DOI: 10.24996/ijs.2026.67.5.14

ISSN: 0067-2904

Immune Checkpoint Gene Polymorphism (CTLA-4 and TIM-3) as Risky Factors of Brain Tumor (from Grade I to IV) Incidence in Iraqi Population

Saba Amer Mughir Al.Ammar*, Shakir H.Mohammed Al.Alwany, Noor Salman Kadhim AL.Khafaji

Department of Biology, College of Science, University of Babylon, Iraq, Babil, Iraq

Received: 13/2/2025 Accepted: 7/5/2025 Published: xx

Abstract

Brain and central nervous system cancer was the fourth most common cause of death in Iraq. Brain tumors can hijack immune checkpoint gene polymorphisms to evade immune surveillance and promote tumor progression. T-cell immunoglobulin and mucin domain 3 (TIM3) are type I membrane proteins that are associated with tumor and immune cells regulation. The cytotoxic T-lymphocyte antigen-4 (CTLA-4) is an inhibitory cell surface receptor that can modulate T-cell proliferation. This study aimed to investigate the impact of *TIM-3* and *CTLA-4* genes polymorphism on brain tumor susceptibility in the Iraqi population. One hundred fifty tissue samples were taken from brains enrolled in current research, including 100 brain tumor patients and 50 brain tissues as control, which were taken directly from the deceased in accidents. The DNA was extracted, and the *TIM-3* and *CTLA-4* genes were amplified using PCR. Then, the *TIM-3* and *CTLA-4* gene polymorphisms were identified using the Sanger sequencing method. A significant difference was found among brain tumour patients according to sex and age group (P=0.04 and 0.004, respectively). A novel mutation in the *TIM3* gene at intron 2 position 157106624 C>A was identified, a statistically significant increase in CA mutant genotype frequencies in patients compared to controls (P=<0.0001). Also, the A allele was significantly increased in the brain tumor patients (34%), P=0.001, OR=0.216(0.081-0.577). A mutation in *CTLA4* gene at exon 4 rs60872763 DEL(AT) was first identified, and the frequencies of Del(AT)21 fragments were increased significantly in patients with a brain tumor (93.3%) P<0.001, OR=0.048(0.014-0.160). The A allele and AC heterozygote genotype in *TIM3* gene at intron 2 position 157106624 and CTLA-4 rs60872763 Del(AT)21 repeats may play roles as risky factors in the pathogenesis of brain tumors in Iraqi patients.

Keywords: TIM-3; CTLA-4; brain tumors; PCR; mutation, sequencing

تعدد أشكال جينات نقاط التفتيش المناعية *CTLA-4* و *TIM-3* كعوامل خطر لحدوث أورام الدماغ لدى السكان العراقيين

صبا عامر مغير العمّار*, شاكر حمّاد محمد العلواني, نور سلمان كاظم الخفاجي

قسم علوم الحياة، كلية العلوم، جامعة بابل، بابل، العراق

الخلاصة

سرطان الدماغ والجهاز العصبي المركزي هو السبب الرابع الأكثر شيوعاً للوفاة في العراق. يمكن لأورام الدماغ استخدام الأشكال الجينية لنقاط التفتيش المناعية للتهرب من المراقبة المناعية وتعزيز تطور الورم.

*Email: sci396.saba.amaar@student.uobabylon.edu.iq

الجلوبيولين المناعي للخلايا التائية ومجال الميوسين3(TIM3) هي بروتينات غشائية من النوع الأول ترتبط بتنظيم الاوارم والخلايا المناعية. مستضد الخلايا اللمفاوية التائية 4 (CTLA-4) السام للخلايا، وهو مستقبل مثبط على سطح الخلية يمكنه تعديل تكاثر الخلايا التائية. هدفت هذه الدراسة إلى دراسة تأثير تعدد أشكال الجينات TIM-3 و CTLA-4على قابلية الإصابة بورم الدماغ لدى السكان العراقيين. تم تسجيل 150 من أنسجة الدماغ في البحث الحالي، تتضمن 100 مريض بورم في الدماغ و50 من أنسجة المخ كمجموعة تحكم. تم استخلاص الحمض النووي وتضخيمه باستخدامPCR ، ومن ثم تم تحديد الأشكال الجينية TIM-3 و CTLA-4باستخدام طريقة تسلسل سانجر . بالنسبة لمرضى أورام الدماغ، تم العثور على اختلاف كبير وفقا للجنس والفئة العمرية 0.04 و0.004، على التوالي .(تم التعرف على طفرة جديدة في جين TIM3 في موقع Intron 2 157106624 C>A، وهي زيادة ذات دلالة إحصائية في ترددات النمط الجيني الطافر CA في المرضى مقارنة مع مجموعة التحكم(P = <0.0001) ، كما تم زيادة الأليل A بشكل ملحوظ في مرضى أورام الدماغ 34٪، P = 0.001، .(OR = 0.216 (0.081-0.577تم التعرف لأول مرة على طفرة في جين CTLA4 عند إكسون rs60872763 DEL(AT)4 ، وتمت زيادة ترددات أجزاء Del(AT)21 بشكل ملحوظ في المرضى الذين يعانون من ورم في الدماغP<0.001 (93.3%) ، .(OR=0.048(0.014-0.160قد يلعب النمط الجيني للأليل A و ACالمتغاير في جين TIM3 في موضع 157106624 Intron 2 وتكرارات CTLA-4 rs60872763 Del(AT)21أدوارًا كعوامل خطرة في التسبب في أورام المخ لدى المرضى العراقيين

Introduction

The brain tumor is an accumulation of aberrant cells proliferating in the brain or central spinal canal [1]. There are two fundamental types of brain cancers: Primary brain tumors originate in the brain and typically remain there, and Metastatic brain tumors originate from cancer in other parts of the body and disseminate to the brain. Also, it can be classified as Benign Tumors (slow growing, distinct borders, and rarely spread) and Malignant Tumors (rapid growing, invasive, and life-threatening)[2, 3]. In 2020, among all malignancies, brain and central nervous system cancer was the fourth most common cause of death in Iraq of all ages (both male and female) [4] and the fifth of the top ten cancers in both genders in Iraqi population in 2022 according to the Cancer Registry Report Iraq 2022 [5]. Brain tumors can hijack immune checkpoint gene polymorphisms (genetic variations) to influence their ability to evade immune surveillance and promote tumor progression [6].

