ORIGINAL ARTICLE

The C/A functional polymorphism of *TGF-β2* gene (rs991967) in primary open angle glaucoma patients

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Abstract

Background Primary Open-angle Glaucoma (POAG) is a functional disease that.leads to blindness globally. The aims of this study are estimation the importance.of transforming growth factor-beta 2 (TGF-β2) in the pathogenicity of POAG and. to evaluate the effect of the C/A SNP of the *TGF-β2* gene (*rs991967)* on POAG development.

Methods Blood samples and some topographic data were collected from POAG.patients and the controls. The serum level of TGF-β2 was estimated by ELISA.and the C/A SNP of the *TGF-β2* gene (*rs991967)* was determined by RFLP-PCR.

Results The males are more susceptible to having POAG (p = 0.0201). The serum.TGF-β2 is higher in POAG patients as compared with the control (p < 0.0001). The.AA (reference) genotype was the most common in the patients (61.7%). While.. CC genotype (45.0%, OR: 0.136, 95%CI: 0.05–0.36, P < 0.0001) and AC..genotypes (41.7%, OR: 0.051, 95%CI: 0.01–0.16, P < 0.001) were most common..in the control group. Moreover, the *TGF-β2* C allele is protective (OR: 0.25,.95%, CI: 0.15–0.44, P < 0.0001). Patients with AA, CC, and AC genotypes have..significantly high levels of TGF-β2 (P < 0.001) than the control.

Conclusions The males were more susceptible to acquiring POAG than females,.. especially the elderly. The TGF-β2 plays important role in the pathogenesis of POAG. The CC and AC genotypes are common in the control and the C allele is a protective factor.

Keywords TGF-β2 · *TGF-β2* gene · Primary Open-angle Glaucoma · SNP

Introduction

Primary Open-angle Glaucoma (POAG) is a functional disease that.. leads to blindness globally. The POAG-specific cause is unknown, but.. it is widely thought that it results from an interaction between genetic.. and environmental factors [1]. The main risk factor for glaucoma is.. family history in addition to age and race factors. POAG is a subset of.. glaucomas defined by an open, normal-appearing anterior chamber.. angle and raised intraocular pressure (IOP). Other health conditions.. like diabetes, pathogenic myopia, and high blood pressure are also..considered risk factors. Many studies noted that previous eye surgery,.. eye injury, and taking corticosteroids especially as eye drops elevated.. the chances of glaucoma incidence or progression [2, 3]. Gene..

mutations in some genes like optineurin and myocilin genes may cause.. POAG. In the last years, the genetic causes of POAG are extensive.. studies, and a huge number of patients are involved to discover the.. most important genetic factors [4]. They found that single nucleotide.. polymorphisms (SNPs) are the most predominant genetic factors [5].

There are a group of immune suppressive cells that down-regulate the.. stimulation and multiplying of the T cells by cell interactions or by.. secretion of some cytokines like interleukin 10, transforming growth.. factor beta (TGFβ), and interleukin 35 thereby dampening immune.. activation and autoreactivity [6]. The TGF-β is a factor that shows a.. central role in the function of the cells, like angiogenesis and..production of the extracellular matrix. There are five of TGF-β..isoforms, which are TGF-β1, TGF-β2, TGF-β3, TGF-β4 TGF-β5[7]. The isoforms, TGFs-β1, β2, and β3, are produced in animals including.. humans, the chief isoform of TGF-β in the human eyes is TGF-β2, [8], and was found in the tears and vitreous of the human eyes [9].

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.. The gene of TGF-β2 is found on chromosome 1, this gene has some. polymorphisms that affect the expression of TGF-β2 [10]. For..example, according to Derakhshan's study [11], the TGFB2 rs991967. Polymorphism has a direct and significant association with POAG. This Polymorphism can reduce the synthesis of TGF-β2 that contribute to POAG pathogenesis. The proteoglycans production in human eyes are induced basically by TGF-β. The increased IOP is the main cause of POAG, this increase is due to the production of proteoglycan in human eyes that induce a reduction in aqueous humor outflow [12]. The Aqueous TGF-β can be a probable POAG sub-type diagnostic marker [13]. In response to infection or injury, the immune cells enter the eyeball due to the Fas-FasL activation [14]. The aim of this study is estimation the role of TGF-β2 in the pathogenicity of POAG as well as evaluation the effect of the C/A SNP of the *TGF-β2* gene (*rs991967)* on POAG development.

