Original Article

# Cytotoxic T-lymphocyte antigen-4 Gene Polymorphism are Associated with High Immune Response to BNT62b2 Coronavirus Disease 2019 Vaccine and Low Level of Soluble Immune Check Point in Iraqi Subjects

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## Abstract

**Background:** One way to stop novel coronavirus pandemic is vaccination. Studying the role of inhibitory immune checkpoint gene polymorphisms is considered an important tool for understanding why the immune response to BNT162b2 vaccine is different among people. The present study aims to demonstrate the role of Cytotoxic T-Lymphocyte antigen-4 (*CTLA-4)* rs231775 gene polymorphism and the level of soluble inhibitory checkpoints in individuals vaccinated with BNT162b2 coronavirus disease 2019 (COVID-19) vaccine following the booster dose. A cross-sectional study was conducted between December 2021 and April 2022. **Methods:** A blood sample was obtained from 180 vaccinated individuals. The level of immunoglobulin G (IgG) toward spike protein-1 and soluble inhibitory checkpoints was measured by Enzyme-linked Immunosorbent Assay. After deoxyribonucleic acid extraction, genotypes were detected by Allele-specific polymerase chain reaction. Statistical analysis used SPSS Version 16 software. Quantitative results are indicated as mean ± standard deviation. The statistical significance level was set at $P < 0.05$. **Results:** A highly significant association in *CTLA-4* rs241775 genotypes distribution and immune response to BNT162b2 COVID-19 vaccine, the IgG titer means of individuals with homozygous wild (AA) lower than the means of individuals with heterozygous (AG) and homozygous mutant (GG) genotypes. Furthermore, there were highly significant difference between inhibitory immune checkpoint serum levels and *CTLA-4* rs231775 genotype frequency. **Conclusion:** *CTLA-4* rs231775 linked with high immune response to BNT162b2 vaccine. Subjects who carry G allele showed a high level of IgG titer than the subjects who carry G allele after vaccination. Soluble immune checkpoint markers are dramatically raised in individuals with low immune response.

**Keywords:** Anti-S1 immunoglobulin G response, BNT162b2, Cytotoxic T-lymphocyte antigen-4 gene polymorphism, messenger RNA vaccine, rs231775

## Introduction

A novel coronavirus disease is a highly transmissible infection caused by a newly discovered strain of coronavirus called "Novel coronavirus,"[1] it caused a global outbreak and a tremendous loss of human life worldwide.[2] Depending on the genomic sequencing results, SARS-CoV-2 is phylogenetically related to severe acute respiratory syndrome bat viruses, suggesting that bats could be the major reservoir.[3] The first case of this infection was found in Wuhan/China in the December 2019 then spreads swiftly throughout the world, including our country.[4] Clinically, it causes respiratory diseases ranging from minor to life-threatening infection even death.[5] Some published studies from worldwide suggested the link between hematology parameters and sociodemographic features with morbidity, severity, or mortality of coronavirus

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disease 2019 (COVID-19).[6] Lymphopenia and increased neutrophils were also effectively correlated with disease progression.[7]

There is a positive connection between T-cell destruction and increased expression of inhibitory immunological checkpoint molecules on their surface after SARS-CoV-2 infection.[8] T-cell destruction has been shown to be regulated by inhibitory immunological checkpoint molecules during malignancies, chronic viral infections, and acute viral infections such as Ebola and Hantavirus. The serum value of the inhibitory checkpoint is significantly greater in patients with severe infection than the mild or asymptomatic infection.[9] CD8+ T cells, additionally to CD4+ T cells and neutralizing antibodies play a vital function in preventing the severe corona infection, despite the fact that their quantity is declining.[10] The CD8+ T-cell subset in patients with COVID-19 changes in both quality and quantity particularly in patients with severe infection, decreased cell number and activation characteristics are observed.[11] The effector activities of an exhausted CD8+ T-cell are gradually lost. Early on, defects in tumor necrosis factor production, interleukin-2 production, and proliferative capability, as well as prolonged presentation of inhibitory checkpoints altered transcriptional and epigenetic landscapes and metabolic re-programming are all affected.[12]

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 messenger RNA (mRNA)-based technologies have been developed.[13] Depending on the platforms on which they were developed, vaccinations can be classified as either classical or new generation according to the most often used classification scheme.[14] Pfizer, AstraZeneca, and Sinopharm vaccines were the most significant and widely used in Iraq.[15]

The Pfizer/BioNTech vaccine candidate, developed with Germany's BioNTech, is mRNA vaccine based on a relatively new technology, which uses a piece of genetic code, mRNA for an important part of the SARS-CoV-2 virus called the "spike protein."[16]

