Methylenetetrahydrofolate Reductase C677T Polymorphism and Plasma Homocysteine Levels in Egyptian Patients with Primary Open-angle Glaucoma

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ABSTRACT

Objectives: *The association between primary open-angle glaucoma (POAG) and cardiovascular disease has recently stimulated interest in the role of homocysteine in the pathogenesis of glaucoma. In the present study, we aimed to measure plasma homocysteine and its relation to C677T methylenetetrahydrofolate reductase polymorphism in patients with POAG.* ***Subjects and Methods:*** *Fasting venous blood samples from one hundred patients with POAG and 50 age and sex matched healthy control subjects were analyzed for plasma homocysteine by high performance liquid chromatography (HPLC) and genotype for C677T polymorphism by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).* ***Results:*** *There was no statistically significant difference in POAG patients and controls for the age and sex (P>0.05). Mean plasma homocysteine was significantly higher in POAG patients (12.37±3.05µmol/l.) compared with controls (10.40 ±1.74 µmol/l.) (P< 0.001). C677T polymorphism did not show significant differences between the POAG patients and control subjects (CC: 46%, CT: 41%, TT: 13%) for patients with POAG; CC: 60%, CT: 32:%, TT: 8% for controls) (P>0.05). Frequency of T allele was 33.5% and 24% for patients with POAG and controls, respectively.* ***Conclusion:*** *In Egyptian patients plasma homocysteine was elevated in POAG patients but no association was found between C677T polymorphism and POAG patients. If homocysteine measurement proves useful for risk stratification, therapies that lower it may aid the management of POAG.*
Keywords: *Primary open-angle glaucoma, Homocysteine, gene C677T polymorphism.*

INTRODUCTION

Primary open-angle glaucoma (POAG) is a slowly progressive multifactorial optic neuropathy characterized by retinal ganglion cell death. It manifests as changes to optic disc, retinal nerve fiber layer, and visual field.[1,2] . It is the second leading cause of irreversible blindness worldwide.[3]

High levels of homocysteine in the aqueous humor and the serum of patients suffering from glaucoma and other ocular diseases have been observed in different studies.[4–8] Homocysteine can induce neuronal cell death by inducing apoptosis or gliotoxicity,[9,10] vascular injuries,[11] thus representing a hypothetical risk factor for POAG.[12] Moreover, it causes dysregulation of matrix metalloproteinases and their inhibitors.[13] Hyperhomocysteinemia has been shown to be involved in the structural remodeling of connective tissues.[14,15] Homocysteine concentrations are influenced by both environmental and genetic factors, such as mutations in the methylenetetrahydrofolate reductase (MTHFR) gene.[16, 17]

MTHFR enzyme catalyzes the reduction of 5`,10`-methylenetetrahydrofolate to 5`-methyltetrahydrofolate, the predominant circulatory form of folate which is the methyl donor for homocysteine remethylation.[18] Polymorphisms in the gene are associated with a reduced enzyme activity and elevated circulating homocysteine levels.[19,20] The C677T polymorphism of MTHFR, is characterized by a C>T substitution at nucleotide 677 which results in a missense mutation due to the substitution of valine for alanine at position 222 of the MTHFR enzyme.[16,21] There are conflicting data regarding the association of polymorphisms with glaucoma. Several studies have reported an association of the C677T polymorphism with glaucoma,[22–25] whereas no association in other studies.[4,26–28]

Since POAG is a disease with a high heritability as shown in population-based studies[29, 30], the present study was conducted to investigate a hypothesized association between C677T polymorphism of MTHFR enzyme and POAG in a sample of Egyptian patients, and their possible association with plasma homocysteine levels hoping that our results will contribute toward a better understanding of the disease's causative agents.

PATIENTS & METHODS

Subjects

This case control study was conducted with 100 Egyptian patients with POAG and 50 control subjects attending the Ophthalmology Clinics of Zagazig University Hospital, Egypt, between April 2010 and July 2011. The study was approved by the ethical committee of Faculty of Medicine, Zagazig University. Informed consent for the experimental use of specimens was obtained from all subjects.