Materials and methods

Samples of the study

The blood samples of control and POAG patients were collected from.. March to October 2021. The patients are 21 females and 39 males..enrolled from Ibn Al-Haitham Hospital. The control is 60 healthy..individuals (20 females and 40 males). The disease was diagnosed by..the physician at the Hospital according to the following criteria..enlarged cornea, raised cup disk ratio, and elevated intraocular.. pressure. The standards that were adopted to diagnose POAG by the..physician are as follows: Age of patients, elevated IOP (measured by..Goldmann applanation tonometry) over 21 mmHg, increased cup-to-..disk (c/d) ratio, (by two databases: The RIMONE v3 and the..DRISHTI-GS) and enlarged cornea bigger than 11 mm as well as..visual field test was also performed. 4 ml of venous blood was taken.. and divided into two parts. The first part (3 ml) was for serum..separation. The serum was divided into aliquots in Eppendorf tubes..and stored at − 40 °C until used for TGF-β2 determination by ELISA. The second part (1 ml) was put into EDTA tubes and stored at – 40 °C until used for genetic analysis.

Compliance with ethical standards

This article does not contain any studies involving animals performed by any of..the authors. All procedures performed in studies involving human participants..were following the ethical standards of the institutional and/or national research..committee and with the 1964 Helsinki Declaration and its later amendments or..comparable ethical standards.

Informed consent was obtained from all individual participants included in the study.

Estimation of human TGF-β2

Serum TGF-β2 was determined by Immunoenzymetric Assay using..the TGF-β2 Elisa kit (Komabiotech, South Korea). The ELISA..procedure was performed according to the manufacturer's instructions.

Molecular methods

Molecular methods include; genomic DNA extraction, measurement of DNA purity and concentration, agarose gel electrophoresis, and detection of TGF-β2 rs991967 polymorphisms by RFLP-PCR. The electrophoresis using 2% agarose gel pre-stained with red safe in TBE buffer was done for all the PCR products to..ensure the presence of the concerning bands. Optimal DNA isolation includes..the lysis of white blood cells. This method involved sample exposure to chemicals and enzymes or binding to matrices which reduce the sample volume, complexity, and variability to get purity for nucleic acid. The DNA extraction was performed by the protocol of the Geneaid company kit (Taiwan). DNA concentration was measured using a nano-spectrophotometer (NanoDrop) by placing 1 µl of the DNA sample on the highly sensitive micro-detector to..measure DNA concentration and the purity was detected by measuring the ratio..of optical density at 260/280. The purity of nucleic acid is estimated by the DNA..ratio of absorbance at 260 and 280 (A260/A280). A pure preparation of nucleic.acid should have an A260/A280 ratio between 1.7 and 2.0 that depend on the content.

The Forward primer of the TGF-β2 gene was 5-TGA CCGAGAAAGTCTGCATT-3 and the reverse primer was 5-AAGGTCTGAAGTTTGACCAGTACA-3. The TGF-β2 amplification..program was mentioned by Derakhshan et al., [11]. The RFLP was performed..as follows: a mixture of 10 µl of 0.5 µl of Bst4Cl and PCR amplicon were..incubated at 65 °C for one hour. Ten microliters of the digestion product were..run on 2% agarose gel electrophoresis for 90 min.

Statistical analysis

The Chi-square test was used to analyze the data of the study while the Mean ± Standard Error was used for numerical data analysis. The Student T-test was performed for two numeric variables comparison. F-test used in this study was also applied to test the relationship between age groups and the study..parameters. The SPSS version 22 and Graph pad prism v.6 programs were used to analyze the data.

Results

The rate of open-angle glaucoma occurrence increased with the age of the patients. The mean age group of the patients was 59 ± 0.9 and the mean age group of the control was 55 ± 1.5. The age group of 60–69 years is about half of the POAG patients (53.3%). The occurrence of POAG was 65.3% in males while in females 34.7%. Many factors were studied for their association with POAG including, family history was a risk factor for POAG (76.0%). Myopia and Diabetes were also associated with the development of the disease (64.0% and 62.7% respectively). The percentage of hypertension in patients was (57.3%). As compared with females, males are more likely to acquire POAG ($p = 0.02$). Both females and males in the age group 60–69 years are significantly ($p = 0.04$) acquired POAG as compared with other age groups (Table 1).

The results of serum TGF-β2 concentration are listed in Table 2. The serum. TGF-β2 level is higher (330.6858 $\pm$ 17.97, $p < 0.0001$) significantly in Primary Open Angle Glaucoma patients.