Lipid nanoparticles that contain modified mRNA molecules act as delivery system, allow the genetic material to cross through the lipid plasma membrane of the cells.[17]

The vaccine is taken by intramuscular injection, where they cause a short localized inflammatory response and attract various immune cells, primarily monocytes, macrophages, and dendritic cells, to the injection site through the large network of blood arteries.[18,19]

After entering the body, the mRNA finds its way into cells, where protein manufacturer's 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 antigen-presenting cells, where it is destroyed in the endosomes and the pieces are loaded onto MHC class 2 molecules. After then, the compound is shown on the cell surface.[20] 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.[21] 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.[22]

## Materials and Methods

This cross-sectional study was done at the College of Medicine/Al-Nahrain University, Baghdad, Iraq. One hundred and eighty healthy adults (above 18 years old) vaccinated with the Pfizer BNT162b2 mRNA COVID-19 vaccine were enrolled in this study in community-dwelling 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. All data were obtained through direct interviews with individuals using a questionnaire sheet that includes the person's age, gender, date of the second dose of vaccine, date of sampling, and if they are immunocompromised or have a history of COVID-19 infection.

### Sample collection

Five milliliters of venous blood was collected from each participant 21–30 days after the second dose: Three milliliters was collected from vaccinated individuals to assess the soluble inhibitory immune checkpoints and anti-SARS-COV-2 spike protein immunoglobulin G (IgG) antibodies level. After being left in a gel tube for 30 min and centrifugation at 3000 rpm for 5 min, the serum was stored at −20°C until used for the serological test by Enzyme-linked immunosorbent assay (ELISA) assay. The remaining 2 mL of whole blood was stored in an ethylenediaminetetraacetic acid tube, which was used to detect Cytotoxic T-lymphocyte antigen-4 (*CTLA-4 gene*) polymorphisms with specific primers after deoxyribonucleic acid (DNA) isolation.

### SNP analysis

Genomic DNA is 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 polymerase chain reaction (PCR) was used for *CTLA-4* rs231775 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

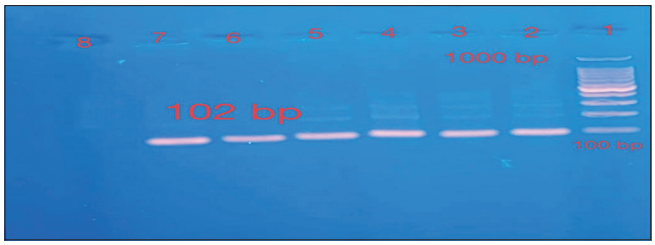


**Figure 1:** Cytotoxic T-lymphocyte antigen-4 rs231775 polymerase chain reaction (PCR) product was electrophoresis on 2% agarose at 70 volt/cm$^2$ × 1 TBE buffer for 1 h. Lane 1: deoxyribonucleic acid ladder (100–1000 bp), lanes: 2–7 successful amplification with 102 bp PCR product. Lane 8: nontemplete negative control

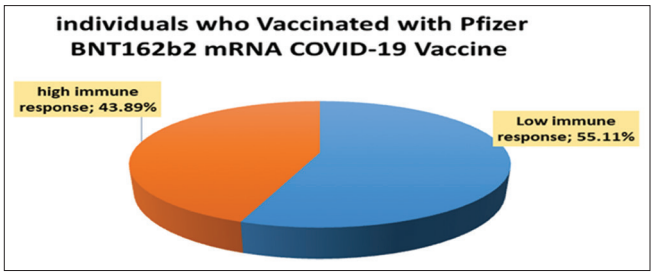


**Figure 2:** The percentage of low and high immune response to Pfizer BNT162b2 mRNA COVID-19 vaccine. mRNA: Messenger RNA, COVID-19: Coronavirus disease 2019

that used for *CTLA-4* (+49A/G) rs241775 polymorphism by PCR were supplied by the company BiONEER/Korea,[23] as Forward wild:(5′-GGCTCAGCTGAACCTGGCCG-3′) Forward mutant: (5′-GGCTCAGCTGAACCTGGCCA-3′) Reverse (5′-ATGCTCCAAAAGTCTCACTC-3′).

### Serological examination

The serum level of the anti-S1 IgG for all participants has been measured using indirect Enzyme-linked immunosorbent assay technique (ELISA). All were done depending on the manufacturer's instructions, MyBioSource/USA Company. Sandwich ELISA technique has been used to measure serum level of soluble markers (*CTLA-4*, program cell death-1 [PD-1], and program cell death-ligand 1 [PD-L1]) for all participants. The entire procedure was carried out according to the manufacturer's instructions, BTLAB/China Company.