Patients with chronic renal impairment, diabetes mellitus, cancer, or a gastrointestinal malabsorption syndrome were excluded from the study. Subjects who had used anticonvulsant, immunosuppressive therapy, postmenopausal hormone replacement, cholesterol-lowering agents, antidepressants, antimicrobial therapy, and vitamin supplements in the preceding 6 months were also excluded. Patients were excluded if they had any other ocular diseases that would have caused an increase in the intraocular pressure (IOP), a period of steroid administration, trauma, or uveitis, or if they had a history of eye surgery before the diagnosis of glaucoma.

All subjects underwent a complete ophthalmic examination including best-corrected visual acuity, slit-lamp examination, IOP measurement using Goldmann applanation tonometer, gonioscopy, and dilated funduscopic examination.

POAG patient was defined by: an IOP before initiation of a pressure-lowering therapy of at least 21 mm Hg, an open anterior chamber angle on a gonioscopic examination, optic disc changes (notching, increased cup/disc ratio), thinning of the neuroretinal rim, glaucomatous visual field defects (inferior or superior arcuate scotoma, nasal step, paracentral scotoma) (assessed by automated static perimetry, Humphrey Visual Field Analyzer 30-2, Humphrey Instruments, San Leandro, CA), and with no other contributing pathology.

The control group consisted of 50 unrelated Egyptian patients with other eye problems such as refractive errors or cataract. All controls had an IOP of less than 21 mmHg, a cup/disc ratio of less than 0.5, no history of IOP lowering medications, and no family history of glaucoma and met the same exclusion criteria of POAG patients. Controls were matched to cases by age, gender, and diagnosis of systemic hypertension to make the groups maximally comparable.

Blood sampling

After overnight fasting (> 12 h), venous blood sample was collected from each participant in ethylene diamine tetra acetic acid (EDTA) treated tubes. Plasma was immediately separated by centrifugation at 1800 × g for 15 min, coded and stored at –20 °C for homocysteine assay, and whole EDTA blood used for DNA extraction.

Total plasma homocysteine

Processing of samples:

Tubes contain 20 mg of 5-Sulfosalicylic acid (SSA) were prepared as follows: a solution of 200g/L SSA in absolute ethanol was divided into 0.1 ml aliquots in 1 ml tubes. After evaporation of the ethanol in an oven (over night at 50°C), the tubes were capped and stored at room temperature. These SSA tubes contain sufficient SSA to deproteinize 0.5 ml of plasma.

Determination of plasma homocysteine:

Chemicals: O-phthaldehyde, 3-mercaptopropionic acid (Fluka-Switzerland), 5-SSA, Boric acid and Potassium dihydrogen phosphate (Scientific limited UK), high performance liquid chromatography (HPLC) grade methanol and acetonitrile (Honil, UK), triethylamine (Reidel-dehaen. Germany).

Homocysteine measurement was carried out by programmable HPLC equipment (GBC model dual head pump, Australia), supplied with autosampler LC 1610, online degasser model GT 104 using spherisorb ODS-2 column with 3 µm particles size (150 × 4.6 mm). On the day of analysis 0.5 ml of plasma was transferred to SSA tubes vigorously mixed and centrifuged for 15 min at 3000 g the clear supernatants were used for dervitization process. Dervitization was started by the transfer of 20 µl of water to an empty reaction vial, followed by the addition of 5 µl of sample and 90 µl of OPA-mercaptopropionic acid reagent. Working solution was prepared on the day of analysis by adding one part of stock to 20 parts of 100 mmol/L borate, pH 10.0. The sample and reagent were mixed and incubated at room temperature for 3 min; 50 µl of neutralizing buffer (400 mmol/L potassium dihydrogen phosphate +10 ml/L triethylamine) was added, after mixing 3 µl of reaction mixture was injected. The mobile phase A was formed of 1000 ml of phosphate buffer + 1000 ml of water + 4ml of tetrahydrofurane. Mobile phase B was a mixture of previous buffer, methanol and acetonitrile (50/35/15 by volume). Flow rate was 1.5 ml/min for 12 min. The composition of the eluent during analysis was shown in Table 1. Perkin Elmer LS 30 luminescence spectrometer was used for detection, the excitation and emission wave length were 230 and 389 nm respectively. Peak analysis was done using Winchrom 1.2 software.[31]