Immune checkpoints (ICs) are critical receptors that suppress and prevent over-activation of the immune responses. Typically, immune checkpoints are involved in the maintenance of homeostasis and prevent inflammatory responses [7], and they can be employed by cancer cells to evade detection and elimination [8]. Cancer cells target immunological checkpoint molecules to suppress T cell activation and enhance negative signaling via cell surface molecules, hence promoting cancer development and metastasis [9].

The immune status of cancer is modulated by many factors, including patients' immune reactivity, which may be affected by single nucleotide polymorphisms (SNPs) of immune-related genes [10]. These SNPs may occur in regulatory regions and cause changes leading to damaging or introducing binding sites for transcription factors (TFs) or miRs, and in that way, exert the influence on the expression level of encoded molecules as well as affect chromatin accessibility or DNA-looping [11]. They may also introduce changes to protein structure, which may affect the function of these molecules. Hence, genetic polymorphism may impair the function of molecules important for the effective activity of anti-tumor response, such as TIM-3 and CTLA-4.

The human T-cell immunoglobulin and mucin domain 3 (*TIM-3*) gene is situated on chromosome 5q33.3. TIM3 are type I membrane proteins that have several unique structural components, including a mucin domain, a transmembrane (TM) domain, IgV domain, a

cytoplasmic region (CR), and a signal peptide (SP) and are expressed in several types of immune cells, encompassing B cells, T cells, regulatory T cells, Dendritic cells, mast cells, macrophages, and Natural killer cells [12].
TIM3 has several immune functions depending on the type and status of the cells; for example, by inhibiting T-cell activation, TIM-3 can dampen (CD4+ and CD8+) T-cell responses and induce peripheral tolerance. However, in other cases, TIM-3 can help eradicate pathogenic stimuli by activating different innate immune cells, such as quiescent macrophages [13].
During the progression of tumor and infection with viruses, it has been found that TIM3 expression is high on the exhausted T-cells. Additionally, previously has been discovered that it is associated with tumor and immune cells regulation, potentially irritating tumor progression [14]. Additionally, TIM-3 has drawn increasing interest as a potential target for cancer immunomodulation. TIM-3 expression has been linked to a number of malignancies, according to studies, and it may be involved in regulating the growth of tumors [15].
TIM-3 has many single nucleotide polymorphisms (SNPs). These TIM-3 polymorphisms have been demonstrated to correlate with TIM-3 expression and activity, subsequently influencing cancer risk across various populations [16], such as increasing the susceptibility and progression of breast cancer [17].

The cytotoxic T-lymphocyte antigen-4 *CTLA-4* gene is found in chromosome 2q33, belonging to many regions of immune regulatory genes[18]. CTLA-4 is expressed on the surface of activated T-cells and can modulate T-cell proliferation[19].
CTLA-4 is one of the immunoglobulin-like receptor superfamily: an inhibitory cell surface receptor similar to the CD28 structure. It consists of four domains, including a transmembrane domain, an extracellular ligand-binding domain, a signal peptide, and a small cytoplasmic tail [20], typically expressed on the surface of T lymphocytes (T-cells) [21]. Functioning as a negative regulator of T-cell-mediated immune responses through an intrinsic mechanism that transmits a negative signal directly to effector T-cells and an extrinsic mechanism mostly associated with the functions of regulatory T-cells (Tregs)[22].
CTLA-4 is a polymorphic gene, and more than 100 single-nucleotide polymorphisms (SNPs) found in the *CTLA-4* gene were thoroughly investigated and found to be associated with the risk of human cancers, including colorectal cancer [23], oral squamous cell carcinoma, cervical, lung cancers, and breast cancer [24].

The current study aimed to inspect the impact of *TIM-3* and *CTLA-4* genes polymorphism on brain tumor susceptibility and the relation with several clinical and demographic features such as sex, age and age strata, types and grades of tumor in Iraqi patients with a brain tumor.

Materials and Methods

1.Study Population

One hundred fifty brain tissue samples were obtained between November/2023- June/ 2024 in the Oncology Department at Imam AL-Sadiq Teaching Hospital, International Hospital and other hospitals. A total of 100 patients with brain tumor and 50 healthy controls without a previous history of brain tumor were included, which were taken directly from the deceased in accidents. Brain tumor patients comprised patients with diverse ages (3-85 years) and different types and grades of brain tumor. All subjects (patients and controls) included in this study were of Iraqi ethnicity.

2. DNA Extraction

Approximately 5-10 mg of brain tissue was collected during brain tumor resection surgery and placed in suitable, clean, and sterile tubes and immediately refrigerated until DNA extraction. About 20 mm sections were excised carefully, transferred immediately to 1.5 ml

Eppendorf tubes, and homogenized using a hand-held homogenizer. Genomic DNA was extracted from brain tissue using the G-Spin Total DNA Extraction Kit (iNtRON Biotechnology Co., Korea) and stored at -20°C until used.

3. Primer selection

Primers were designed for the detection of SNPs located in genes that have been reported to be associated with immune-related diseases, including *TIM-3* and *CTLA-4*. The 2 pairs of primers were designed to amplify the genomic DNA fragments covering SNPs in intron and exons. *TIM3* gene primer sequence P F- "GCCCTCCTCCAGGGCTATAA', R- "GCTTGAGTCTTGGCTCTCCTT'. And the primers for *CTLA4* gene F- "AGTTAGGGAATGGCACAGCC', R-"GCCCCAAAGCACATGTCAAC'

4. PCR Experiments:

The conventional thermal cycler (Prime/Germany) was used for PCR amplification. The total volume was about 25µL, which included 12.5µL of master mix, DNA template 5µL, 1µL of each forward and reverse primer, and 5.5µL of nuclease-free water.

5. DNA genotyping and sequencing

The condition summarized in Table 1 was used to carry out the PCR amplification procedure. After completing the amplification process, an agarose gel (1.5 percent) was used for the gel electrophoresis, and a transilluminator (gel-documentation-system/ Igene) was used for analysis.