### Ethics statement

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.

### Statistical analysis

IBM SPSS Version 24 (IBM Corp. Released 2016. IBM SPSS Statistics for Windows, version 24.0. Armonk, NY:IBM Corp) program was used for all statistical analyses. A normality test was conducted on all continuous variables (Shapiro–Wilk test). The *t*-Student's *t*-test or analysis of variance was employed to compare means if the data had a normal distribution. The mean, standard deviation, were used to express these variables. A $P < 0.05$ indicated a difference that was statistically significant.

## Results

Overall 180 individuals, 101 (56.11%) individuals had a low immune response to the vaccine, and 79 (43.88%) individuals had a high immune response according to the mean of IgG titer 29.44 ± 1.97 IU/ml, as shown in Table 1 and Figure 2.

### Immune response to BNT162b2 vaccine according to age groups

Results showed no differences in the immunological response to the vaccine. In the age group <30 years, the mean age of individuals with high immune response was 24.2 ± 3.0 years, while it was 23.7 ± 3.2 years in low immune response group. On the other hand, in the age group ≥30 years, the mean age of individuals with high immune response was 39.2 ± 7.7 years, while it was 38.0 ± 6.7 years in the low immune response group. There were no significant statistical differences between age groups and immune response to vaccine, as shown in Table 2.

**Table 1: Descriptive statistics of BNT162b2 vaccine immunoglobulin G titer among study population**

| Statistics | IgG titer (IU/mL) |
|---|---|
| Mean | 29.44 |
| SD | 1.97 |
| Median | 28.40 |
| Range | 118.8 |
| Minimum | 6.31 |
| Maximum | 125.14 |

IgG: Immunoglobulin G, SD: Standard deviation

### Immune response to BNT162b2 vaccine according to sex groups

This study included 76 females and 104 males. The IgG titer means of males in the high immune response group was 43.9 ± 19.3 IU/mL, while it was 46.18 ± 18.3 IU/mL in female. In addition, The IgG titer means of males in the low immune response group was 16.9 ± 8.5 IU/mL, while it was 17.2. ± 8.5 IU/mL in female. There were no significant statistical differences between both sexes, as shown in Table 3.

### Cytotoxic T-lymphocyte antigen-4 rs231775 gene polymorphism among study groups

Allele-specific PCR was used for gene amplification and analysis of this SNP. Gel electrophoresis of PCR products [Figure 1] showed that this SNP had three genotypes, AA (wild type), GA, and GG (mutant type).

### Relationship between cytotoxic T-lymphocyte antigen-4 rs231775 gene polymorphism and immune response to BNT162b2 vaccine according to immunoglobulin G titer

According to the immune response to a vaccine, results showed the IgG titer means of individuals with homozygous wild (AA)

was 26.5 ± 15.8 IU/ml lower than the means of individuals with heterozygous (AG) and homozygous mutant (GG) genotypes were 31.8 ± 24.0 IU/ml versus 35.5 ± 20.2 IU/ml, respectively. Strong significant differences existed between the immunological response to the vaccine and *CTLA-4* rs231775 genotypes frequency.

Allele's analysis revealed that the IgG titer means of individuals with wild allele (A) was 32.4 ± 2.0 IU/ml lower than the IgG titer mean of individuals with mutant allele (G) was 26.7 ± 13.3 IU/. A strong significant difference existed between IgG titer and *CTLA-4* rs231775 alleles frequency, as shown in Table 4.

**Table 2: Relationship between BNT162b2 vaccine immune response and age group**

| Age (years) | Individuals vaccinated with Pfizer BNT162b2 mRNA COVID-19 vaccine, mean±SD | | *P* |
|---|---|---|---|
| | Low immune response (*n*=101) | High immune response (*n*=79) | |
| <30 | 23.7±3.2 | 24.2±3.0 | 0.47 |
| ≥30 | 38.0±6.7 | 39.2±7.7 | 0.41 |

SD: Standard deviation, COVID-19: Coronavirus disease 2019, mRNA: Messenger RNA

**Table 3: Relationship between BNT162b2 vaccine immune response and sex group**

| Sex | Individuals vaccinated with Pfizer BNT162b2 mRNA COVID-19 vaccine, mean±SD (IU/mL) | |
|---|---|---|
| | Low immune response (*n*=101) | High immune response (*n*=79) |
| Male (*n*=104) | 16.9±8.5 | 43.9±19.3 |
| Female (*n*=76) | 17.2±8.5 | 46.3±18.3 |
| *P* | 0.86 | 0.58 |

