/C) and rs733618(C/C) genotypes significantly related with high level of s-CTLA-4, while rs733618 T/T genotype related with low level of s-CTLA-4.

Keywords: Anti-S1 IgG response, BNT162b2, CTLA-4 Gene Polymorphism, mRNA Vaccine, rs733618

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1. Introduction

COVID-19 is a highly contiguous and highly transmissible infectious disease caused by a virus belonging to the family of Coronaviridae **(1, 2)**. The first case was discovered in Wuhan, China in late 2019 **(3)**. The World Health Organization (WHO) acknowledged COVID-19 disease as a pandemic in March 2020 **(4)**. Patients with COVID-19 experience a broad range of clinical symptoms, varying from mild or moderate to severe cases even death **(5, 6)**. The most common cases are mild **(7)**, infected patients with influenza-like symptoms such as fever, dry cough, headache, and diarrhea, not specific symptoms of disease **(8)**. People with underlying, chronic, cardiovascular, and respiratory disorders, as well as the elderly, are at a higher risk **(9)**.

The most crucial diagnostic indicator in COVID-19 patients is lymphocyte reduction, especially T

lymphocytes (10). Because of the stimulation of interferon-γ (INF-γ) secretion by monocytes and neutrophils they are widely distributed throughout blood circulation of the patients with COVID-19 disease, the appearance of "CTLA-4 and PD-1/PD-L1" across the surface of T-cells is stimulated (11).

To control this pandemic, it is imperative to immunize the whole world, there for a variety of safe and efficient vaccine platforms such as viral vector and mRNA-based technologies have been developed (12). Depending on the platforms on which they were developed, according to the most commonly used classification scheme, immunizations can be categorized as either classical or new generation (13). Pfizer, AstraZeneca, and Sinopharm vaccines were the most significant and widely used in Iraq (14).

The Pfizer/BioNTech vaccine candidate, developed by Germany's BioNTech, is an mRNA vaccine based on a relatively new technology, which uses a piece of genetic code, messenger RNA (mRNA) for an important part of the SARS-CoV-2 virus called the "spike protein" (15). Lipid nanoparticles that contain modified mRNA molecules act as the delivery system, allowing the genetic material to cross through the lipid plasma membrane of the cells (16). The vaccine is taken by intramuscular injection, where it causes a short localized inflammatory response and attracts various immune cells, primarily monocytes, macrophages, and dendritic cells, to the injection site via the large network of blood arteries (17, 18). After entering the body, the mRNA finds its way into cells, where protein manufacturers decode the genetic code and produce a vast number of viral proteins, a process known as translation. The S protein produced can be broken in the cytoplasm into pieces that form a compound with major histocompatibility complex class-1 molecules. This combination is delivered to the cell surface, where it induces antigen-specific CD8+ T cells. Otherwise, the" S protein" produced by the host cells can be released and picked up by additional APCs, where it is destroyed in the endosomes and the pieces are loaded onto MHC class 2 molecules. After that, the compound is shown on the cell surface (19). Although B cells-induced antibody production is the key mechanism for SARS-CoV-2 protection, the coordination of CD8+ and CD4+ T cells with the antibody response contributes significantly to the protection (18). Participants who received the Pfizer vaccination had the highest antibody concentration when compared to other vaccines (20). Pfizer and AstraZeneca had a much greater rate of protection against SARS-CoV-2 infection, according to study results. Furthermore, they greatly minimize the occurrence of severe infection, resulting in less hospitalization and mortality (21).

2. Materials and Methods

Study Design

This cross-sectional study was done in Baghdad, the capital of Iraq. One hundred and eighty (180) healthy adults vaccinated with BNT162b2 (mRNA) vaccine over the age of 18 years were enrolled in this study in community-dwelling during the period from December 2021 to April 2022. Patients with autoimmune diseases, cancer, patients on immunosuppressant or chemotherapy, pregnant females, and any individual with acute or chronic inflammation or infection were excluded from the study. Before participating in the study, each subject provided informed consent and had their information obtained through direct conversation using a questionnaire form with the following information: demographic characteristics, date of the second dose of vaccine, date of sampling, their mobile numbers, and if they are immune-compromised or have a history of COVID-19 infection.

Sample Collection

Five milliliters of venous blood were collected from each participant 21-30 days after the second dose. The whole blood was put in an EDTA tube and kept at -20ºC for genomic DNA isolation. From the remaining part of the blood, the serum was separated and kept at -20˚C to be used in serological testing.