Table 1: PCR conditions used for genome amplification

gene	Initial denaturation	denaturation	Annealing	Extension	Final extension	Hold
TIM3	95 Cº /5 min	95 Cº / 1 min	55 Cº /45 sec	72 Cº /2 min	72 Cº /5min	4 Cº
CTLA-4		95 Cº / 30 sec	60 C0/45 sec	72Cº/3 min		
		35 cycles				

Then, the PCR products were sequenced via automated sequencing utilizing the PCR forward (F) primer as the sequencing primer for both *TIM3* and *CTLA-4*. Sequencing was conducted at Macrogen Company in Geumcheon, Seoul, South Korea, and the sequences were analyzed using Geneious Bioinformatics software version 2 for sequence data analysis and alignment, relying on the DNA sequences available in the NCBI reference database.

6. Statistical analysis

For statistical analysis, SPSS version 24 software was used to analyze the genotypes and allele frequencies of *TIM3* and *CTLA-4* SNP polymorphism between brain tumor patients and control groups. To estimate the relative risk of polymorphism on tumor, the significant differences between *TIM3* and *CTLA-4* SNP polymorphism were calculated, with a P value ≤0.05 being deemed statistically significant. The odds ratios and 95% confidence intervals (CIs) were also calculated. To ascertain the variations in demographic factors, we employed the chi-squared test. For demographical figures, GraphPad Prism 8 was used to draw figures. BioEdit version 7.7 was used for sequence alignment.

7. Ethical certification:

The present study followed the principles of the Declaration of Helsinki. Before sample collection, verbal and written consent was obtained from the patients. A local ethics commission reviewed and approved the study protocol, consent form, and subject information

on October 15, 2023, under project number M231002 provided by the Ethical Approval Committee at the University of Babylon.

Results

Age distribution of study groups:

The age characteristics of the 100 patients with brain tumors and the 50 healthy controls were investigated. The mean ages of brain tumor patients and control group were 49.9±20.43 years and 47.64 ± 13.53 years, respectively, according to the age; there were no significant differences between the brain tumor patients and control group.

Demographic characteristics of brain tumor patients

Depending on the grades of brain tumor, Grade I was found in 46 (46%) of brain tumor cases, which involved 20 males and 26 females; Grade IV was found in 25 (25%) of brain tumor cases, which included 12 male cases and 13 female cases, while Grade II was found in 20 (20%) of brain tumor cases that involved 8 males and 12 females; lastly, Grade III was found in 9 (9%) patient's cases of brain tumors, which involved 4 cases of male and 5 cases of female as reported in Table 2. No significant differences (*P value* =0.1) between patient groups of brain tumors depending on their grade comparison were found.

Table 2: Brain tumor distribution according to the Grades

	Grades	Male		Female		p-value
		No.	%	No.	%	
Brain tumor patients	*Grade I*	20	45.5%	26	46.4%	0.1
	Grade II	8	18.2%	12	21.4%	
	Grade III	4	9.1%	5	8.9%	
	Grade IV	12	27.3%	13	23.2%	
	total	44	100%	56	100%	

In studied brain tumor patients and in the terms of age, 8% of patients cases were in the age stratum of 2 to 18 years (3 men and 5 women), 22% of cases were in the age stratum of 19 to 35 years (10 men and 12 women), 27% of cases were at the aged stratum of 36 and 52 years (12 men and 15 women), 24% of cases were in the age stratum of 53 to 69 years (11 men and 13 women), and 19% of them were in the age stratum of 70 to 85 years (8 men and 11 women). Importantly, the highest frequency of males (12) was found in the age group 36–52 years, while the highest female frequency (15) was also found in the age group 36–52 years. There were significant differences between male and female brain tumor patients according to age strata (*P*=0.004) (Table 3).

According to the type of brain tumor, Table 3 showed that there were 34 cases of brain tumor with meningioma (16 male and 18 female), 7 cases with Pilocytic Astrocytoma (2 male and 5 female), 2 with Chardomas (1 male and 1 female), 3 cases with craniopharyngioma (1 male and 2 female), 15 cases with Diffuse Fibrillary Astrocytoma (7 male and 8 female), 5 cases with Planum sphenoidale meningioma (1 male and 4 female), 7 cases with Anaplastic Oligodendroglioma (2 males and 5 females), 2 male cases with Anaplastic Astrocytoma, 20 cases with Glioblastoma Multiforme (7 male and 13 female), and 5 male cases with medulloblastoma. There were no significant differences between the groups of patients with brain tumors according to their type (*P*=0.19).

Table 3: Demographic characteristics of brain tumor patients

Variables		Male	Female	*P*
Sex	*patients*	44 (44%)	56 (56%)	**0.04***
	control	20 (40%)	30 (60%)	
Age				
2-18		3 (6.8%)	5 (8.9%)	**0.004***
19-35		10 (22.7%)	12 (21.4%)	
36-52		12 (27.3%)	15 (26.8%)	
53-69		11 (25%)	13 (23.2%)	
70-85		8 (18.2%)	11 (19.7%)	
Types				
Meningioma		16 (36.4%)	18 (32.1%)	0.19
Pilocytic Astrocytoma		2 (4.5%)	5 (8.9%)	
Chardomas		1 (2.3%)	1 (1.8%)	
Craniopharyngioma		1 (2.3%)	2 (3.6%)	
Diffuse Fibrillary Astrocytoma		7 (15.9%)	8 (14.3%)	
Planum sphenoidale meningioma		1 (2.3%)	4 (7.1%)	
Anaplastic Oligodendroglioma		2 (4.5%)	5 (9%)	
Anaplastic Astrocytoma		2 (4.5%)	0 (0%)	
Glioblastoma Multiforme		7 (15.9%)	13 (23.2%)	
Medulloblastoma		5 (11.4%)	0 (0%)	

Detection of TIM3 gene polymorphisms

A novel mutation in the *TIM3* gene at intron 2 position 157106624 C>A was identified in a homozygous (C/C) pattern as well as a heterozygous (C/A) pattern, as shown in Figure 1. After that, the sample sequences were submitted in NCBI and recorded under accession numbers: PQ869007; PV092543; PV092544; PV092545; PV092546; PV092547; PV092548; PV092549; PV092550; PV092551; PV092552; PV092553; PV092554; PV092555; PV092556; PV092557; PV092558; PV092559; PV092560; PV092561; PV092562.

