

GENE POLYMORPHISM OF TRANSFORMING GROWTH FACTOR Beta 1(*TGF-β1*) IN AUTISM SPECTRUM DISORDER ASD IN BASRAH

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ABSTRACT

Transforming growth factor-β1 (TGF-β1) is an important immune regulator critical for immune homeostasis. Accumulating evidence suggests that TGF-β1 has a crucial regulatory role in CNS development and potential implications for neurogenesis in a variety of TGF-β1-related CNS diseases: so the aim of the study to investigate the association of the *TGFβ-1* gene polymorphism with its plasma protein plasma level (TGFB-1) in ASD patients, It's a case – control study at total of 94 patients with ASD, their age ranging from 2 to 13 years and 100 apparently healthy children were used as a control which were matched by age and sex. TGFβ-1 levels was measured by ELISA and *TGF-β1*(Codon 10 +869 C/T) and *TGF-β1*(Codon 25: +915*G/C) Gene Polymorphism were detected by specific sets of primers. The mean value of TGF-β1 was significantly low in the autistic group (95.91pg / mm) as compare with the control one (117.08 pg / mm) *TGF-β1*(Codon 10: +869*T/C) gene polymorphism showed heterogeneous results between autistic group and control group

INTRODUCTION

Autism Spectrum Disorder (ASD) etiology has long been a widely debated topic and stills weakly understood (1). Double studies supply proof that ASD susceptibility may have genetic, heritability, in addition to significant environmental components (2,3). A strong inflammatory state associated with ASD reported increasingly in the recent years(4). The inflammatory disorder is also associated with dysfunction of the immune system (5).

There is abundant knowledge that disturbance normal levels of cytokine plays a major role as a risk factor for many neurodevelopmental disorders, including autism and schizophrenia(6). A significant immune regulator critical to immune homeostasis is the transformation growth factor- β 1 (TGF- β 1). Transforming growth factor- β 1 (TGF- β 1) has been found to play a crucial role in early central nervous system development. Several studies have illustrated decreased TGF- β 1 levels in sera and brains of autistic children. Two point mutations in the TGF- β 1 signal peptide at 869T/C and 915G/C have been reported to influence TGF- β 1 expression (7). TGF- β 1 has therefore been generally accepted as a cytokine that make a response to injury of brain. Many researches have documented increases in TGF- β 1 levels in brain and autistic serum (8,9).

Transforming growth factor Beta (TGFB) represents a family of cytokines with closely related isoforms, encoded by three different genes. *TGFB1*, *TGFB2* and *TGFB3* are expressed in several central nervous system (CNS) cell types, including neurons, astrocytes, and microglia (10). The TGF- β s are involved in a variety of biological functions in cellular activities, fibrosis, and immune responses, in addition to their crucial roles in tissue homeostasis (11). Accumulating evidence suggests that TGF- β 1 has a crucial regulatory role in CNS development and potential implications for neurogenesis in a variety of TGF- β 1-related CNS diseases (12). TGF- β 1 has been extensively investigated in immunity, because TGF- β 1 is expressed predominantly by various immune cells (13) and deficiency of TGF β -1 results in fatal systemic autoimmune disease(14).

TGF- β controls the magnitude and type of immune responses against microbes, and has fundamentally important roles in maintaining immune tolerance and homeostasis against self- and benign antigens at steady state (15) so the aim of this study to investigate the association of the *TGF β -1* gene polymorphism with its plasma protein level in ASD patients.

MATERIAL AND METHODS

This is a case – control study in which (94) Autistic children (patients group) and(100) apparently healthy control individual (control group) were enrolled from

the same population living in Basra providence in Iraq during the period between December 2018 and October 2019. they were matched by age and sex.

Ethical manifestation:

This study received approval by the Primary Health Care Centers manager and family of both patients and control children. Its procedures and purpose were explained to all studied population, and every individual member of the family has obtained written informed consent.

