ORIGINAL REPORT

Lack of Association between the C677T Single Nucleotide Polymorphism of the *MTHFR* Gene and Glaucoma in Iranian Patients

Navid Nilforoushan[1], Sevil Aghapour[1,3], Reza Raoofian[3], Samira Saee Rad[3,] Wayne K. Greene[4], Ghasem Fakhraie[2], and Mansour Heidari[3]

[1] *Department of Ophthalmology, Eye Research Center of Rasoul Hospital, Tehran University of Medical Sciences, Tehran, Iran*

[2] *Eye Research Center, Farabi Eye Hospital, Tehran University of Medical Sciences, Tehran, Iran*

[3] *Department of Medical Genetics, Tehran University of Medical Sciences, Tehran, Iran*

[4] *School of Veterinary and Biomedical Sciences, Faculty of Health Sciences, Murdoch University, Perth WA 6150, Australia*

Received: 22 Jun. 2011; Received in revised form: 19 Nov. 2011; Accepted: 17 Dec. 2011

Abstract- Glaucoma is a major cause of blindness worldwide. A single nucleotide polymorphism of the *MTHFR* gene (C677T) has been associated with susceptibility to this disease, although this is controversial in the last decade. In this study, the possible association between the *MTHFR* C677T polymorphism and the risk of developing primary open angle (POAG) and pseudoexfoliation glaucoma (PEXG) was investigated. For this, a prospective study consisting of 73 POAG, 85 PEXG and 90 matched controls was undertaken in an Iranian population. Genomic DNA was extracted from whole blood. Genotyping of all individuals for the *MTHFR* C677T polymorphism was conducted using the PCR-RFLP technique. Our findings revealed no significant association between the *MTHFR* C677T polymorphism in POAG and PEXG compared with controls. Consistent with several other studies, our analysis suggests that the *MTHFR* C677T polymorphism is unlikely to be a factor contributing to the risk of developing specific forms of glaucoma.

Acta Medica Iranica, 2012; 50(3): 208-212.

Keywords: Primary open angle glaucoma; Pseudoexfoliation glaucoma; MTHFR; PCR-RFLP

Introduction

Glaucoma is the second leading cause of blindness, accounting for 70 million cases worldwide (1). Approximately 10% of bilateral blindness cases have been reported to be associated with glaucoma (1,2). Glaucoma is defined as a retinal ganglion cell disease characterized by atrophy and cupping of the optic nerve head (3,4). This disease often has no warning symptoms in the early stages. Therefore, many patients do not even know their disease until it progresses into irreversible blindness (5). There is no cure for glaucoma and the main treatment involves lowering intraocular pressure using drugs or surgery (6).

The most common form of glaucoma is the primary open angle (POAG) type, defined as a chronic and progressive optic neuropathy (4,7). Pseudoexfoliation glaucoma (PEXG) has been reported to be the most common identifiable cause of open-angle glaucoma, accounting for about 25% of all and most cases of glaucoma in some countries (8). Pseudoexfoliation syndrome (PEXS) is a systemic microfibrillopathy characterized by pathologic accumulation of abnormal fibrillar material in various organs of the body including lung, heart, liver, gallbladder, skin, kidney, and cerebral meninges (5,9). The most prominent accumulations of PEX material has been demonstrated in the anterior segment of the eye, leading to important eye manifestations such as secondary glaucoma (10).

Homocysteine, a sulphur-containing excitotoxic amino acid, induces neuronal cell death in the brain due to overstimulation of *N* -methyl- D-aspartate (NMDA) receptors. It also, selectively activates NMDA receptors of retinal ganglion cells. Several lines of evidence indicate an involvement of homocysteine in the process of cellular apoptosis (5). The levels of plasma homocysteine are controlled by the interaction of environmental and genetic factors (6). The enzyme 5, 10 methylenetetrahydrofolate reductase (*MTHFR*) catalyzes the conversion of 5, 10 methylenetetrahydrofolate into 5-methylentetrahydrofolate, which acts in the remethylation of homocysteine to methionine.