In the current study, the *TIM3* genetic sequence is located in chromosome 5. Regarding the currently investigated 483 bp amplicons of *TIM-3* gene, as shown in Figure 2, the NCBI BLASTn engine exhibited about 99.9% similarities in sequences between the intended target reference sequences and the samples gene sequences, which cover total sequence of the exon-2 sequences for the hepatitis A virus cellular receptor 2 (TIM3) and a part of the adjacent introns sequences, based on the previous explanations of the human genome (GenBank acc. NC_000005.10). By comparison of the detected sequences of the current investigated samples by the retrieved target DNA reference sequences (GenBank acc. NC_000005.10). A novel mutation in *TIM3* gene at intron 2 position 157106624 C>A was identified.

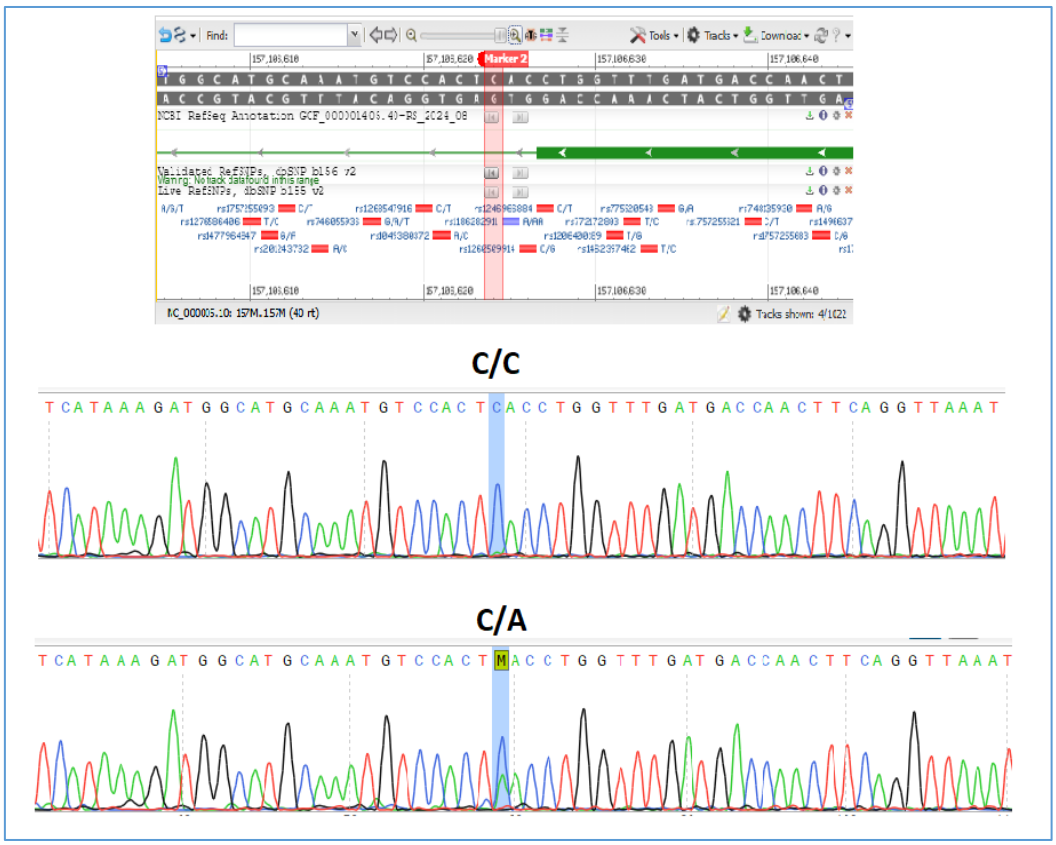

Figure 1: A Novel SNP was detected at 157106624 C>A (ref|NC_000005.10), with homozygous (C/C) pattern as well as a heterozygous (C/A) pattern. BioEdit version 7.7

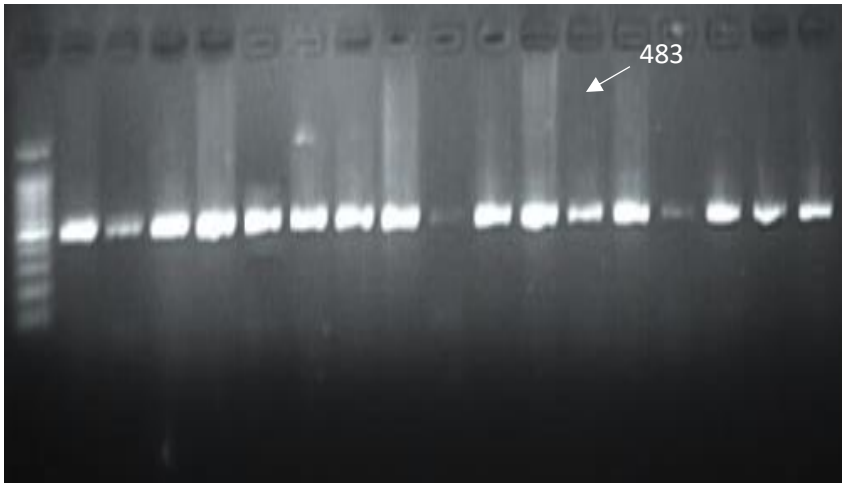

Figure 2: Gel electrophoresis of an amplified product pattern (483bp) of *TIM-3* gene, with conditions, 1.5% agarose, 75 V, for 50min, 5 µl in each well with a red safe solution for staining.

Genotyping of TIM3 SNP Polymorphism

In order to summarize the results derived from the sequenced 483 bp fragments, the specific positions of the identified differences are reported in the NCBI reference sequences, as shown in Table 3. However, the substitutions of C to A in this position have not yet been reported in any previous publication.

According to our study, the results exhibited that the distribution of gene polymorphism was according to CC and CA, which were 32% and 68%, respectively, in brain tumors patients and 80% and 20%, respectively, in the healthy control group. A statistically significant difference

in genotype frequencies of polymorphism distribution between brain tumors patients and control *P*=<0.0001, OR= 0.118 (0.039-0.351).

In addition to genotyping, the Allele frequency was investigated. A significant increase in the A allele in the brain tumor patients of 34% was found by comparing with the control group of only 10%, which may be associated with an increase in the risk of brain tumors, while the C allele was significantly increased in the control group 90%, *P*=0.001, OR =0.216 (0.081-0.577) which may associate with providing protection or decreasing the risk of tumor, the results summarized in Table 4.

Table 4: Genotyping of TIM3 (NC_000005.10) gene in brain tumor patients and control groups.