Receiver operating characteristic curve analysis

According to the Receiver Operating Characteristic Curve (ROC) results, as shown in Fig. 1 positive true results were observed in each age and TGF-β2 and may describe the disease case of patients, Table 3 shows the area under the curve (AUC) values and Cut of value (The value by which a person is considered to have the disease) for age and TGF-β2.

Molecular results

Genomic DNA extraction

The purity and concentration of the extracted genomic DNA were.. checked by the Nanodrop instrument, and the integrity of the..extracted DNA was checked by agarose gel

Table 2 The serum level of TGF-β2 in primary open-angle glaucoma patients and control

Parameter	Patients	Mean ± Std. Error (pg/ml)	Controls	Mean ± Std. Error (pg/ml)	P-value
TGF-β2	60	330.6858 ± 17.97***	60	129.8475 ± 2.308	< 0.0001***

***Highly significant difference (student t-test)

electrophoresis. The..range of DNA concentration was 11.2 – 58.1 µg/ml and the purity was 1.5 – 1.9.

The C/A *TGF-β2* gene polymorphism

The PCR was used to amplify and detect the SNP of the *TGF-β2 rs991967*..gene**.** The C/A SNP of the *TGF-β2* gene (*rs991967)* is shown in Supplement..file 1 as displayed in the National Center of Biotechnology Information..(NCBI). The amplicons were resolved on agarose gel, with a product size of 239 bp.

The digested products of restriction fragment length polymorphism were..resolved on an agarose gel. The RFLP bands shown in Fig. 2 revealed a 239..bp band for A (homozygous for allele A; AA) and 139 + 100 bp bands for C/C.(homozygous for allele C; CC) and 239 + 139 + 100 bands for A/C (heterozygous).

The AA genotype of *TGF-β 2* is significantly common in patients (61.7%, P < 0.000) as compared with the control (13.3%). While AC genotype is significantly occurring in control (41.7%, P = 0.001) as compared with patients (10.0%). Both CC and AC genotypes are common in control (45.0%, 41.7%, P = 0.004) as compared with the AA genotype (13.3%). The A allele is significantly common in patients (66.7%, P = 0.00039). While the C allele was in control (65.8%, P = 0.00035) when compared with POAG patients (33.3%, Table 4).

Table 1 Male and female age groups of POAG patients

Age group	Male		Female		Total		P-value
	N	%	n	%	N	%	
40–49	4	10.3	2	9.5	6	10.0	0.41
50–59	9	23.1	5	23.8	14	23.3	0.28
60–69	21	53.8	10	47.6	31	51.7*	0.04*
> 70	5	12.8	4	19.0	9	15.0	0.73
Total	39	100	21	100	60	100	0.02*
P-value	0.0003**		0.085		0.02 *		

Chi-square test. *Significant different, **and ***Highly significant. POAG: primary open-angle glaucoma.
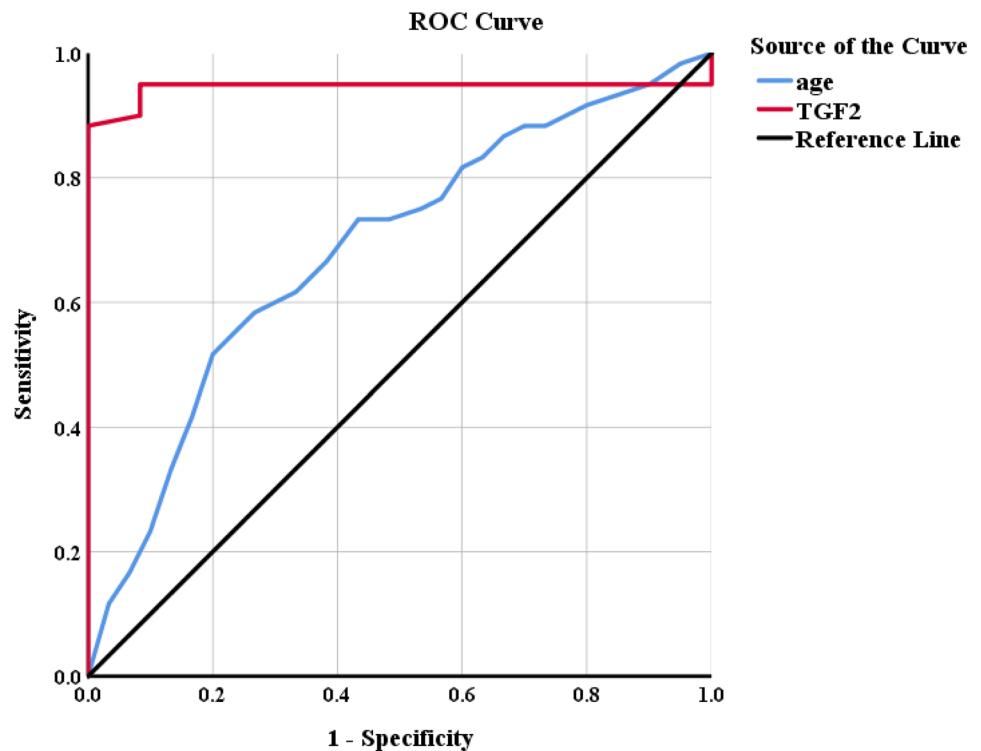
Fig. 1 ROC Curve of age and TGF-β2 of POAG patients