Collection of Blood Samples:

Three mL of venous blood was drawn from each Participants using a sterile disposable syringe and divided as following :1 ml of the sample was emptied into an anticoagulant EDTA tube (Ethyline Diamine Tetra Acetic acid) which used in whole DNA extraction to detected *TGF β -1* gene polymorphisim, and remaining blood emptied in other EDTA tube , last tube was centrifuged at 3000 rpm for 10 mints and plasma transferred to sterile plane tube to use in ELISA technique.

Assessment of plasma TGF β -1 levels:

Plasma levels of TGF β -1 were evaluated using an ELISA (Elabscience, USA, Catalog No: E-EL-0162), according to manufacture protocol.

Extraction of Whole DNA:

From patients and control samples, Whole DNA was extracted by using DNA Blood Mini Kit (Favorgen -Europe Cat.No: HB10.03.10 UK) depend on kit manufacture supplied with kit, the DNA extraction stored at -20 until use.

Primers used to detect *TGF β -1* Genes :

Eight specific primers was used to detect *TGF- β 1*(Codon 10 +869 *C/T) and *TGF- β 1*(Codon 25: +915*G/C)Gene Polymorphism according to reference (Bazzaz *et al.*, 2014) which listed in table below: primers were prepared depending on manufacturer instruction (alpha DNA Company).

Table (1): Oligonucleotide Primers Sequences Used for PCR Amplification of *TGF β1* Gene Polymorphism(26).

Primer name	Sequence (5'-3')
<i>TGF-β1</i> (Codon 10: +869*C/T) (Generic)	5'- TCCGTGGGATACTGAGACAC-3'
<i>TGF-β1</i> (Codon 10: +869*C/T) (C allele)	5'- GCAGCGGTA GCAGCAGCG-3'
<i>TGF-β1</i> (Codon 10: +869*C/T) (T allele)	5'- AGCAGCGGTAGCAGCAGCA-3'
<i>TGF-β1</i> (Codon 25: +915*G/C) (Generic)	5'- GGCTCCGGTTCTGCACTC-3'
<i>TGF-β1</i> (Codon 25: +915*G/C) (G allele)	5'- GTGCTGACG CCTGGCCG-3'
<i>TGF-β1</i> (Codon 25: +915*G/C) (C allele)	5'- GTGCTGACG CCTGGCCC-3'
<i>TGF-β1</i> - Internal control (Forward)	5'-GCCTTCCCAACCATTCCTTA- 3'
<i>TGF-β1</i> - Internal control (Reverse)	5'-TCACGGATTTC TGTTGTGTTTC- 3'

Amplification of *TGF β-1* Gene Polymorphism:

The *TGF-β1*(Codon 10 +869 *C/T) and *TGF-β1*(Codon 25: +915*G/C)Gene Polymorphism were studied by using Eppendrofe thermo cycler with specific primers (Table 1). The PCR reaction mixture was prepared according to reference (26) as in table (2) below:

Table (2) The Reaction mixture (25 μl) for *TGF β1* gene polymorphism.

DNA templates	2 μl
Master mix	12.5 μl
Primers(forward, reverse of gene and forward, reverse of internal control) (50 picomole)	2μl for each primer
Free nuclease D.W	2.5 μl
Final volume	25 μl

For each single sample the following steps were processed: For detection of *TGF-β1* gene polymorphism codon 10+869 C/T, and codon 25+915 G/C four tubes were prepared, for detection of (T) allele / (C) allele, (C) allele and G one respectively.

Internal control Forward and Reverse primers were used to amplify *The glyceraldehyde 3-phosphate dehydrogenase (GAPDH)* that was counted as control for the purpose of comparison. All tubes then shaken well by vortex for 10 seconds. The PCR tubes were transferred to the thermalcycler to start the amplification reaction according to specific program table (3).

Table(3) PCR amplification program for *TGFβ-1* Gene Polymorphism detection.