Hyperhomocysteinemia is defined as homocysteine level > 15 µmol/l.. [32]

DNA extraction

EDTA peripheral blood samples were obtained from all participants coded and analyzed in a blind manner for genomic DNA extraction using QIA amp DNA Blood Mini Kit supplied by *Qiagen GmbH (Hilden, Germany)* as described in the user manual. The quality of the genomic DNA was tested using agarose gel electrophoresis.

Genetic analysis using polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP):

The C677T polymorphism was genotyped by PCR-RFLP as described by **Papoutsakis *et a l*** [33] using forward primer 5'-TGA AGG AGA AGG TGT CTG CGG GA-3' and reverse primer 5'-AGG ACG GTG CGG TGA GAG TG-3'. The PCR was carried out in final volume of 25 µl containing 30 pmol of each primer (*Biosource Europe SA, Belgium*), 100 ng of genomic DNA template and 12.5 µl of 2 × Dream Taq™ Green PCR Master Mix (MBI Fermentas, Germany).

Table 1 High performance liquid chromatography gradient conditions

Time, min	Mobile phase B in eluent, %
0.0	2
3.5	25
5.2	44
6.9	52
10.0	100
11	2

The amplification protocol was as follow: 95 °C for 10 min; then 35 cycles of 95°C for 1 min, 65 °C for 30 s, and 72 °C for 1 min; then 72 °C for 7 min using thermal cycler PTC-100 machine (MJ Research, Inc., Watertown, Mass. USA).

PCR products of C677T polymorphism were subjected to digestion with 5 U *Hinf*I (New England Biolabs, Beverly, MA, USA), for at least 2 h at 37 °C. The digested products were separated in a 3% agarose gel electrophoresis system (Maxicell, EC 360 M-E-C apparatus Cooperation. St Petersburg Florida USA) and visualized with ethidium bromide staining under UV transillumination with 100-bp ladder (Pharmacia Biotech, USA).

Statistical Analysis:

Statistical analysis was conducted using the Statistical Package for the Social Sciences version 13 (SPSS Inc., Chicago IL version 13, USA). Continuous variables were expressed as means ± standard deviation. Categorical variables were expressed as percentages. Differences between continuous variables were analyzed using the Student *t* test, while those between categorical variables were analyzed using the Chi square (χ^2) test. Hardy-Weinberg equilibrium analysis was performed to compare observed and expected genotype frequencies using χ^2 test. The association between genotypes and glaucoma risk were estimated by the odds ratios (OR) and their 95% confidence intervals (CI). *P* value of <0.05 was considered statistically significant.

RESULTS

Demographic and clinical data characteristics of the study Subjects

Table 2 shows the demographic and clinical characteristics of the POAG and controls. Controls and patients showed no statistically significant differences as regards gender (P = 0.81), age (P = 0.26), hypertension (P = 0.96), diabetes mellitus (P = 0.64), or cardiovascular diseases (P = 0.41).