Table 3 Area under the Curve of age and TGF-β2 of primary open-angle glaucoma patients

Criteria	AUC	P-Value	Cut of value
age	0.686	0.0004**	64.50 year
TGF-β2	0.945	< 0.00001**	198.67 pg/ml
Pearson correlation			
		r- value	P-value
Age *vs*. TGF-β2		0.303	0.001**

**Highly significant difference

Supplement file 2 shows the Hardy–Weinberg equilibrium results, the observed.. and expected numbers of POAG patients, and control where there is no significant association between observed and expected numbers in control. While there is a significant difference between observed and expected numbers in POAG patients (p < 0.001). Table 5 showed that AA (reference) genotype was the most common in the patients (61.7%) while AC was less frequent (10.0%, OR: 0.051,95% CI: 0.01–0.16, P < 0.001) in the same group. While CC genotype (45.0%, OR: 0.136, 95%CI: 0.05–0.36,
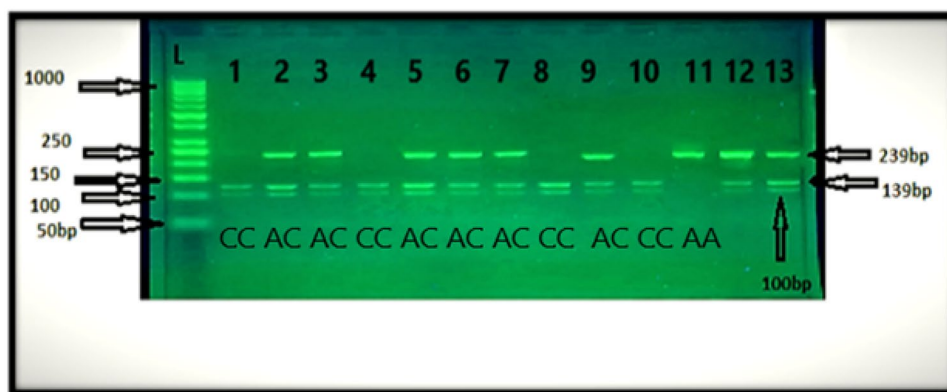

Fig. 2 The electrophoreticogram of RFLP products of the *TGF-β2* gene after Bst4Cl enzyme digestion. The cleaved C allele at the polymorphic site, generating two fragments (139 bp and 100 bp respectively), the enzyme did not cut the A allele generating three geno- types: AA (239 bp), AC (239, 139, 100 bp), and CC (139, 100 bp). L: 100 bp DNA ladder. The electrophoresis was carried out on 2% Red safe-stained agarose gel, at 7 V/Cm for 90 min

P < 0.0001) and AC genotypes (41.7%, OR: 0.051, 95%CI: 0.01–0.16, P < 0.001) were most common in the control group. Moreover, the *TGF-β2* C allele is protective (OR: 0.25, 95%, CI: 0.15–0.44, P < 0.0001).

TGF-β2 concentration according to genotypes

Patients with AA, CC, and AC genotypes have higher levels of TGF-β2 (337.33.. ± 24.49, P = 0.0003; 332.22 ± 33.1, P < 0.0001; 285.33 ± 32.0, P < 0.0001 respectively), than levels of TGF-β2 of control (132.52 ± 8.02, 131.63 ± 3.95, 127.06 ± 2.56) respectively (Table 6). No significant association was found between the three genotypes and the level of serum TGF-β2.

Discussion

This study was conducted in Baghdad city, particularly on Ibn Al-Haitham Hospital POAG patients. Despite the limited sample size, the serum level of TGF-β2 was significantly more than the serum level of TGF-β2 in controls and these findings agree with Kuchtey et al. [15], and Guo et al., [16]. The excess extracellular matrix was proposed to increase aqueous outflow resistance in trabecular meshwork in eyes with glaucoma. POAG patients have been reported to have a higher concentration of TGF-β2 in a recent study [13].