Stage	Steps	Temperature(C°)	Time(sec)	
First	Denature template	95	1	
Second	I	Initial denaturation	95	
	II	Annealing	65	
	III	Extension	72	
			10 cycles	15
				15
				40
	I	Initial denaturation	95	
	II	Annealing	56	
	III	Extension	72	
		20 cycles	20	
			20	
			50	
Third	Final Extension	72	7 min	

PCR product was analyzed by gel electrophoresis in 1.5% agarose containing ethidium bromide 0.5mg/ml.

Statistical analysis:

The Statistical Package of Social Science (SPSS, version 23) were utilized to examine and processed data. The relation between polymorphism of *TGFβ-1* with its protein level in the plasmas was analyzed by utilizing χ^2 test, and *TGFβ-1* concentration was tested for autistic and control group utilizing the T-test.

RESULTS

Plasma Concentration TGF-β- 1 in Study Groups:

The mean value of TGF-β1 was significantly low in the autistic group (95.91pg / mm) as compare with the control one (117.08 pg / mm) as illustrate in Table (4). The P value was < 0.05 as estimated by Independent T test.

Table (4) Plasma Concentration of TGFβ-1 in the study Population

	Autistic group	Control group	
Test	No.=94	No. =100	P value
	Mean ± SD	Mean ± SD	
TGFβ-1	95.91 ± 6.37	117.08 ± 7.58	0.035

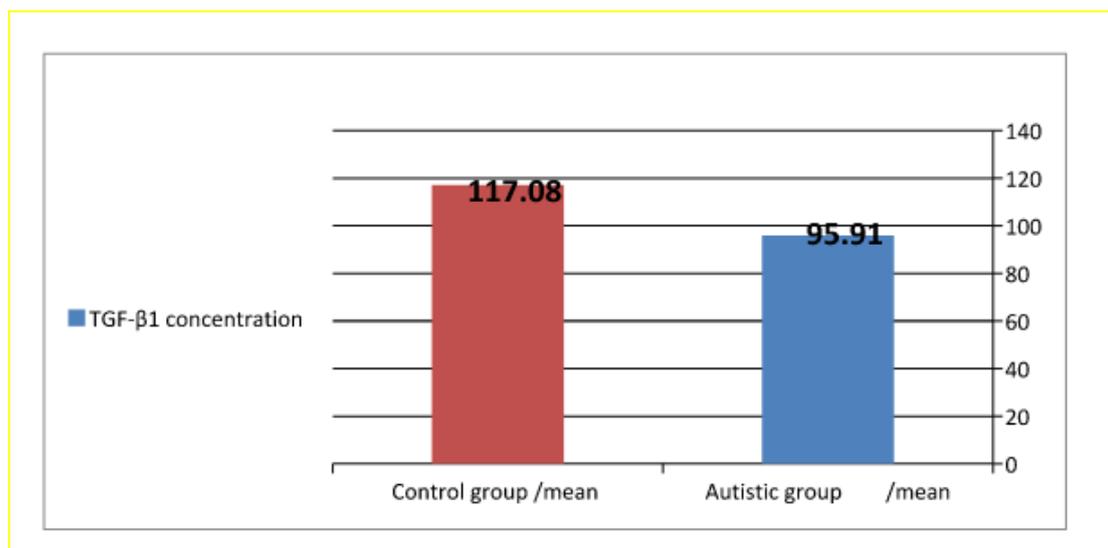


Figure (1) Plasma TGFβ-1 Concentration in the Study Population Genetic polymorphism of TGF-β1 A-(Codon 10: +869*T/C).

The results of the electrophoresis of TGF-β1(Codon 10: +869*T/C) gene polymorphism amplification were shown two alleles T/ C and 3 genotyping (TT, TC, CC) in patients and control samples.