Corresponding Author: Mansour Heidari
Department of Medical Genetics, Tehran University of Medical Sciences, Pour Sina Ave, Tehran, Iran
Tel: +98 21 88953005, Fax: +98 21 88953005, E-mail: mheidari@sina.tums.ac.ir

Concentrations of homocysteine have been demonstrated to be affected by a single base pair polymorphism, C677T, in the *MTHFR* gene. Transition of C>T at codon 222 results in substitution of valine for alanine and causes a mild enzymatic dysfunction. Patients that are homozygous for this polymorphism (TT) have slight elevation in plasma homocysteine concentration and are at increased risk for premature vascular disease (7,8).

Based on the literature, the prevalence of *MTHFR* C677T variants may be different in various ethnic populations (9, 10). The main goal of this study was to ascertain whether there is any significant association with *MTHFR* C677T polymorphism and two types of glaucoma, POAG and PEXG in an Iranian population.

Materials and Methods

Subjects

In this prospective case–control study, we investigated a total of 248 Iranian subjects comprising 73 primary open angle glaucoma (POAG) cases, 85 pseudoexfoliation glaucoma (PEXG) cases, and 90 controls. All participants were seen at the Department of Ophthalmology, Rasoul Hospital, Tehran. All participants gave their written informed consent, and the study was approved by the TUMS ethics review board as part of the reviewing process of TUMS research projects. The study was conducted according to the principles of the Helsinki Declaration.

All individuals underwent a complete ophthalmologic examination comprising best-corrected visual acuity, slit-lamp biomicroscopy, Goldmann applanation tonometry, gonioscopy, and dilated fundoscopic examination. POAG was defined by the following criteria: an intraocular pressure (IOP) of more than 21 mm Hg before initiation of a pressure-lowering therapy with a Goldman applanation tonometery, open anterior chamber angles on gonioscopy; glaucomatous optic disc changes (increased cup/disc ratio, thinning of the neuroretinal rim, notching) on ophthalmoscopy and visual field defects characteristic of glaucoma by standard automated perimetry (Humphrey Visual Field Analyzer 30-2, Humphrey Instruments, San Leandro, CA). Patients included in the POAG group were shown not to have any systemic or local condition causing secondary glaucoma. Diagnosis of PEXG was based on pseudoexfoliation material on the lens capsule or near the pupil. Inclusion criteria for control subjects was an increased IOP of below 21 mm Hg, no glaucomatous changes in the optic disc, no visual field loss characteristic for glaucoma and no pseudoexfoliation material in the lens capsule or near the pupil.

Genotyping

Genomic DNA was extracted from peripheral blood (0.5 ml) using a DNGPLUS kit (Cinnagen, Tehran, Iran). Genotyping of all individuals for the *MTHFR* C677T polymorphism was conducted using the PCR-RFLP technique. PCR amplification was performed using specific primers (MTRF: 5'-TGTCATCCCTATTGGCAGGT-3'; MTRR: 5'-ACTCAGCACTCCACCCAGAG-3') 1U *Taq* DNA polymerase (Roche, Mannheim, Germany), 10 pM of each primer, 200 µM of each dNTPs, 0.67 µl of 50 mM $MgCl_2$, 60 ng DNA and 2.5µl of 10X PCR buffer in 25 µl PCR reactions. The PCR conditions included an initial denaturation step for 3 min at 95ºC, 30 sec at 95ºC, 45 sec at 64ºC with a 1ºC decrease every second cycle down to 55ºC, then 55ºC for 20 cycles, 1 min at 72ºC for extension, and finally 10 min at 72ºC. In order to detect the C677T polymorphism the PCR products (305bp) were subjected to RFLP using *Hinf* I restriction enzyme (Promega, UK). The reactions comprised 12 µl of crude PCR product, 16 µl of dH_2O, 2 µl of 10X buffer R and 1 µl of *Hinf* I enzyme in a total volume of 31 µl. The digested PCR products were separated on 2% agarose gels and visualized with ethidium bromide.

Direct genomic sequencing

In order to confirm the identified genotypes from PCR-RFLP, nine genotypes in a random selection of the samples were subjected to PCR sequencing. Sequence data searches were carried out in non-redundant nucleic acid and protein databases BLAST (http://www.ncbi.nlm.nih.gov/BLAST).

Statistical analysis

The Statistical Package for Social Sciences (SPSS) version 11.2 was used for data entry and analysis. Correlations between *MTHFR* variants and glaucoma (POAG and PEXG) were examined using the Fisher test and an alpha level of 0.05 was used to indicate statistical significance.