SD: Standard deviation, COVID-19: Coronavirus disease 2019, mRNA: Messenger RNA

**Table 4: Relationship between cytotoxic T-lymphocyte antigen-4 rs231775 gene polymorphism and immune response to BNT162b2 vaccine according to immunoglobulin G titer**

| *CTLA-4* (+49 A/G rs231775) | Immune response to Pfizer BNT162b2 mRNA COVID-19 vaccine (IgG titer), mean±SD (IU/mL) | *P* |
|---|---|---|
| rs231775 genotypes | | |
| Homozygous wild AA | 26.5±21.8 | 0.004 |
| Heterozygous AG | 31.8±11.0 | |
| Homozygous mutant GG | 42.5±15.2 | |
| rs231775 alleles | | |
| A wild | 26.7±13.3 | 0.001 |
| G mutant | 33.0±18.4 | |

SD: Standard deviation, COVID-19: Coronavirus disease 2019, *CTLA-4*: Cytotoxic T-lymphocyte antigen-4, IgG: Immunoglobulin G, mRNA: Messenger RNA

### Relationship between cytotoxic T-lymphocyte antigen-4 rs231775 gene polymorphisms and soluble immune checkpoint markers among study groups

The level of *CTLA-4*, PD-1, and PDL-1 in serum changed according to *CTLA-4* rs231775 genotypes and alleles. The means of *CTLA-4*, PD-1, and PDL-1in individuals with homozygous wild (AA) were 67.1 ± 14.9 ng/mL, 311.7 ± 73.7 ng/L, and 733 ± 384 ng/L, respectively, higher than the means of *CTLA-4*, PD-1, and PDL-1in individuals with heterozygous (AG) and homozygous mutant genotypes (GG). Strong significant differences existed between *CTLA-4*, PD-1, and PD-L1 serum levels and *CTLA-4* rs231775 genotype frequency.

Allele's analysis revealed that the level of *CTLA-4*, PD-1, and PDL-1 in serum of individuals with wild allele (A) was 62.2 ± 13.1 ng/mL, 333.8 ± 63.5 ng/L, and 733 ± 384 ng/L, respectively, higher than the individuals with G allele were 45.01 ± 16.7 ng/mL, 240.5 ± 169.3 ng/L, and 612.7 ± 414.5 ng/L, respectively. A strong significant difference between *CTLA-4*, PD-1, and PDL-1 serum value and *CTLA-4* rs231775 allele frequency is shown in Table 5.

## Discussion

COVID-19 vaccinations have been developed to combat the pandemic; however, the first vaccines approved by global health authorities and used in Iraq were the BNT162b2 vaccine, the Oxford-AstraZeneca followed by Sinopharm. The efficacy and adverse effect of each vaccine were studied.[24] Regarding vaccine status in Iraq, a total dose of vaccine administrated 19.557.364 up to August 9, 2023, according to the World Health Organization data.[25]

The current study hypothesized that the polymorphism (SNP) in the inhibitory immune checkpoint *CTLA-4* gene may be affected the immunological response to the vaccine. The research was carried out in Iraq on the group of volunteers who received a second dose of the BNT162b2 vaccine.

Regarding demographic characteristics, no significant difference between sex or age groups with high and low immunological response to vaccine is shown in Tables 2 and 3, which agrees with Alameri and Kadhim, 2022 who reported that sex and age were not significantly associated with the immune response to the BNT162b2 vaccine,[26] while this result disagrees with Abu Jabal *et al*., in 2021, who said that ethnicity and age no sex might significantly associated with the immunological response to vaccine (anti-SARS-CoV-2 spike IgG antibodies titer decrease with age).[27] Our study restricted subject's age below 60 years may have reduced the impact of age on the immunological response to the vaccine.

In the molecular assay, regarding the functional SNP rs231775 in exon-1 detected by allele-specific PCR, the A → G mutation at position + 49 leads to a nonsynonymous amino acid substitution from threonine to hydrophobic alanine in codon 17. The developing *CTLA-4* peptide's ability to move from the

**Table 5: The influence of cytotoxic T-lymphocyte antigen-4, program cell death-1and program cell death-ligand 1 serum value by cytotoxic T-lymphocyte antigen-4 rs231775 polymorphism among study groups**

| *CTLA-4* (+49 A/G rs231775) | Soluble inhibitory immune checkpoints, mean±SD | | |
|---|---|---|---|
| | Serum *CTLA-4* (ng/mL) | Serum PD-1 (ng/L) | Serum PD-L1 (ng/L) |
| rs231775 genotypes | | | |
| Homozygous wild AA | 67.1±14.9 | 311.7±73.7 | 733±384 |
| Heterozygous AG | 60.19±14.6 | 267.3±123.5 | 660.2±430.8 |
| Homozygous mutant GG | 52.03±15.9 | 208.5±171.3 | 612.7±414.5 |
| *P* | 0.001 | 0.004 | 0.040 |
| rs231775 alleles | | | |
| A wild | 62.2±13.1 | 333.8±63.5 | 733±384 |
| G mutant | 45.01±16.7 | 240.5±169.3 | 612.7±414.5 |
| *P* | 0.0001 | 0.0001 | 0.01 |