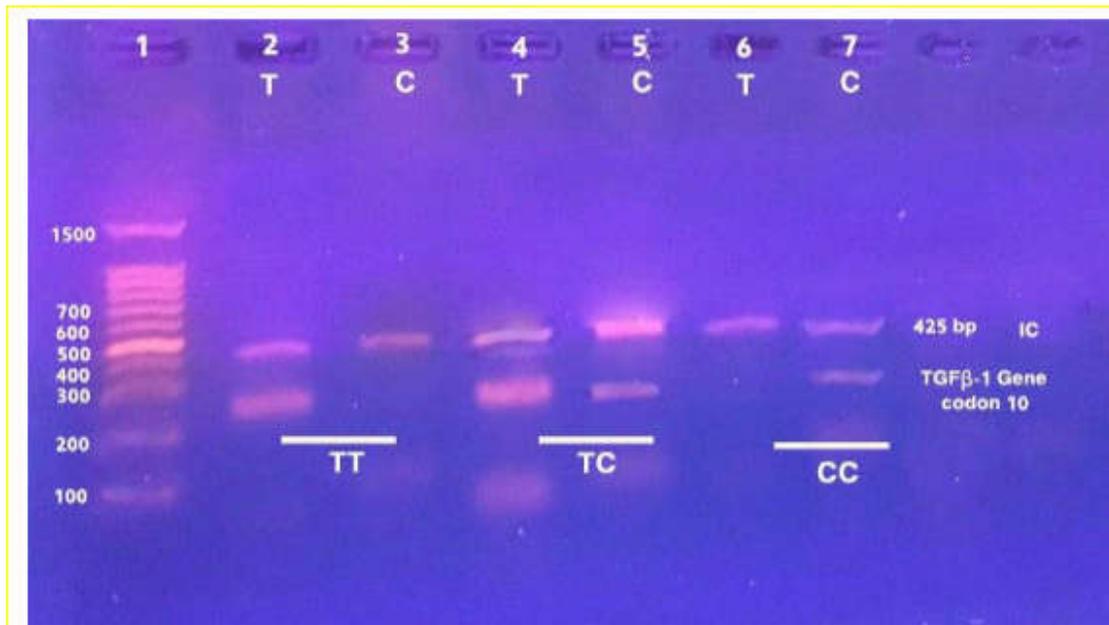


Figure (2) 2.5% Gel Electrophoresis of *TGF-β1*(Codon 10 T/C): lane 1 Ladder(1.5kb), lane 2&3 patient1, lane 4&5 patient 2 and lane 6&7 patient 3

Results of iterative distribution of *TGF-β1*(Codon 10: +869*T/C) gene polymorphism showed heterogeneous results between autistic group and control group, as the T allele was scored in autistic group 85.1% in compared with C allele which scored 76.6%, while T allele was scored 34% in compared with C allele which scored 67% in control group figure 3. The difference in repetitive distribution of T allele between patients and control group was statistically significant (p value = 0.000). While no significant differences (p value = 0.000) in the iterative distribution of C when compared with control group, also table 4 showed significant differences in repetitive distribution of T allele between patients and control group by using Fisher's test, and the OR of T allele was 0.249 (less than one). While OR of C allele was 1.2. (more than one) could be considered as preventive fraction (not associated with the disease).

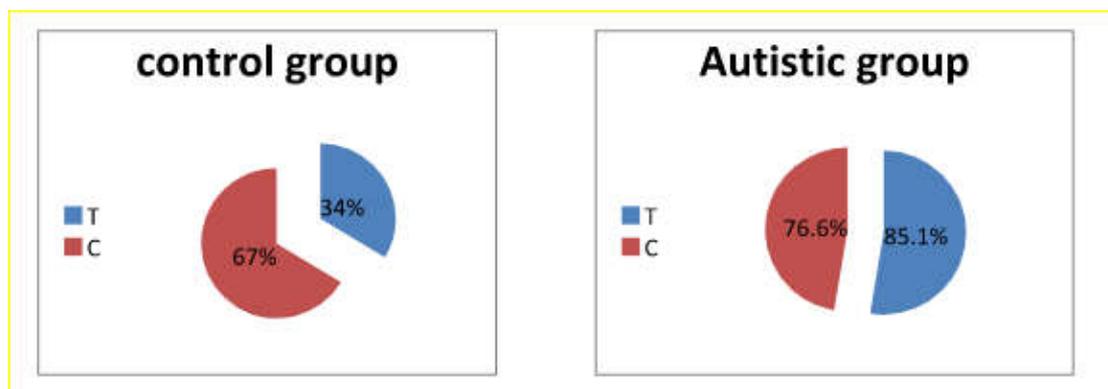


Figure (3) Frequency of *TGFβ-1* Codon 10 T/C alleles in Study Population.