Results

To examine a possible association of the *MTHFR* C677T polymorphism with glaucoma, a total of 248 individuals comprising 73 POAG patients, 85 PEXG patients and 90 matched controls were enrolled in this study.

Table 1. Demographic characteristics of the participants.

Characteristics	POAG (n=73)	PEXG (n=85)	Control (n=90)
Age (year): Mean	67.8	69.3	67.5
Minimum	50	51	50
Maximum	80	80	80
Gender: Female	19	20	25
Male	54	65	65

The mean age of patients with POAG and PEXG was 67.9 years and 69.1 years, respectively, versus 67.5 years in the control group. The demographic characteristics of all study individuals are summarized in Table 1. Our PCR-RFLP findings showed no significant association between the genotype distribution (Table 2) or allele frequency (Table 3) of the C677T single nucleotide polymorphism within the *MTHFR* gene in either POAG or PEXG patients compared with the matched controls.

Genotypes in our study were identified using PCR-RFLP approach. In spite of the fact that this assays is very rapid and inexpensive it is also highly susceptible to error, especially if the PCR products are not completely digested. Therefore, we confirmed the genotypes in a random selection of the samples by PCR sequencing.

Discussion

Several reports have described increased levels of plasma homocysteine in patients with POAG and PEXS, suggesting that this metabolite may be associated with the pathogenesis of these diseases (17,20). Consistent with this, Moore *et al*., (2001) demonstrated that homocysteine is toxic to neurons of the ganglion cell layer and that retinal neurons die by apoptosis (11). More recent studies have shown an association between elevated homocysteine levels and vascular disease (2,12). Homocysteine can also affect extracellular matrix remodeling by interfering with matrix metalloproteinases (MMPs) and their regulation of inhibitors. This process has been suggested to be a significant factor in the pathogenesis of PEXG (13).

A number of factors have been implicated in elevated homocysteine levels, including a common polymorphism of the *MTHFR* gene, C677T, which has been shown to reduce the activity of the encoded *MTHFR* enzyme via substitution of valine for alanine at codon 222 (14,15). As a result, several studies have sought to investigate whether the *MTHFR* C677T variant is itself a genetic risk factor for POAG and PEXG (16-19). Notably, Junemann *et al.,* (2005) showed a positive association between POAG and the *MTHFR* 677C>T polymorphism, but not for PEXG. Using 76 POAG cases, 71 patients with PEXG, and 71 controls, they found 9% TT homozygosity and 49% heterozygosity (CT) in patients with POAG and 3% and 34% in controls, therefore, hypothesized that the *MTHFR* 677C>T variant itself may contribute to development of POAG (16).

By contrast, a larger group of studies using diverse patient cohorts from Austria, Turkey, Japan, Australia and the U.S.A. have reported that the *MTHFR* 677C>T polymorphism is not a genetic risk factor for POAG or PEXS (17-22). Michael *et al.,* (2009) also reported no association between of this polymorphism and POAG in Pakistani patients, but interestingly did find an association between C677T and primary closed angle glaucoma (PCAG) (23). In agreement with these studies, our results revealed no significant association between the *MTHFR* 677C>T variant and POAG or PEXS in our cohort of Iranian patients.

Table 2. Genotype frequency of *MTHFR* 677 in glaucoma patients and controls.

MTHFR 677 Genotype	POAG (%)	*P*-Value	PEXG (%)	*P*-Value	Control (%)
CC	39 (53.42)	0.578	46 (54.12)	0.626	53 (58.89)
CT	28 (38.36)	0.825	31 (36.47)	0.979	33 (36.67)
TT	6 (8.22)	0.88	8 (9.41)	0.194	4 (4.44)
Total	73		85		90

Table 3. Allele frequency of *MTHFR* 677 in glaucoma patients and controls.

MTHFR 677 Alleles	POAG (n=146)	*P*-Value	PEXG (n=170)	*P*-Value	Control (n=180)
C	106	-	123	-	139
T	40	0.337	47	0.294	41

Taken these results together and those of previous studies strongly suggest that the *MTHFR* 677C>T polymorphism is not a genetic risk for the development of POAG or PEXG, including within the Iranian population. Although the *MTHFR* 677C>T variant is associated with increased plasma homocysteine levels, other mechanisms involved in raising homocysteine levels would appear to be more important in POAG and PEXS glaucoma cases, and this warrants further research. Several studies have shown that the plasma homocysteine levels alter in human disorders such as renal disease (24), Behçet disease (BD) (25), retinal vein thrombosis (26,27) and early coronary artery disease (28). Also, it has been shown that various factors such as smoking (29), genetic and lifestyle (30) can have an effect.