Infection / TIM3	Brain tumor patients		Control		*P*	OR	CI
	No.	%	No.	%			
CC	24	32%	20	80%	Reference		
CA	51	68%	5	20%	<0.0001*	0.118	(0.039-0.351)
Allele Frequency							
C Allele	99 (66%)		45 (90%)		Reference		
A Allele	51 (34%)		5 (10%)		0.001*	0.216	(0.081-0.577)

*Statically significant

Detection of CTLA4 gene polymorphisms

A mutation in the *CTLA4* gene at exon 4 rs60872763 DEL(AT)n ((Del(AT)12, Del(AT)21)) was first identified in the Iraqi population, as shown in Figure 3. After that, the sample sequences were submitted in NCBI and recorded under accession numbers: PV067702; PV067703; PV067704; PV067705; PV067706; PV067707; PV067708; PV067709; PV067710; PV067711; PV067712; PV067713; PV067714; PV067715; PV067716; PV067717; PV067718; PV067719; PV067720; PV067721.

The current study shows the *CTLA4* genetic sequences in chromosome 2 (NC_000002.12). Regarding the currently investigated 537 bp gene amplicons of *CTLA-4* gene, as shown in Figure 4, the NCBI BLASTn engine exhibited about 99.9% similarities in sequences between the intended target reference sequences and the samples gene sequences, which cover total sequence of the exon-4 sequences for the cytotoxic T-lymphocyte associated protein 4 (CTLA4), based on the previous explanations of the human genome (GenBank acc. NC_000002.12). By comparison of the detected sequences of the current investigated samples by the retrieved target DNA reference sequences (GenBank acc. NC_000002.12).

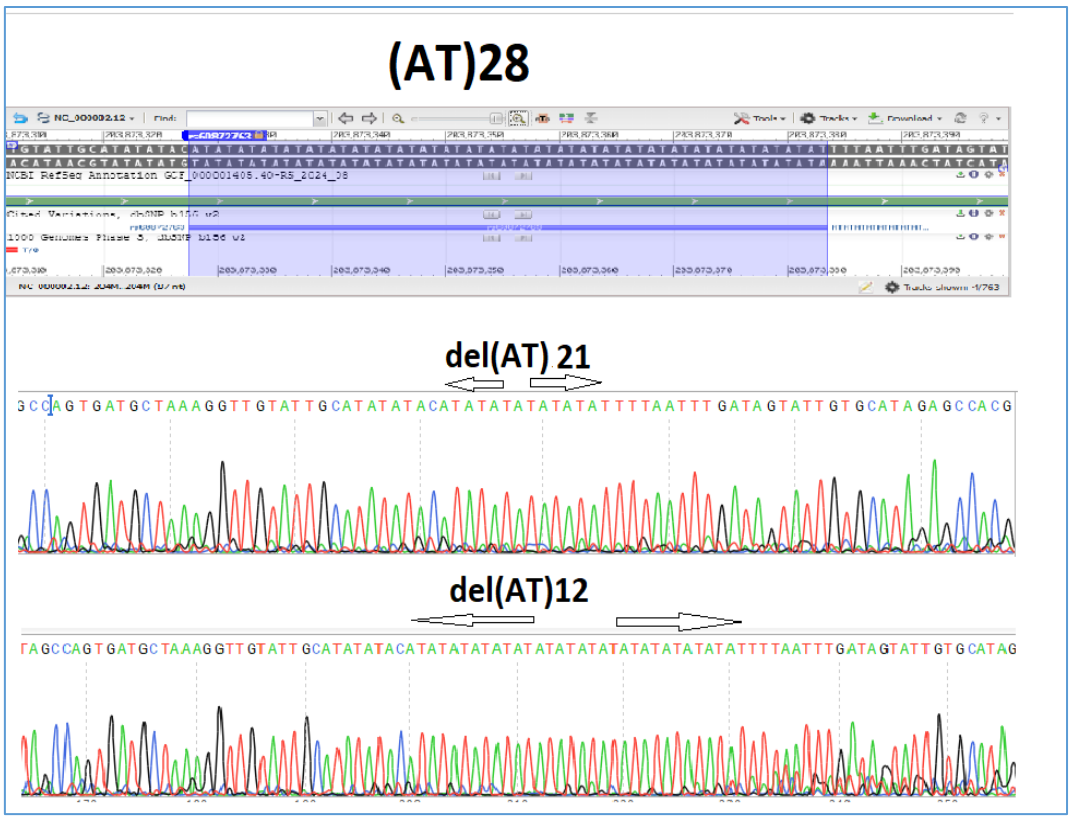

Figure 3: A Novel SNP was detected at 157106624 C>A (ref|NC_000005.10), with homozygous (C/C) pattern as well as a heterozygous (C/A) pattern. BioEdit version 7.7

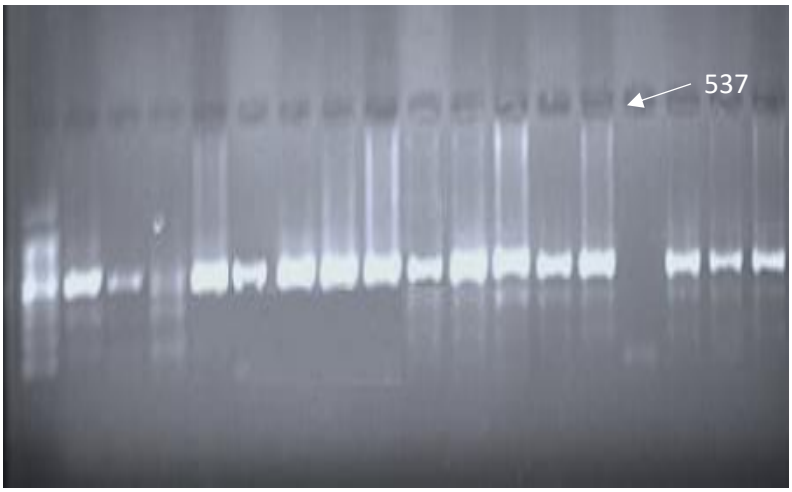

Figure 4: Gel electrophoresis of an amplified product pattern (537bp) of CTLA-4 gene, with conditions, 1.5% agarose, 75 V, for 50min, 5 µl in each well with a red safe solution for staining.