SNP Analysis

Genomic DNA was extracted depending on the procedure of (Geneaid, Taiwan) company. Gel electrophoresis was used to check the integrity of the DNA. The purity and concentration of the DNA were determined using Nanodrop. Allele-specific PCR was used for CTLA-4 rs733618 analysis. PCR results were then seen by electrophoresis on agarose gel (2%) and compared to a 100 bp DNA ladder as shown in (Figure 1). The Primers used for CTLA-4 rs733618 polymorphism by PCR were supplied by the company BiONEER /Korea (22), as:

Forward wild :(5'-ATGATCATGGGTTTAGCTGT-3')

Forward mutant :(3'GTGATCATGGGTTTAGCTGC-5')

Reverse: (3'-CCATGTTGGTGGTGATGCAC-5')

Serological Examination

The serum level of the anti-S1 IgG for all participants has been measured using the Indirect Enzyme-linked immunosorbent assay technique (ELISA). All that was carried out according to the manufacturer's instructions, MyBioSource/USA Company. The Sandwich ELISA technique has been used to measure serum level of CTLA-4 for all participants. The

procedure was carried out in accordance with the manufacturer's instructions, BT LAB/China Company.

Statistical Analysis

Version 24 of the Statistical Package for Social Science (SPSS Inc., Chicago, IL., USA) program was used for all statistical analyses. A normality test was conducted on all continuous variables (Shapiro-Wilk test). The Student t-test or analysis of variance (ANOVA) was employed to compare means if the data had a normal distribution. The mean, and standard deviation (SD), were used to express these variables.

3. Results

3.1. Demographic Characteristics of the Study Population

This study included 76 women and 104 men were randomly selected. The subjects were between the ages of 18 and 60, with the highest frequency between the ages of 21 and 30, as shown in Table 1.

Table 1. Demographic characteristics of the study population

		Frequency	Percentile
Sex	Male	104	57.3
	Female	76	42.7
Age	≤20 years	17	9.4
	21-30 years	78	43.3
	31-40 years	54	30.0
	41-50 years	23	12.8
	51-60 years	8	4.4
Total		180	100

3.2. Relationship Between Immune Response to BNT162b2 Vaccine and Age Groups

Regarding the age groups, the low immune response was measured as 62.50% in the 51-60 years age group, while it was 43.50% in the 41-50 years age group. On the other hand, the high immune response was measured as 56.50% in the 41-50 years age group, while it was 37.50% in the age group 51-60 years. There were no statistically significant differences between age groups and immune response to the BNT162b2 vaccine, as shown in Table 2.

Table 2. Vaccine immune response according to age groups

		Age groups				
		≤ 20 years	21-30 years	31-40 years	41-50 years	51-60 years
Immune Response	Low	20	80	50	20	10
	%	58.80%	51.30%	46.30%	43.50%	62.50%
	High	14	76	58	26	6
	%	41.20%	48.70%	53.70%	56.50%	37.50%
Total		34	156	108	46	16
P-value				0.472^{NS}		

NS: statistically non-significant

3.3. Relationship Between Immune Response to BNT162b2 Vaccine and Sex Groups

Regarding the sex groups, 48 (47.52%) females had a low immune response and 28 (35.44%) of them had a high immune response. Moreover, 53 (52.4%) of males exhibited a low response to the BNT162b2 vaccine, and 51 (64.55%) exhibited a high immune response. Statistically, no significant difference was observed between immune response to the vaccine and sex groups, as shown in Table 3.

Table 3. Vaccine immune response according to sex

		Sex	
		Female (76)	Male (104)
Immune Response	Low	48(47.52%)	53(52.47%)
	High	28 (35.44)	51 (64.55%)
Total		80(100%)	
P-value		0.103^{NS}	

NS: statistically non-significant

3.4 CTLA-4 rs733618 Gene Polymorphism Among Study Groups

For the analysis of this SNP, allele-specific PCR was employed. Gel electrophoresis of PCR products (Figure 1) showed that this SNP had three genotypes in the high and low immunological response to the Vaccine. TT (wild type), TC, and CC (mutant type).