Table 2: Demographic and clinical characteristics of the primary open-angle glaucoma (POAG) patients (n = 100) and controls (n = 50)

Variables	**POAG patients**	**Controls**	**P value**
Male, No (%)	64 (64)	33 (66)	0.81*
Age, yr; range mean ±SD	42-88 65.3±12.5	42-88 62.8±13.3	0.26**
Hypertension, No (%)	59 (59)	27 (54)	0.96*
Diabetes mellitus, No (%)	40 (40)	18 (36)	0.64*
Cardiovascular diseases, No (%)	10 (10)	3 (6)	0.41*

* χ^2 test; ** student *t* test

Plasma homocysteine levels and glaucoma risk

The mean plasma levels of homocysteine were 10.40 ±1.74 µmol/l. in controls, and 12.37±3.05 µmol/l. in the POAG patients with significant increase in POAG patients (*P*< 0.001) and there was significant increase in the frequency of hyperhomocysteinemia in POAG patients than controls (P = 0.03) Table 3.

C677T polymorphism analysis and glaucoma risk

PCR-RFLP analysis of *Hinf*I digestion of MTHFR C677T polymorphism is shown in the figure 1.

The distributions of genotype and allele frequencies for C677T polymorphism in POAG patients and controls are represented in Table 4. The genotype frequencies were conformed to the Hardy-Weinberg equilibrium in controls (*P* = 0.912). In patients with POAG, the frequency of CC, CT, and TT genotypes were 44, 41, and 13% respectively; and in controls, the frequencies were 60, 32, and 8% respectively. As regard the risk of development of glaucoma, the CC genotype and C wild allele were taken as references, there was no significant difference in the distribution of CT and TT genotypes or T allele between POAG and the controls (P value was 0.37, 0.43, and 0.11 respectively.

Table 3: Plasma homocysteine levels (µmol/L) in primary open-angle glaucoma (POAG) patients (n = 100) and controls (n = 50)

Homocysteine values	**Control**	**POAG**	**P-value**
Homocysteine levels (mean ± SD)	10.40 ±1.74	12.37±3.05	<0.001*
Hyperhomocysteinemia[a] , No (%)	6 (12.0)	29 (29.0)	0.03**

[a] homocysteine level > 15 µmol/l
*student unpaired "*t*" test.
**χ^2 test.

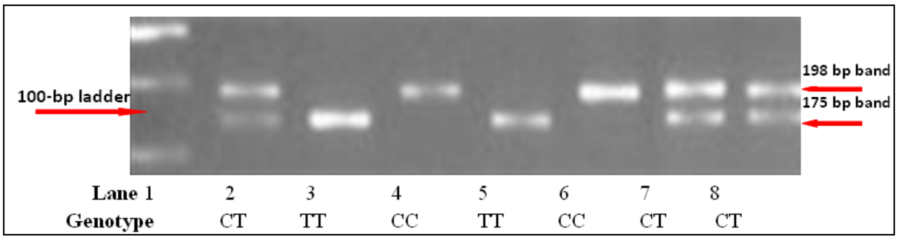

Figure 1 Polymerase chain reaction restriction fragment length analysis of *Hinf*I digestion of C677T polymorphism. Lane 1; 100 bp ladder, lane 2,7,8; three fragments of 198, 175, and 23 bp (not shown) were identified as heterozygous (CT), lane 3,5; two fragments of 175 and 23 bp (not shown) were identified as homozygous mutant (TT) genotype, lane 4,6; a single fragment of 198 bp was identified as homozygous (CC).

C677T *gene polymorphism and homocysteine concentrations*

Patients with POAG were divided according to the genotype of C677T polymorphism; there was no significant difference in the plasma homocysteine levels among the three genotypes (*P* = 0.83); figure 2.

DISCUSSION

The present study has demonstrated significant elevation of plasma homocysteine in Egyptian patients with POAG, which lend support to the hypothesis that there is an association between POAG and elevated plasma homocysteine levels.

Homocysteine measurement is exquisitely sensitive to many environmental factors including inter-current diseases and use of certain medications.[34]. So, all our participants met the chosen exclusion criteria in an attempt to control for the main influences on homocysteine level including vitamin use, renal impairment, diabetes mellitus, and specific medication.