Distribution of genotype and allele frequencies of CTLA-4 in patients with brain tumor and in healthy controls

In order to summarize the results derived from the sequenced 537 bp fragments, the specific positions of the identified differences are reported in the NCBI reference sequences, 2 alleles (Del(AT)12, Del(AT)21) were found in the exon 4 at 3UTR of the *CTLA-4* gene in Iraqi population.

The distribution of genotype and allele frequencies of (AT)n repeats of the *CTLA-4* gene (rs60872763) in patients with brain tumor and in healthy controls is shown in Table 5. However, genotype distributions were in Hardy–Weinberg equilibrium for the (AT)n repeat (Del(AT)12,

Del(AT)21) (P<0.0001, OR. = 0.048 (0.014-0.160)) the frequencies of the Del(AT)21 fragments were much increased in patients with brain tumor whereas Del(AT)12 fragments were decreased. Conversely, in the control group, the frequencies of the Del 21 fragments decreased, whereas the Del 12 fragments increased.

Table 5: Genotype and allele frequencies of CTLA-4 gene (rs60872763) (AT)n repeats in brain tumor patients and control groups.

Infection / CTLA-4 rs60872763	Brain tumor patients		Control		*P*	OR	CI
	No.	%	No.	%			
Del (AT) 12	5	6.6%	15	60%	Reference		
Del (AT) 21	70	93.3%	10	40%	<0.001*	0.048	(0.014-0.160)

*Statically significant

Discussion

TIM-3 is an immune checkpoint molecule which has gained great attention for different cancers and have been uncovered on several types of immune cells, such as CD8+ T cells, CD4+ T cells, Th1, Th17, and Treg cells [25], and it is involved in modulate the immune responses because it is expressed on tumor-infiltrating lymphocytes and tumor antigen-specific T-cells in the peripheral blood [26], expression of TIM-3 results in limitation of antitumor immune responses and is associated in T cells exhaustion [27]. The genetic polymorphism of *TIM-3* may contribute to cancer development and be involved intimately in malignant tumors pathogenesis and several cancer types progression [28]. Interestingly, in the present study, A novel mutation in *TIM3* gene at intron 2 position 157106624 C>A was identified, and a heterozygous (C/A) pattern was significantly increased in brain tumor patients P<0.0001, OR.(0.118 (0.039-0.351)), suggesting that this new polymorphism (mutant genotype) in *TIM3* may contribute to the increased risk of brain tumor.

SNPs located at introns potentially influence at all levels of gene expression, as well as via epigenetic changes [29]. At the molecular level, the majority of SNPs are found in non-coding regions, which eventually influences oncogene and/or tumor-suppressor regulation in a way that is unique to cancer. Notably, decades before the disease manifests, inherited non-coding mutations can predispose to cancer [30]. Such SNP could influence the gene expression levels by altering binding sites, creating new sites, or modifying the degree of affinity of different transcription factors for particular DNA binding [31]. Intron regions' SNPs cause transcript splice variations and can enhance or impair long non-coding RNA (lncRNA) binding and function [32]. Moreover, as the disease progresses, the accumulation of more non-coding driver mutations leads to genomic instability, which serves as the catalyst for the establishment of neoplastic development and malignant transformation [30].

Despite the growing interest in CTLA-4 function as a new target in the control of inflammation in several tumors, relatively little is known about the interaction between brain tumor and *CTLA-4* genetic polymorphism. According to the current study, the frequencies of rs60872763 Del(AT)21 and Del(AT)12 bp polymorphisms in the *CTLA-4* gene in brain tumor patients were compared to that in the control group. The study suggests that the Del(AT)21 repeats might be associated with an increased risk of brain tumor occurrence and may clinically affect the features of the diseases during development.

Interestingly, in the present study, a new Del(AT)n repeats (Del(AT)21, Del(AT)12) was first identified in the Iraqi population. The frequencies of Del(AT)21 fragments were increased significantly in patients with brain tumor (93.3%). In contrast, the Del(AT)12 fragments were

decreased than the control group, P<0.001, OR= 0.048 (0.014-0.160), our results further corroborate the intriguing discovery that the longer alleles of the CTLA-4 gene's (AT)n repeats were frequently linked to the diseases. At the same time, the shorter variants were frequently seen in healthy individuals.

The 3′ untranslated region (3′-UTR) is a post-transcriptional regulatory domain that critically governs gene regulation and encompasses numerous regulatory elements that modulate various mRNA fate-related activities, including mRNA processing, stability, translation initiation, and localization [33]. Mutations in the 3′-UTR may lead to alterations in mRNA expression and recurrence. Mutations in the 3′-UTR of cancer genes have been identified using whole-genome sequencing (WGS) as crucial factors in carcinogenesis [34].

The impact of *CTLA-4* rs60872763 (AT)n repeats deletion in the 3 UTR region on mRNA stability and maybe protein synthesis is assumed to account for this phenomenon. The more (AT)n repeats deletion in the 3 UTR, the more unstable the mRNA. Similar effects on RNA stability have been noted for the granulocyte macrophage colony-stimulating factor gene due to (AU)-rich regions in the 3 UTR[35].

Previous studies found that the longer alleles (AT)n of the rs60872763 CTLA-4 microsatellite polymorphism were associated with diseases such as Ulcerative colitis (UC) [35], and Crohn's disease (CD)[36]. This is consistent with the current results.

Conclusion

In conclusion, this study is the first to identify a novel mutation (SNP) in the *TIM3* gene at intron 2 position 157106624 and Del(AT)n repeats (Del21, Del12). And determine the impact of *TIM3* intronic SNP and Del(AT)n repeats on the risk of brain tumor incidence in the Iraqi population. The current case-control study of brain tumor patients showed a significant increase in the A allele and AC heterozygote genotype in the *TIM3* gene and CTLA-4 rs60872763 Del(AT)21 repeats in patients with brain tumors, suggesting that the *TIM3* gene polymorphism at this newly diagnostic (intron variant) polymorphisms and the *CTLA-4* rs60872763 Del(AT)21 repeats might be associated with risk factors in patients with brain tumors.

Acknowledgments

A big thank you to the patients who contributed to our study by allowing samples to be taken for the study purpose, and thanks to the doctors in the Oncology Surgery Department at Al-Qimma Hospital in Baghdad and Imam Al-Sadiq Hospital in Babylon for helping us collect brain tumor samples.

Conflict of Interest

The authors declare that they have no conflicts of interest.