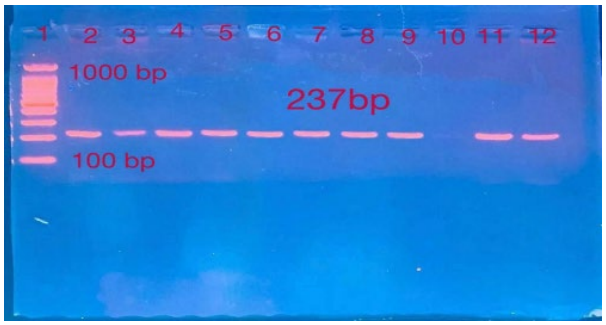

Figure 1. CTLA-4 rs733618 PCR product was electrophoresis on 2% agarose at 70 volt/cm 2. 1x TBE buffer for 1 hour. Lane 1: DNA ladder (100-1000 bp), lanes: 2-9, 11, and 12 successful amplification with 237 bp PCR product. Lane 10:non-template negative control.

3.5. Vaccine Immune Response in Association with CTLA-4 rs733618 Genotypes and Alleles

According to the immune response to a vaccine, results showed the IgG titer means of individuals in the study group with different genotypes and alleles of this SNP were very close and statistically no significant differences existed between the immune response to the vaccine and *CTLA-4 rs733618* genotypes and alleles, as shown in Table 4.

Table 4. Vaccine immune response in association with CTLA-4 rs733618 genotypes and alleles

CTLA-4 (-1722 T/C rs733618)		Immune response to Pfizer BNT162b2 mRNA COVID-19 Vaccine (IgG titer) (mean±SD) IU/mL	P-value
rs733618 Genotypes	Homozygous wild TT	33.3±17.5	0.40^{NS}
	Heterozygous TC	30.4±20.7	
	Homozygous mutant CC	29.5±21	
rs733618 Alleles	T wild	31.5± 19.5	0.23^{NS}
	C mutant	29.1±19.6	

NS: statistically non-significant

3.6. Impact of *CTLA-4 rs733618* Gene Polymorphisms on Serum CTLA-4 in Study Groups

Data regarding the serum level of circulating CTLA-4 changed according to CTLA-4 rs733618 genotypes and alleles. The mean of CTLA-4 in individuals with homozygous wild (TT) was 54.17±15.22 ng/mL, lower than the mean of CTLA-4 in individuals with heterozygous (TC) and homozygous mutant genotypes (CC). Significant statistical differences existed between CTLA-4 serum value and CTLA-4 rs733618 genotype frequency. Allele's analysis revealed that the serum level of CTLA-4 in individuals with wild allele (T) was (52.29±10.31 ng/mL) lower than the mean of individuals with mutant allele (C) (65.42±15.34 ng/mL). A significant statistical difference existed between CTLA-4 serum value and CTLA-4 rs733618 allele frequency, as shown in Table 5.

Table 5. The influence of CTLA-4 serum value by *CTLA-4 rs733618* polymorphism in study group

CTLA-4 -1722T/C (rs733618)		sCTLA-4 ng/mL (mean±SD)
rs733618 Genotypes	Homozygous Wild TT	54.17±16.22
	Heterozygous TC	58.64±11.69
	Homozygous Mutant CC	62.87±11.09
P-value		0.02*
rs733618 Alleles	T Wild	52.29±10.31
	C Mutant	65.42±15.34
P-value		0.001**

*: Statistically significant. **: statistically highly significant.

3.7. Correlation Between Circulating CTLA-4 and IgG Titer

Pearson's correlation was used to explore the possible correlation between IgG titer and serum value of CTLA-4. IgG titer demonstrated a negative correlation with circulating CTLA-4 (r= -0.23, *P*=0.001). There was a significant association between IgG titer and circulating CTLA-4, as shown in Table 6.

Table 6. Pearson's correlation between circulating CTLA-4 and IgG titer

Variables	s-CTLA-4	
	r	P-value
IgG titer	-0.23	0.001**

**: statistically highly significant.

4. Discussion

Data regarding the high level of serum CTLA-4 significantly associated with low production of anti-S1 IgG and low levels of serum CTLA-4 associated with high production of anti-S1 IgG are shown in Table 1. However, this result was expected, and it indicated that inhibitory immune checkpoints have an important role in immune response after BNT162b2 vaccination which is in line with a recent study according to which the immune checkpoint inhibitors (ICIs) have shown promise in enhancing vaccine immunogenicity and effectiveness when combined with licensed or unlicensed vaccines **(23)**. Another study showed that the cell-mediated immunogenicity of the influenza vaccine is robust in cancer patients receiving ICIs **(24)**.