Results of genetic analysis of *TGF-β1*(Codon 10 T/C) for SNP-PCR were showed 3 genotypes (TT,TC and CC) in patients and control samples. The results showed contrast in genotypes repetition between autistic group and control group. As heterogeneous genotype TC showed higher frequency among autistic patients when compared to control subjects,(60.6% vs 1% respectively),while homogenous genotype CC showed lower frequency among autistic patients when compared to control subjects,(14.9% vs 66% respectively),and TT genotypes scored 24.4% in autistic patients and 33% was scored in control group figure 4.

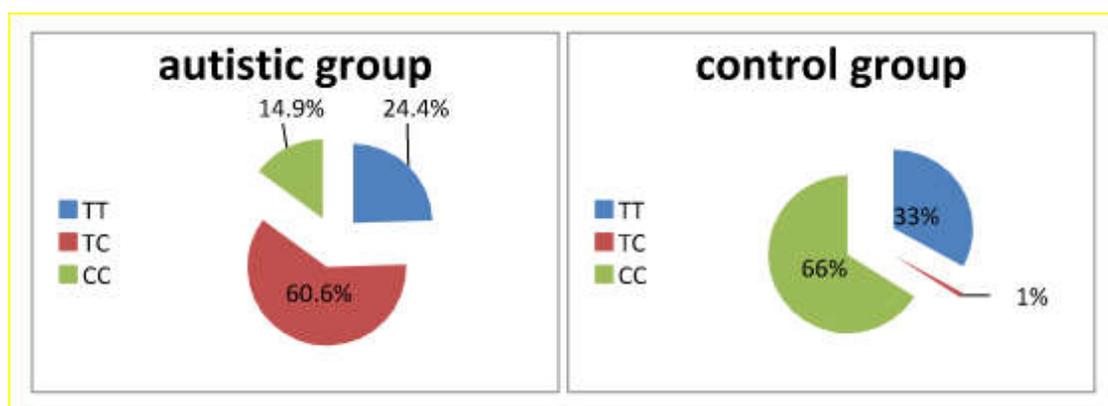


Figure 4 Frequency of *TGFβ-1* Codon 10 Genotyping in Study Population .

On the other hand the results of the electrophoresis of *TGF-β1*(Codon 25 G/C) gene polymorphism amplification were shown two alleles G/ C and 3 genotyping (GG,GC,CC) in patients and control samples,