Acknowledgement

We are thankful to patients for their cooperation.

References

1. Quigley HA. Number of people with glaucoma worldwide. Br J Ophthalmol 1996;80(5):389-93.
2. Quigley HA, Broman AT. The number of people with glaucoma worldwide in 2010 and 2020. Br J Ophthalmol 2006;90(3):262-7.
3. Osborne NN, Wood JP, Chidlow G, Bae JH, Melena J, Nash MS. Ganglion cell death in glaucoma: what do we really know? Br J Ophthalmol 1999;83(8):980-6.
4. Quigley HA, Green WR. The histology of human glaucoma cupping and optic nerve damage: clinicopathologic correlation in 21 eyes. Ophthalmology 1979;86(10):1803-30.
5. Lipton SA, Kim WK, Choi YB, Kumar S, D'Emilia DM, Rayudu PV, Arnelle DR, Stamler JS.. Neurotoxicity associated with dual actions of homocysteine at the N-methyl-D-aspartate receptor. Proc Natl Acad Sci U S A 1997;94(11):5923-8.
6. Miner SE, Evrovski J, Cole DE. Clinical chemistry and molecular biology of homocysteine metabolism: an update. Clin Biochem 1997;30(3):189-201.
7. Christensen B, Frosst P, Lussier-Cacan S, Selhub J, Goyette P, Rosenblatt DS, Genest J Jr, Rozen R. Correlation of a common mutation in the methylenetetrahydrofolate reductase gene with plasma homocysteine in patients with premature coronary artery disease. Arterioscler Thromb Vasc Biol 1997;17(3):569-73.
8. Ma J, Stampfer MJ, Hennekens CH, Frosst P, Selhub J, Horsford J, Malinow MR, Willett WC, Rozen R. Methylenetetrahydrofolate reductase polymorphism, plasma folate, homocysteine, and risk of myocardial infarction in US physicians. Circulation 1996;94(10):2410-6.
9. Franco RF, Araujo AG, Guerreiro JF, Elion J, Zago MA. Analysis of the 677 C-->T mutation of the methylenetetrahydrofolate reductase gene in different ethnic groups. Thromb Haemost 1998;79(1):119-21.
10. Pepe G, Camacho Vanegas O, Giusti B, Brunelli T, Marcucci R, Attanasio M, Rickards O, De Stefano GF, Prisco D, Gensini GF, Abbate R. Heterogeneity in world distribution of the thermolabile C677T mutation in 5,10-methylenetetrahydrofolate reductase. Am J Hum Genet 1998;63(3):917-20.
11. Moore P, El-sherbeny A, Roon P, Schoenlein PV, Ganapathy V, Smith SB. Apoptotic cell death in the mouse retinal ganglion cell layer is induced in vivo by the excitatory amino acid homocysteine. Exp Eye Res 2001;73(1):45-57.
12. Leibovitch I, Kurtz S, Shemesh G, Goldstein M, Sela BA, Lazar M, Loewenstein A. Hyperhomocystinemia in pseudoexfoliation glaucoma. J Glaucoma 2003;12(1):36-9.
13. Schlötzer-Schrehardt U, Lommatzsch J, Küchle M, Konstas AG, Naumann GO. Matrix metalloproteinases and their inhibitors in aqueous humor of patients with pseudoexfoliation syndrome/glaucoma and primary open-angle glaucoma. Invest Ophthalmol Vis Sci 2003;44(3):1117-25.
14. Chango A, Potier De Courcy G, Boisson F, Guilland JC, Barbé F, Perrin MO, Christidès JP, Rabhi K, Pfister M, Galan P, Hercberg S, Nicolas JP. 5,10-methylenetetrahydrofolate reductase common mutations, folate status and plasma homocysteine in healthy French adults of the Supplementation en Vitamines et Mineraux Antioxydants (SU.VI.MAX) cohort. Br J Nutr 2000;84(6):891-6.
15. Goyette P, Frosst P, Rosenblatt DS, Rozen R. Seven novel mutations in the methylenetetrahydrofolate reductase gene and genotype/phenotype correlations in severe methylenetetrahydrofolate reductase deficiency. Am J Hum Genet 1995;56(5):1052-9.
16. Jünemann AG, von Ahsen N, Reulbach U, Roedl J, Bönsch D, Kornhuber J, Kruse FE, Bleich S. C677T variant in the methylentetrahydrofolate reductase gene is a genetic risk factor for primary open-angle glaucoma. Am J Ophthalmol 2005;139(4):721-3.
17. Turacli ME, Tekeli O, Ozdemir F, Akar N. Methylene-tetrahydrofolate reductase 677 C-T and homocysteine levels in Turkish patients with pseudoexfoliation. Clin Experiment Ophthalmol 2005;33(5):505-8.