Regarding demographic characteristics, the current study showed no statistical differences in age or sex with serum level of soluble CTLA-4, as shown in Tables 2 and 3, which disagrees with Leng Q *et al.* (2002), who discovered that prolonged immunological activation contributes to immune senescence associated with aging, which is accompanied by a decline in CD28 co-stimulatory molecules and an increase in inhibitory CTLA-4 molecules **(25)**. However, another study conducted by Chen Y *et al*. (2011) reported that the serum concentration of sPD-1 vs. sPD-L1 increased in an age-dependent manner, rendering older adults sensitive to apoptotic signals compared with younger adults **(26)**.

In the molecular assay, regarding the SNP rs733618 found in the promoter part of the CTLA-4 gene identified by allele-specific PCR, the T→C mutation at position -1722 may alter the transcriptional regulation of CTLA-4 **(27)**. According to Hudson *et al*. (2002), the rs733618 -1772(T) allele was found in the promoter to reduce the CTLA-4 gene transcription by affecting the binding of transcription factors **(28)**. This study demonstrated no significant association between CTLA-4-1722T/C rs733618 and anti-S1IgG titer. Likewise, there is no significant association between allele distribution and anti-S1-IgG titer, as shown in Table 4. A recent study conducted by Talib and Kadhim (2022) reported no significant difference between COVID-19 infection and rs733618 genotypes and alleles frequency but the (T/T) genotype was more frequent in the mild (moderate) COVID-19 infection group than the severe infection, and (T/C) was high frequent in sever group than mild (moderate) group **(29)**. A study conducted by Mahdi and Kadhim (2020) explained that the (T/T) genotype was more frequent

in the uncomplicated hepatitis B infection than complicated group, while the (T/C) genotype was more frequent in the complicated group than the uncomplicated infection group **(30)**.

Regarding the effect of rs733618 on the serum concentration of circulating CTLA-4, there was a significant association between the serum concentration of circulating CTLA-4 and rs733618 genotypes. The result of ELISA in this work showed that the serum level of CTLA-4 in individuals with rs733618(T/C), and rs733618(C/C) genotypes was higher than the individuals with rs733618(T/T), as shown in Table 5. This result explains high levels of inhibitory immune checkpoints induce T-cell suppression, which agrees with the study by Beserra et al. The aforementioned study showed that the increased serum level of s-CTLA-4 selectively prevents CD80/CD86 from interacting with the co-stimulatory receptor CD28 to prevent early T-cell activation **(31)**. According to this study, by preventing T cell activation or promoting its death, circulating PD-L1 has immunosuppressive effects **(32)**.

Regarding the correlation between IgG titer and serum value of circulating CTLA-4, this study demonstrates a negative correlation, and a highly significant association between them, as shown in Table 6. The current study result agrees with the research that reported that immune inhibitory molecules, including CTLA-4, TIM-3, TIGIT, PD-1, and LAG-3, normally inhibit immune responses via negatively regulating immune cell signaling pathways to prevent immune injury.

Limitations of our clinical study include the small sample size and its restriction to participants below 60 years of age, which may limit the effect of age on the immune response to the vaccine. Another limitation is that we did not perform further analysis to detect cellular immune response to BNT162b2 and whether this SNP impacts CMI; instead, we limited our study to only an antibody response. Due to limited access to all participants at different times, it was difficult to evaluate anti-S1 IgG, measuring before and after vaccination and following the primary and the booster dose.

5. Conclusion

The serum level of soluble inhibitory immune checkpoint marker cytotoxic T-lymphocyte antigen-4 (s-CTLA-4) was higher in participants that produced a high level of IgG antibody against spike protein (high immune response) after the second dose of Pfizer BNT162b2 mRNA COVID-19 vaccine. There was no relation between (age or sex) groups and immune response to vaccine (IgG titer). CTLA-4 -1722 (T/C) rs733618 is not significantly associated with an immune response to the BNT162b2 vaccine. The serum level of s-CTLA-4 may be affected by rs733618 polymorphism. The rs733618 (T/C) and rs733618(C/C) were significantly related to high serum levels of soluble CTLA-4 while rs733618 (T/T) was related to low levels of soluble CTLA-4.

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Ethics Approval

The study was reviewed and approved by the Institutional Review Board's (IRB) ethical of the College of Medicine, Al-Nahrain University on 2021/12/05 under the number 20211053.

Conflicts of Interest

There are no conflicts of interest.

Patients Consent

Before participating, participants provided informed consent.

Author contribution

Selda Sabah Ezzaldeen contributed to the preparation of the manuscript. Haider Sabah Kadhim and Atheer Juad Abdulameer contributed to the revision of the manuscript.

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