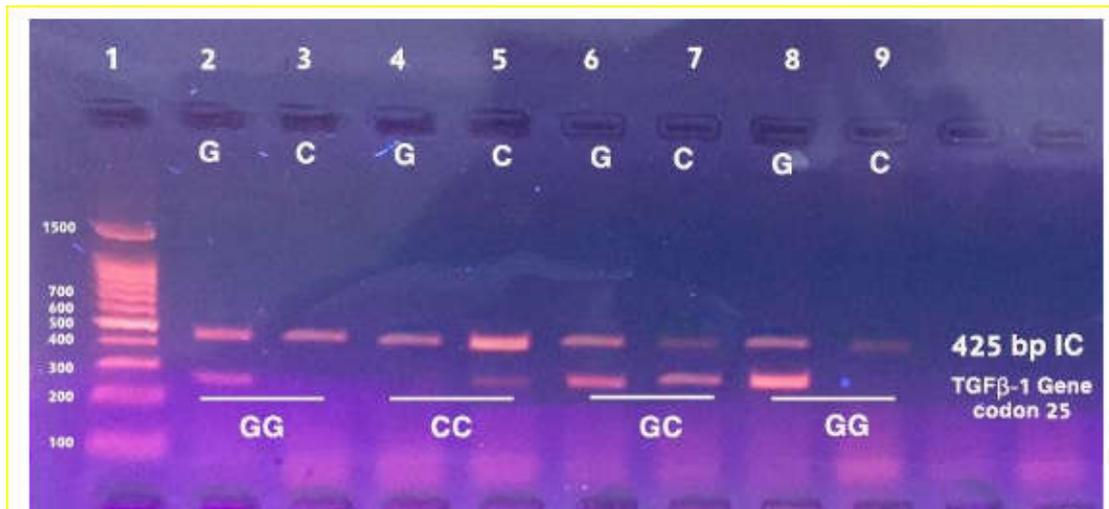
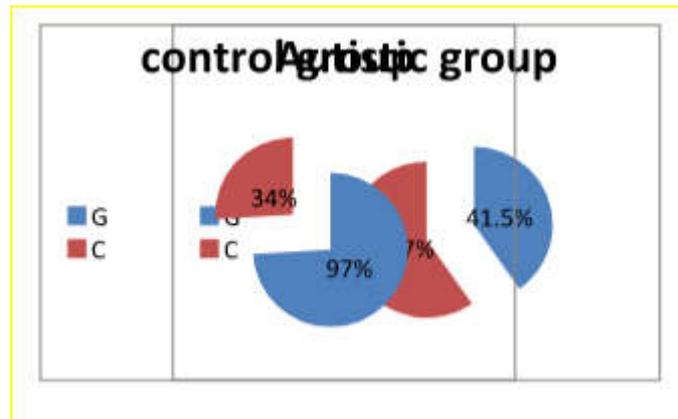


Figure (5) 2.5% Gel Electrophoresis of *TGF-β1*(Codon 25 G/C): lane 1 Ladder(1.5kb), lane 2&3 patient1, lane 4&5 patient 2, lane 6&7 patient 3 and lane 8&9 patient 4.

Results of iterative distribution of *TGF-β1*(Codon 25 G/C) gene polymorphism showed heterogeneous results between autistic group and control group, as the G allele was scored in autistic group 41.5% in compared with C allele which scored 61.7%, while G allele was scored 97% in compared with C allele which scored 34% in control group figure 5 .The difference in repetitive distribution of G allele and C allele between patients and control group was statistically significant (p value = 0.000) by chi square test which scored (41.5%, 97% and 61.7%, 34%) respectively . Also table 5 showed significant differences in repetitive distribution of G allele between patients and control group by using Fisher's test, and the OR of G allele was 0.45 and its preventive fraction and not associated with the disease. While OR of C allele was 0.349 so this allele could be considered as preventive fraction.



Figure(6) Frequency of *TGFβ-1* Codon 25 G/C alleles in Study Population

Results of genetic analysis of *TGFβ-1*(Codon 25 G/C) for SNP-PCR were showed 3 genotypes (GG,GC and CC) in patients and control samples. The results showed contrast in genotypes repetition between autistic group and control group. As homogeneous genotype CC showed higher frequency among autistic patients when compared to control subjects,(58.5% vs 3% respectively),while heterogeneous genotype GC showed lower frequency among autistic patients when compared to control subjects,(3.1% vs 31% respectively),and GG genotypes scored 38.3% in autistic patients and 66% was scored in control group. figure (7).