Table 4: Genotype distributions and allelic frequencies of C677T polymorphism the primary open-angle glaucoma (POAG) patients (n = 100) and controls (n = 50)

C677T polymorphism	POAG patients, n (%)	Controls, n (%)	Odds Ratio (95% Confidence interval)	*P* - value*
Genotypes				
CC	46 (46)	30 (60)	1 (reference)	-
CT	41 (41)	16 (32)	1.48 (0.72-3.02)	0.37
TT	13 (13)	4 (8)	1.7 (0.53-5.57)	0.43
Alleles				
C	133 (66.5)	76 (76)	1 (reference)	-
T	67 (33.5)	24 (24)	1.60 (0.93-2.75)	0.11

* χ^2 test.

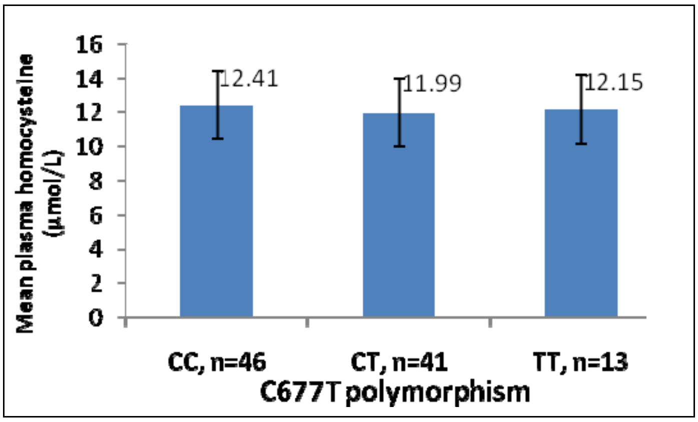

Figure 2 Plasma homocysteine levels in C677T polymorphism carriers in primary open-angle glaucoma patients (n = 100). P = 0.83 (single factor analysis of variance).

The rate of hyperhomocysteinemia varies in different studies. In control subjects the rate of hyperhomocysteinemia in this study was 12% which is comparable to the result of **Leibovitch et al.** (10%).[35] The reported rates in united state population were 29.2% [36] and 36% [37]; in Australian normal subjects, the rate was very low (2.4%) [4]; and in Turkish normal subjects, it was as high as 67.6%, with a mean plasma homocysteine level of 17.43 µmol/l in one study [38], and 25% in with a mean plasma homocysteine level of 11.64 µmol/l in another study. [39] This difference may be due the method of assessment of homocysteine, the cut of level of hyperhomocysteinemia, and it may also relate to socioeconomic status with good vitamin B/folate status in some population causing low levels of hyperhomocysteinemia. Nevertheless, it is interesting to note the strong correlation between elevated plasma homocysteine and low serum folate in glaucoma patients. [40]

POAG patients were either on topical glaucoma medications and/or had previously undergone laser trabeculoplasty and/or glaucoma filtration surgery. This was in contrast to the control group who had no history of glaucoma treatment. The impacts of topical glaucoma medications on plasma homocysteine levels are unknown.

Reports on plasma homocysteine in POAG[6,22,37,39–41] have yielded different results. A small number of studies has

investigated the association between homocysteine and POAG with inconsistent results. **Bleich et al.**[5,6] have demonstrated that serum homocysteine is elevated in patients with either POAG or pseudoexfoliation glaucoma. **Bleich et al.**[6] were the first to demonstrate an association between increased plasma homocysteine levels and POAG, which suggested evidence of its involvement in the pathogenesis of glaucomatous optic neuropathy.

Leibovitch et al.[35], **Vessani et al.**[36] and **Altintaş et al.**[39] have independently demonstrated elevation in the levels of total homocysteine in patients with pseudoexfoliative glaucoma. **Vessani et al.**[36] have found no association between serum homocysteine and normal tension glaucoma. Also, **Wang et al.**[37] failed to find raised homocysteine levels in POAG patients.