18. Fingert JH, Kwon YH, Moore PA, Johnston RM, Kim KY, Sheffield VC, Alward WL, Stone EM. The C677T variant in the methylenetetrahydrofolate reductase gene is not associated with disease in cohorts of pseudoexfoliation glaucoma and primary open-angle glaucoma patients from Iowa. Ophthalmic Genet 2006;27(2):39-41.
19. Mossbock G, Weger M, Faschinger C, Steinbrugger I, Temmel W, Schmut O, Renner W, Hufnagel C, Stanger O. Methylenetetrahydrofolatereductase (MTHFR) 677C>T polymorphism and open angle glaucoma. Mol Vis 2006;12:356-9.
20. Mabuchi F, Tang S, Kashiwagi K, Yamagata Z, Iijima H, Tsukahara S. Methylenetetrahydrofolate reductase gene polymorphisms c.677C/T and c.1298A/C are not associated with open angle glaucoma Mol Vis 2006;12:735-9.
21. Clement CI, Goldberg I, Healey PR, Graham SL. Plasma homocysteine, MTHFR gene mutation, and open-angle glaucoma. J Glaucoma 2009;18(1):73-8.
22. Fan BJ, Chen T, Grosskreutz C, Pasquale L, Rhee D, DelBono E, Haines JL, Wiggs JL. Lack of association of polymorphisms in homocysteine metabolism genes with pseudoexfoliation syndrome and glaucoma. Mol Vis 2008;14:2484-91.
23. Micheal S, Qamar R, Akhtar F, Khan MI, Khan WA, Ahmed A. MTHFR gene C677T and A1298C polymorphisms and homocysteine levels in primary open angle and primary closed angle glaucoma. Mol Vis 2009;15:2268-78.
24. Ferechide D, Radulescu D. Hyperhomocysteinemia in renal diseases. J Med Life 2009;2(1):53-9.
25. La Regina M, Orlandini F, Prisco D, Dentali F. Homocysteine in vascular behcet disease: a meta-analysis. Arterioscler Thromb Vasc Biol 2010;30(10):2067-74.
26. Sottilotta G, Oriana V, Latella C, Luise F, Piromalli A, Ramirez F, Mammi C, Occhiuto A, Lombardo VT. Role of hyperhomocystinemia in retinal vascular occlusive disease. Clin Appl Thromb Hemost 2007;13(1):104-7.
27. Sottilotta G, Siboni SM, Latella C, Oriana V, Romeo E, Santoro R, Consonni D, Trapani Lombardo V. Hyperhomocysteinemia and C677T MTHFR genotype in patients with retinal vein thrombosis. Clin Appl Thromb Hemost 2010;16(5):549-53.
28. Blacher J, Montalescot G, Ankri A, Chadefaux-Vekemans B, Benzidia R, Grosgogeat Y, Kamoun P, Thomas D. Hyperhomocysteinemia in coronary artery diseases. Apropos of a study on 102 patients. Arch Mal Coeur Vaiss 1996;89(10):1241-6.
29. Marszall ML, Makarowski R, Hinc S, Klos M, Czarnowski W. Hiperhomocysteinemia in active and passive smokers and the levels of folate and vitamin B6 in plasma. Przegl Lek 2008;65(10):486-90.
30. Summers CM, Mitchell LE, Stanislawska-Sachadyn A, Baido SF, Blair IA, Von Feldt JM, Whitehead AS. Genetic and lifestyle variables associated with homocysteine concentrations and the distribution of folate derivatives in healthy premenopausal women. Birth Defects Res A Clin Mol Teratol 2010;88(8):679-88.