A study investigated the association between some cytokines and the aging process, but the results of the study are conflicting because it is difficult to distinguish

Table 4 Genotypes of *TGF-β2* and allele frequency in Primary Open Angle Glaucoma patients

Group	Patients Number = 60		Control Number = 60		χ^2	*P*-value
TGF Genotypes	No	%	No	%		
AA	37	61.7	8	13.3	18.68	0.00001**
CC	17	28.3	27	45.0	2.273	0.132 N.S
AC	6	10.0	25	41.7	11.64	0.001**
Total	60	100	60	100		
χ^2	24.7		10.9			
P-value	0.00004**		0.004**			
A	80	66.7	41	34.2	12.57	0.00039**
C	40	33.3	79	65.8	12.78	0.00035**
Total	120	100	120	100		
χ^2	13.33		12.03			
P-value	0.00026 **		0.00052 **			

χ^2 Chi-square

**Significant at < 0.01

Table 5 The Statistical evaluations of *TGF-β2* SNP of the POAG patients and healthy control

TGF-β2 C/A (rs991967)				
Genotype	Patients N = 60 (%)	Controls N = 60 (%)	*P*	OR (95% CI)
AA	37 (61.7)	8 (13.3)	Reference	
CC	17 (28.3)	27 (45.0)	<0.001**	0.136 (0.05–0.36)
CA	6 (10.0)	25 (41.7)	<0.001**	0.051 (0.01–0.16)
A	80 (66.7)	41 (34.2)	Reference	
C	40 (33.3)	79 (65.8)	<0.0001**	0.25 (0.15–0.44)

Table 6 The serum level of TGF-β2 according to genotypes

Group	Patients Number = 60	Control Number = 60	*P*-value
TGF-β2 Genotypes	TGF-β2 concentration Mean ± Standard Error (pg/ml)	TGF-β2 concentration Mean ± Standard Error (pg/ml)	
AA	337.33 ± 24.49	132.52 ± 8.02	<0.0003
CC	332.22 ± 33.1	131.63 ± 3.95	<0.0001
AC	285.33 ± 32.0	127.06 ± 2.56	<0.0001
P-value	0.704 N.S	0.599 N.S	

the pathological aging process from the natural aging process [17]. Moreover, no significant difference between males and females with measured TGF-β2, and maybe this result is due to low estrogen levels in old women. A study proposes that the differences between females and males could be a result of decreased estrogen production as well as a reduction in inflammatory cytokines production [18]. Any similarity between parameters is because of many factors, including gender, sex hormones, reproductive status, variations in immune responses during the life of individuals, environmental factors, and age. When considering the significant variations between males and females in different diseases, it's becoming increasingly vital to identify gender differences in immune responses.

In this study, the possible relationship between POAG development and the *TGFB2* rs991967 polymorphism was investigated. The CC genotype and AC genotypes were most common in the control group. These results were near to the Iranian study, which demonstrated that the AA genotype of *TGFB2* rs991967 was common in POAG Iranian patients [11]. In the eyes of POAG patients the two forms of TGF-β2, total and active forms, are increased Many studies were directed to inspect the role of SNP of some genes in the pathogenesis of POAG. The *TGF-β2* AA genotype was found to be predominant in POAG patients. Several models have been suggested to clarify the role of TGF-β2 SNPs in POAG development. However, the TGF-β2 plays a major role in the stimulation of elastin and actin of smooth muscle, angiogenesis inhibition of endothelium, and proteoglycan syntheses inhibition leading to some complications, like the development of POAG [19]. According to the results of this study, the *TGF-β2* rs991967 polymorphism is associated with POAG development.

The results of this study also showed a slight reduction in TGF-β2 level by the AC genotype in POAG patients with no significant difference among other genotypes (AA and CC) in the same group. So the AC genotype could be a protective factor. It was previously found that the functional single nucleotide polymorphisms of some regulatory gene promotors are associated with the profile of a certain cytokine and thus could be used as disease-associated markers [20]. The polymorphisms of cytokine genes mainly affect the transcription of the particular cytokine [21].

In conclusion, the males were more susceptible to acquiring POAG than females, especially the elderly. The TGF-β2 plays important role in the pathogenesis of POAG. The CC and AC genotypes are common in the control and the C allele is a protective factor.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11033-023-08503-4.

Declarations

Conflict of interest The author reports no conflict of interest in this work.

Ethical approval The study was approved by the Ethics Committee of the College of Science/Mustansiriyah University (Ethical approval number: 2021–117). All study subjects provided informed consent.

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