References

[1] H. M. Al-Dabbas and M. S. Mahdi, "Classification of Brain Tumor Diseases Using Data Augmentation and Transfer Learning," *Iraqi Journal of Science*, vol. 65, no. 4, pp. 2275–2286, 2024, doi: 10.24996/ijs.2024.65.4.41.

[2] American Brain Tumor Association, "Brain Tumors Tumors," *About Brain Tumors: A Primer for Patients and Caregivers*, p. 86, 2015, [Online]. Available: http://www.abta.org/secure/about-brain-tumors-a-primer.pdf

[3] R. Adnan Omar, J. Mohammed Najim, and I. H Abood, "Comparison of Some Statistical Measurements Extracted From Benign, Malignant and Normal MRI Brain Images," *Iraqi Journal of Science*, vol. 58, no. 4, pp. 2112–2117, 2021, doi: 10.24996/ijs.2017.58.4b.15.

[4] I. Ilic and M. Ilic, "International patterns and trends in the brain cancer incidence and mortality: An observational study based on the global burden of disease," *Heliyon*, vol. 9, no. 7, p. e18222, 2023, doi: 10.1016/j.heliyon.2023.e18222.

[5] C. Registry and A. Report, "Iraqi Cancer Board CANCER REGISTRY OF IRAQ," 2022.

[6] W. W. Chen, T. S. M. Chu, L. L. Xu, C. N. Zhao, W. S. Poon, G. K. K. Leung, and F. M. (Spring)

Kong, "Immune related biomarkers for cancer metastasis to the brain," *Experimental Hematology and Oncology*, vol. 11, no. 1, pp. 1–19, 2022, doi: 10.1186/s40164-022-00349-z.

[7] T. Vilela, S. Valente, J. Correia, and F. Ferreira, "Advances in immunotherapy for breast cancer and feline mammary carcinoma: From molecular basis to novel therapeutic targets," *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer*, vol. 1879, no. 5, p. 189144. 2024, doi: 10.1016/j.bbcan.2024.189144.

[8] M. Wagner, M. Jasek, and L. Karabon, "Immune Checkpoint Molecules—Inherited Variations as Markers for Cancer Risk," *Frontiers in Immunology*, vol. 11, no. pp. 1–29, 2021, doi: 10.3389/fimmu.2020.606721.

[9] S. Dutta, A. Ganguly, K. Chatterjee, S. Spada, and S. Mukherjee, "Targets of Immune Escape Mechanisms in Cancer: Basis for Development and Evolution of Cancer Immune Checkpoint Inhibitors," *Biology*, vol. 12, no. 2, 2023, doi: 10.3390/biology12020218.

[10] Y. Kawakami, S. Ohta, M. A. Sayem, N. Tsukamoto, and T. Yaguchi, "Immune-resistant mechanisms in cancer immunotherapy," *International Journal of Clinical Oncology*, vol. 25, no. 5, pp. 810–817, 2020, doi: 10.1007/s10147-019-01611-x.

[11] C. M. Vockley, A. Barrera, and T. E. Reddy, "Decoding the role of regulatory element polymorphisms in complex disease," *Current Opinion in Genetics and Development*, vol. 43, pp. 38–45, 2017, doi: 10.1016/j.gde.2016.10.007.

[12] J. Wang, Y. Wang, X. Jiang, M. Xu, M. Wang, R. Wang, B. Zheng, M. Chen, Q. Ke, and J. Long, "Unleashing the power of immune checkpoints: Post-translational modification of novel molecules and clinical applications," *Cancer Letters*, vol. 588, p. 216758. 2024, doi: 10.1016/j.canlet.2024.216758.

[13] H.-S. Kim, C. Y. Chang, H. J. Yoon, K. S. Kim, H. S. Koh, S. S. Kim, S.-J. Lee, L. P. Kane, and E. J. Park, "Glial TIM-3 Modulates Immune Responses in the Brain Tumor Microenvironment," *Cancer Research*, vol. 80, no. 9, pp. 1833–1845, 2020, doi: 10.1158/0008-5472.CAN-19-2834.

[14] C. Chen, F. Zhao, J. Peng, D. Zhao, L. Xu, H. Li, S. Ma, X. Peng, X. Sheng, Y. Sun, T. Wang, H. Dong, Y. Ding, Z. Wu, X. Liang, L. Gao, H. Wang, C. Ma, and C. Li, "Soluble Tim-3 serves as a tumor prognostic marker and therapeutic target for CD8+ T cell exhaustion and anti-PD-1 resistance," *Cell Reports Medicine*, vol. 5, no. 8, 2024, doi: 10.1016/j.xcrm.2024.101686.

[15] J. E. Kim, M. A. Patel, A. Mangraviti, E. S. Kim, D. Theodros, E. Velarde, A. Liu, E. W. Sankey, A. Tam, H. Xu, D. Mathios, C. M. Jackson, S. Harris-Bookman, T. Garzon-Muvdi, M. Sheu, A. M. Martin, B. M. Tyler, P. T. Tran, X. Ye, A. Olivi, J. M. Taube, P. C. Burger, C. G. Drake, H. Brem, D. M. Pardoll, and M. Lim, "Combination Therapy with Anti-PD-1, Anti-TIM-3, and Focal Radiation Results in Regression of Murine Gliomas," *Clinical Cancer Research*, vol. 23, no. 1, pp. 124–136. 2017, doi: 10.1158/1078-0432.CCR-15-1535.

[16] H. Fang, C. Yuan, X. Gu, Q. Chen, D. Huang, H. Li, and M. Sun, "Association between TIM-3 polymorphisms and cancer risk: a meta-analysis," *Annals of Translational Medicine*, vol. 7, no. 20, pp. 550–550,. 2019, doi: 10.21037/atm.2019.09.101.

[17] C. Shan, Y. Zheng, M. Wang, S. Lin, T. Tian, Y. Deng, P. Xu, Q. Hao, Y. Wu, T. Yang, Y. Guo, and Z. Dai, "Polymorphisms in HIFs and breast cancer sutarsceptibility in Chinese women: A case-control study," *Bioscience Reports*, vol. 38, no. 5, 2018, doi: 10.1042/BSR20180950.

[18] Q. Jiao, Y. Ren, A. N. Ariston Gabrie, Q. Wang, Y. Wang, L. Du, X. Liu, C. Wang, and Y. shan Wang, "Advances of immune checkpoints in colorectal cancer treatment," *Biomedicine and Pharmacotherapy*, vol. 123, no. March, pp. 1–27, 2020, doi: 10.1016/j.biopha.2019.109745.