SD: Standard deviation, *CTLA-4*: Cytotoxic T-lymphocyte antigen-4, PD-1: Program cell death-1, PD-L1: Program cell death-ligand 1



ribosome to the endoplasmic reticulum lumen may be affected by changes in the signal peptide's hydrophobicity and helix propensity, which could lead to improper *CTLA-4* targeting to the cell surface and inhibiting activated T-cell proliferation.[28] This study appeared a strong significant relationship between *CTLA-4* rs231775 and anti-spik1 IgG titer. That is, there is a significant relationship between rs231775 homozygous (G/G) and heterozygous (A/G) with high immune response, while rs231775 (A/A) is associated with low immune response. Furthermore, this research identified a high significant association between rs231775 alleles and immune response to the vaccine. The IgG titer means in individuals with the (G) allele higher than the IgG titer means of individuals with the (A) allele, as shown in Table 4. Our results suggest that the genotype (A/A) wild affects the CTLA4 (driven down) regulation of T-cell activation; this means that this genotype may be a risk factor in the low immune response to vaccine or infection. On the contrary, the genotypes (A/G) versus (G/G) mutant affect the CTLA4 (driven up) regulation of T-cell activation; this means that this genotype may be a beneficial factor in the high immune response to the vaccine. The rs231775 (A/A) was previously reported to be related to low T-cell proliferation due to high *CTLA-4* protein function. In contrast, rs231775 (G/G) is associated with low *CTLA-4* protein function, this ultimately results in increasing T-cell proliferation and impaired regulatory T-cell function.[29] A recent study by Talib *et al*., in 2022 reported that the (A/A) genotype is more common in the severe COVID-19 infection group than the mild–moderate group. At the same time, the genotypes (A/G) and (G/G) were more common among the mild–moderate group than the severe group, with significant differences. On the other hand, patients with severe COVID-19 infection have a higher prevalence of the A allele than those with mild-to-moderate disease, while the G allele is more common in mild–moderate conditions than the severe infection.[30]

Regarding the effect of rs231775 on the serum value of soluble *CTLA-4*, PD-1, and PD-L1, this study showed a high significant difference between *CTLA-4* rs231775 genotypes distribution and serum levels of inhibitory immune checkpoints. The mean levels of *CTLA-4*, PD-1, and PD-L1in individuals carrying A/A were higher than the mean level of this marker in individuals carrying A/G and G/G with high significant difference between *CTLA-4* rs231775 genotypes distribution and serum levels of inhibitory immune checkpoints. Furthermore, the mean level of soluble *CTLA-4*, PD-1, and PD-L1in serum of participants with A allele was higher than the mean of individuals carrying G allele with high significant difference between *CTLA-4* rs231775 alleles distribution and serum levels of inhibitory immune checkpoints, as shown in Table 5. A high levels of *sCTLA-4*, sPD-1, and sPD-L1in individuals with low level of IgG titer explain over expression of inhibitory checkpoint markers may induce T-cell s suppression, which agrees with another study that showed that the increased serum level of *sCTLA*-4specifically inhibits the early T-cell activation by blocking the interaction of CD80/CD86with co-stimulatory receptor CD28.[31]

Finally, this study was sufficient to know the role *CTLA-4* gene polymorphism in the humoral immune response to Pfizer BNT162b2 mRNA COVID-19, but it is not sufficient to know the role of polymorphism in the gene of other immune checkpoint markers.

## Conclusion

*CTLA-4 rs231775 gene* related with a high immune response to vaccine. In other words, a significant fraction of people who have the A allele (wild type) had inadequate antibody responses after vaccination, but people with the G allele (mutant type) had a strong correlation with high anti-S1 IgG titers. Serum level l of soluble immune checkpoints are significantly raised in individuals with low antibody titer after vaccination.

### Limitation of the study

Our study has several limitations, and we advise caution in the interpretation of the findings. First, the sample size was small. Second, The IgG concentration in each participant needs to be followed after vaccination of the first and second doses. In addition, this study included only one type of COVID-19 vaccine (BNT62b2 vaccine).

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## Conflicts of interest

There are no conflicts of interest.



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