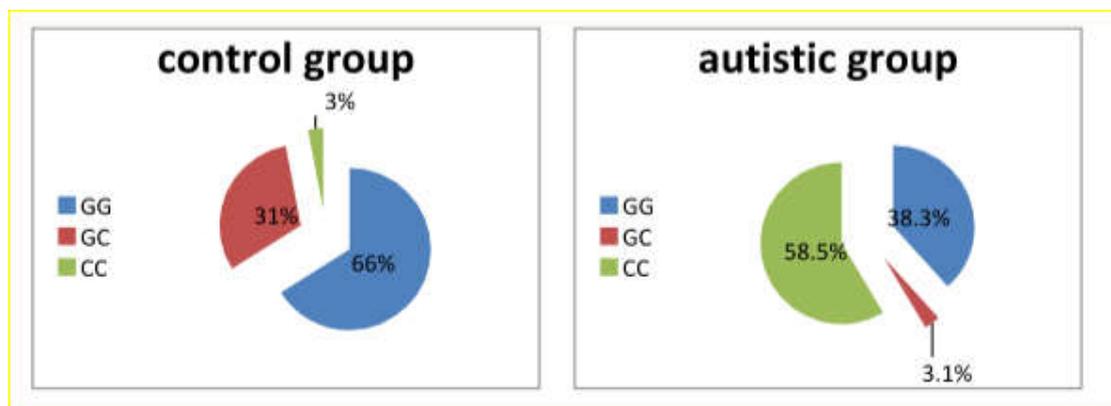


Figure (7) Frequency of *TGFβ-1* codon 25 G/C Genotyping in Study Population

Table (5) Distribution of *TGF-β1*(Codon 10 and codon 25) alleles in study groups.

Allele	Autistic group	Control group	X ²	df	OR	P value
Codon 10 T/C						
T allele	80 (85.1 %)	34 (34 %)	52.2	1	0.249	0.000
C allele	71 (75.5%)	67 (67%)	2.195	1	1.2	>0.05
Codon25 G/C						
G allele	39 (41.5%)	97 (97%)	71.23	1	0.45	0.000
C allele	58 (61.7%)	34 (34%)	12.8	1	0.349	0.000

Table (6) Correlation between *TGF-β1* Plasma Level and *TGF-β1*(Codon 10 and codon 25) Genotyping among the Study Groups

Genotyping	Autistic group		Control group		P value
	No.	TGFB-1 Median	No.	TGFB-1 Median	
Codon 10 T/C					
TT	23	75.0	33	88.0	0.000
TC	57	79.0	1	55.0	0.000
CC	14	101.0	66	97.0	0.000
Codon 25 G/C					
GG	36	74.5	66	86.5	0.000
GC	3	63.0	31	184.0	0.000
CC	55	84.0	3	66.0	0.000

The association of the plasma levels of (TGF β -1) with its genotypes of the study population summarized in table 6 which demonstrated that there were significance differences in the quantity of *TGF- β -1* genotypes and its patients plasma concentration in in the study groups.

DISCUSSION

Over the past decade, numerous reports have noted abnormalities or alterations of immune system activity in autistics; these include increased serum levels of inflammatory cytokines and factors such as tumor necrosis factor- α (TNF- α), interferon- γ (INF- γ) and high sensitivity C-reactive protein .TGF- β 1 is considered to be one of the critical immunosuppressive cytokines in immune homeostasis and T cell activated unresponsiveness (16).

In this study the mean value of TGF β -1 significantly low in ASD than in control group which was (95.91 pg/mm, 117.08 pg/mm) respectively. The result was in agreement with (8, 17), that suggesting that immune responses in ASD may be inappropriately regulated due to reductions in TGF β -1 .Low TGF β -1 levels may lead to an inappropriate control of the immune response in these children (8).

Such immune dysregulation may predispose to the development of possible autoimmune responses and/ or adverse neuroimmune interactions during critical windows in development. Overall, the data suggested that immune dysfunction and excessive inflammation play important pathophysiologic roles in autism disorders.

Transforming growth factor- β 1 (TGF- β 1) has been found to play a crucial role in early central nervous system development. Furthermore, during the brain development, glial and neuronal cells produce TGF- β 1, which plays a crucial role in the regulation of early CNS development such as astrocyte differentiation (18). Several studies have illustrated decreased TGF- β 1 levels in sera and brains of autistic children (7,19). (17) found that serum of ASD patients had decreased level of TGF- β 1as well as increased other inflammatory markers. This suggests wide range of immune dysregulation in ASD, with an improper balance between regulation and activation, which could have wide reaching consequences for many systems in the body.