It is not clear whether elevated plasma homocysteine contributes to POAG development or progression. **Moore et al.**[10] suggested that an elevation in one's homocysteine level may cause changes in the optic nerve head microvasculature and impair optic nerve blood flow via a vasoconstrictive effect, endothelial injury, smooth muscle proliferation, platelet activation, thrombogenesis, and apoptotic cell death in retinal ganglion cells which may cause neuronal cell death, but direct toxic action of homocysteine on retinal ganglion cells are another possible mechanisms.

Some studies, including our study have found no association between the MTHFR C677T gene polymorphism and POAG[4,23,38]. **Micheal et al.**[42] have reported in Pakistanian population that no association of the MTHFR C677T genotype with POAG but a very strong association with primary closed-angle glaucoma which is probably due to the presence of the TT genotype in the disease cohort and its complete absence from the control this population. In contrast to our results, **Bleich et al.**[6] and **Junemann et al.**[22] have reported increased gene mutation frequencies in POAG patients. These different results may be due to the ethnic difference in the study populations.

The present study revealed that no association between C677T polymorphism and the plasma homocysteine levels, a result that agreed with that of **Clement et al.**[4]. Similarly, both branch retinal vein occlusion[43] and non-arteritic ischemic optic neuropathy[44] are significantly associated with elevated plasma homocysteine levels, but not the frequency of C677T MTHFR polymorphism. The influence of C677T MTHFR polymorphism on homocysteine is complex and dependent on the interaction of many factors as homocysteine in MTHFR homozygote that do not smoke is lower than in those that do.[45] These findings should be interpreted cautiously, bearing in mind the small sample size which may have masked the relationship between the MTHFR polymorphism and POAG.

In conclusion, the current study has demonstrated elevated plasma homocysteine levels in POAG patients. This association was observed in the setting of MTHFR C677T polymorphism frequency not significantly different from controls. The relationship between homocysteine levels, MTHFR polymorphisms and glaucoma warrants further study.

Increased levels of homocysteine in patients with POAG may have important implications for understanding the pathogenesis of glaucomatous optic neuropathy. The results of the study highlight the need for further research to study whether neuroprotection against elevated homocysteine or the lowering of homocysteine will protect patients with glaucoma from glaucomatous optic nerve damage.

Conflict of interest

We declare that we have no conflict of interest.

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التعدد الشكليC677T لجين مختزل رابع هيدرو مثيلين الفولات وهوموسيستايين البلازما في المرضى المصريين المصابين بالجلوكوما مفتوحة الزاوية

تهدف هذه الدراسة استقصاء مدى علاقة التعدد الشكلي C677T لجين مختزل رابع هيدرو مثيلين الفولات وهوموسيستايين البلازما بحدوث الجلوكوما مفتوحة الزاوية في المرضى المصريين.

تم إجراء هذه الدراسة في قسمي طب وجراحة العيون والكيمياء الحيوية الطبية بكلية الطب جامعة الزقازيق ، وقد شملت الدراسة ١٠٠ مريضا بالجلوكوما مفتوحة الزاوية ومجموعة ضابطة شملت ٥٠ أصحاء لا يعانون من أمراض ولا يتعاطون أدوية تتعارض مع الدراسة وقد تم قياس مستوى الهوموسيستايين في بلازما الدم واستخدم التفاعل التسلسلي عديد البلمرة للكشف عن التعدد الشكلي C677T لجين مختزل رابع هيدرو مثيلين الفولات

تشير نتائج الدراسة إلى .وجود زيادة ذات دلالة إحصائية في مستوى الهوموسيستايين في مرضي الجلوكوما مفتوحة الزاوية عنه في المجموعة الضابطة. وليس هناك اختلاف ذو دلالة إحصائية في معدل وجود التعدد الشكلي C677T لجين مختزل رابع هيدرو مثيلين الفولات في مرضى الجلوكوما مفتوحة الزاوية عنه في المجموعة الضابطة .