[19] G. R. Kim and J. M. Choi, "Current Understanding of Cytotoxic T Lymphocyte Antigen-4 (CTLA-4) Signaling in T-Cell Biology and Disease Therapy," *Molecules and Cells*, vol. 45, no. 8, pp. 513–521, 2022, doi: 10.14348/molcells.2022.2056.

[20] R. Yadav, R. Khatkar, K. C. H. Yap, C. Y. H. Kang, J. Lyu, R. K. Singh, S. Mandal, A. Mohanta, H. Y. Lam, E. Okina, R. R. Kumar, V. Uttam, U. Sharma, M. Jain, H. Prakash, H. S. Tuli, A. P. Kumar, and A. Jain, "The miRNA and PD-1/PD-L1 signaling axis: an arsenal of immunotherapeutic targets against lung cancer," *Cell Death Discovery*, vol. 10, no. 1, 2024, doi: 10.1038/s41420-024-02182-1.

[21] S. V. A. N. Nguyen, L. Shamoun, K. Landerholm, R. E. Andersson, D. Wagsater, and J. Dimberg, "Cytotoxic T-lymphocyte antigen-4 (CTLA-4) gene polymorphism (rs3087243) is related to risk and survival in patients with colorectal cancer," *In Vivo*, vol. 35, no. 2, pp. 969–975, 2021, doi: 10.21873/INVIVO.12339.

[22] L. Lisi, P. M. Lacal, M. Martire, P. Navarra, and G. Graziani, "Clinical experience with CTLA-4 blockade for cancer immunotherapy: From the monospecific monoclonal antibody ipilimumab to probodies and bispecific molecules targeting the tumor microenvironment," *Pharmacological Research*, vol. 175, no. pp. 1–11, 2022, doi: 10.1016/j.phrs.2021.105997.

[23] C. Zou, H. Qiu, W. Tang, Y. Wang, B. Lan, and Y. Chen, "CTLA4 tagging polymorphisms and risk of colorectal cancer: A case–control study involving 2,306 subjects," *OncoTargets and Therapy*, vol. 11, pp. 4609–4619, 2018, doi: 10.2147/OTT.S173421.

[24] N. Al-Harbi, M. H. Abdulla, M. A. Vaali-Mohammed, T. Bin Traiki, M. Alswayyed, O. Al-ObeedI. Abid, S. Al-Omar, and L. Mansour, "Evidence of Association between CTLA-4 Gene Polymorphisms and Colorectal Cancers in Saudi Patients," *Genes*, vol. 14, no. 4, 2023, doi: 10.3390/genes14040874.

[25] R. Tang, M. Rangachari, and V. K. Kuchroo, "Tim-3: A co-receptor with diverse roles in T cell exhaustion and tolerance," *Seminars in Immunology*, vol. 42, pp. 1–8, 2019, doi: 10.1016/j.smim.2019.101302.

[26] Y. Wolf, A. C. Anderson, and V. K. Kuchroo, "TIM3 comes of age as an inhibitory receptor," *Nature Reviews Immunology*, vol. 20, no. 3, pp. 173–185, 2020, doi: 10.1038/s41577-019-0224-6.

[27] R. Yang and M. C. Hung, "The role of T-cell immunoglobulin mucin-3 and its ligand galectin-9 in antitumor immunity and cancer immunotherapy," *Science China Life Sciences*, vol. 60, no. 10, pp. 1058–1064, 2017, doi: 10.1007/s11427-017-9176-7.

[28] F. Pu, F. Chen, Z. Zhang, J. Feng, and P. Xia, "Functional variants of TIM-3/HAVCR2 3′UTR in lymphoblastoid cell lines," *Future Science OA*, vol. 4, no. 5, pp. 1–6, 2018, doi: 10.4155/fsoa-2017-0121.

[29] N. Schweighofer, M. Strasser, A. Obermayer, O. Trummer, H. Sourij, C. Sourij, and B. Obermayer-Pietsch, "Identification of Novel Intronic SNPs in Transporter Genes Associated with Metformin Side Effects," *Genes*, vol. 14, no. 8, 2023, doi: 10.3390/genes14081609.

[30] M. Lange, R. Begolli, and A. Giakountis, "Non-coding variants in cancer: Mechanistic insights and clinical potential for personalized medicine," *Non-coding RNA*, vol. 7, no. 3, 2021, doi: 10.3390/ncrna7030047.

[31] E. A. Blinova, V. S. Nikiforov, M. A. Yanishevskaya, and A. Akleyev, "Single nucleotide polymorphism and expression of genes for immune competent cell proliferation and differentiation in radiation-exposed individuals," *Vavilovskii Zhurnal Genetiki i Selektsii*, vol. 24, no. 4, pp. 399–406, 2020, doi: 10.18699/VJ20.632.

[32] N. Deng, H. Zhou, H. Fan, and Y. Yuan, "Single nucleotide polymorphisms and cancer susceptibility," *Oncotarget*, vol. 8, no. 66, pp. 110635–110649, 2017, doi: 10.18632/oncotarget.22372.

[33] S. L. Schuster and A. C. Hsieh, "The Untranslated Regions of mRNAs in Cancer," *Trends in Cancer*, vol. 5, no. 4, pp. 245–262,. 2019, doi: 10.1016/j.trecan.2019.02.011.

[34] C. Mayr, "Regulation by 3′-Untranslated Regions," *Annual Review of Genetics*, vol. 51, no. 1, pp. 171–194. 2017, doi: 10.1146/annurev-genet-120116-024704.

[35] Y. Jiang, B. Xia, L. Jiang, M. Lv, and Q. Guo, "Association of CTLA-4 Gene Microsatellite Polymorphism," vol. 12, no. 5, pp. 369–373, 2006.

[36] Z. Chen, F. Zhou, S. Huang, T. Jiang, L. Chen, L. Ge, and B. Xia, "Association of cytotoxic T lymphocyte associated antigen-4 gene (rs60872763) polymorphism with Crohn's disease and high levels of serum sCTLA-4 in Crohn's disease," *Journal of Gastroenterology and Hepatology (Australia)*, vol. 26, no. 5, pp. 924–930, 2011, doi: 10.1111/j.1440-1746.2011.06662.x.