The role of TGF β -1 with respect to the immune response is complex. Early during an immune response, TGF β -1 has the ability to enhance inflammation. Conversely, TGF β -1 has a number of profound down-regulatory effects on T and B cell development and function, as well as the ability to modulate the differentiation and activation status of NK cells, dendritic cells, monocytes/macrophages, granulocytes and mast cells (20). All these facts can give a hint of the effect of low level of TGF β -1 on the immunity and the general health of ASD children.

Given the key role of *TGF- β 1* in brain development and inflammation, we investigated the association between *TGF- β 1* gene polymorphisms and autism. Consequently, estimated alleles and genotypes frequencies were compared between autistic patients and normal controls in Basrah providence population. We found association between the *TGF- β 1* gene polymorphisms and autism. To date, several functional single-nucleotide polymorphisms (SNPs) of *TGF- β 1* have been reported. Particularly, the -509C/T (rs1800469), codon 25G/C (rs1800471), and codon 10 T/C (rs1982073).

SNPs are the most widely evaluated polymorphisms (21). It has been demonstrated that these functional SNPs are associated with the inter-individual differences of *TGF- β 1* expression (22, 23). The above facts suggest that -509C/T, codon 25G/C, and codon 10 T/C SNPs may contribute to TGF- β 1-mediated immune response.

In our study there is a moral repetition result of TC genotype in TGF- β 1 gene polymorphisms codon 10 +860 T/C in autistic patients compared with control samples (the differences highly significance): this may give a hint that there is an association between this genotype and other predisposing factors in causing the disease, TC genotype can be considered as genetic marker associated with autism disease, in addition to that interestingly, the TC allele has been associated with higher *TGF- β 1* expression so we found a significant correlation between the plasma level of (TGF- β) and the TC genotype of Codon 10 in autistic group

In compared to the control group, so the result is in harmony with (23) who published that Transforming growth factor β (TGF- β) signaling pathways are ubiquitous and essential regulators of cellular processes including proliferation, differentiation, migration, and survival, as well as physiological processes, including

embryonic development, angiogenesis, and wound healing. However, alterations in these pathways, including either germ-line or somatic mutations or alterations in the expression of members of these signaling pathways often result in human disease.

While (7) reported that the polymorphisms in *TGF-β1* gene may not play an important role in the development of autism. The understanding of mechanisms behind various signaling pathways in the etiology of ASD may help to facilitate the identification of potential therapeutic targets and design of new treatment methods (25).

Another crucial SNP locus is codon 25G/C which may impact the production of TGF-β1. we found that the CC genotype significantly higher in autistic patients than the control group and the frequency of allele C at *TGF-β1* codon 25 was significantly higher in patients with ASD than in healthy controls.

Also we found that The plasma level of TGF-β1 in autistic patient with the G allele were significantly lower than those with the C allele this finding followed the role of (25) which believed that, the transition of G to C may be correlated with the reduced level of TGF-β1 in vitro (26).

Results of *TGF-β1* gene polymorphism in both codon showed that C allele strongly associated with risk of autistic disease and interestingly, the C, allele have been associated with higher TGFβ-1 expression which may link with the immunological dysregulation . It has also been demonstrated that the TC of codon 10 and CC of codon 25 haplotypes are associated with ASD.

On the other hand, the G allele of *TGFβ-1* codon 25 polymorphism may be interpreted as a protective allele against ASD, Additionally, this polymorphism may be in linkage disequilibrium with other SNPs within or outside the *TGFβ-1* gene, only acting as a marker of an extended haplotype.

Theoretically, it is possible that subjects with the high TGF-β1 producer phenotype which is associated with the codon 25 G allele present over suppression in the human immune response. This mechanism may result in the correlation of the polymorphism of codon 25G/C and disease (27)

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