

## GENETIC ANALYSIS OF PATIENTS WITH PRIMARY OPEN ANGLE GLAUCOMA (POAG) IN NORTH INDIAN POPULATION

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### ABSTRACT

**Aim:** To investigate the role of the methylenetetrahydrofolate reductase (MTHFR) 677C>T polymorphism in a large cohort of patients with POAG. **Methods:** genetic analysis of 80 patients of POAG and 80 controls was done for MTHFR 677C>T polymorphism using PCR. **Results:** The C677T genotype is found to be very significantly associated with POAG) as compared to the controls. There is a significant difference in the frequency of distribution of genotype. The homozygous mutant genotype (TT) is also 1.25% more in cases as compared to controls.

**Conclusion:** There is significant association of MTHFR 677C>T polymorphism with POAG.

**Key words:** Glaucoma, Intraocular pressure, Primary open angle glaucoma

### Introduction

Primary open angle glaucoma: Primary open angle glaucoma (POAG) is the most common variant of glaucoma comprising nearly half of the estimated 67 million people with glaucoma worldwide. POAG is one of the leading causes of irreversible blindness in the world. Worldwide POAG is the most common form of glaucoma and about 1.5 million people are blind due to glaucoma. Primary open angle glaucoma (POAG) is a complex inherited disease that is likely to result from defects in multiple susceptibility genes as well as environmental factors. The disease causes a characteristic degeneration of the optic nerve that is frequently associated with elevated intraocular pressure (high-tension

glaucoma) but can occur even if the intraocular pressure is low (low tension glaucoma) or normal (normal-tension glaucoma). The manifestation of this group of eye conditions could start at birth or may appear in later age, depending on the type of glaucoma present in an individual. Juvenile onset open angle glaucoma (JOAG), a form of POAG may manifest clinically between the ages of 3 and 30. The late onset form of this condition usually manifests clinically before the age of 40 and is the most prevalent type. Besides, differences in age of onset, there are other features that may help differentiate between these two subgroups of POAG. The disease is more severe in JOAG and subjects with significantly higher intraocular pressures (IOP) may be more

refractory to treatment with medicines. In contrast, the late onset form usually has a milder presentation with progressive development, moderate elevation of IOP and satisfactory outcomes with medical treatment.

### **Current understanding of pathology of POAG:**

POAG is typically diagnosed by the presence of the "classical triad": characteristic pattern of visual field defects, morphological loss of optic disc substance and increased IOP. Central visual acuity is relatively resistant to glaucomatous damage in the initial stages of the disease, with the peripheral vision mainly affected. Selective perimetry and visual field testing can effectively identify changes to the peripheral field. Examination of the optic disc is perhaps the most valuable method in early diagnosis, characterized by a concomitant decrease in the width of the neuroretinal rim and enlargement of the optic disc cup. Increased IOP of greater than 21 mm Hg (mean IOP 15-21 mm Hg of adult population) is not required for diagnosis, though it has traditionally been suspected to cause glaucoma. Patients with POAG typically exhibit increased resistance to the outflow of aqueous humor through the trabecular meshwork, which can result in an increase in IOP and subsequent cell death from compression of the optic nerve axons.

### **Risk Factors:**

Risk factors are clinically useful to assess the risk of glaucoma based on unique characteristics of the patients. These may be general risk factors (age, race, and family history), ocular risk factors (Intra ocular pressure, optic nerve head features, Myopia), systemic risk factors (Diabetes mellitus, Hypertension) and

drugs (steroids). Other mechanisms, besides the increase in IOP, have been associated with degeneration of Retinal Ganglion Cells (RGCs). These include ischemia, glutamate excitotoxic stress, high reactive oxygen species production, and the loss of mitochondrial function. Recent mechanistic studies have focused on immunological changes during glaucomatous pathogenesis and possible preventive therapies.

### **Genetic association with polymorphism in MTHFR gene:**

A lot of work has been done to find genetic association of POAG worldwide. In Indian population studies are being carried out mainly on South Indian population as indicated by following evidences. Many things are still to be investigated. Kumar et. al., 2007<sup>1</sup> first reported the association of MYOC, OPTN, CYP1B1, OPTC genes with POAG in South Indian population. This is the first report to document the involvement of the CYP1B1, MYOC, and OPTN genes in the etiology of POAG in the same set of Indian patients. Their study shows that mutations in these genes are rare in Indian population. Ray et. al., 2007<sup>2</sup> identified a novel mutation in MYOC (Gly399 Asp) which is associated with POAG in Indian population. Sripriya et. al., 2006<sup>3</sup> have reported the association of OPTN gene polymorphism in patients with POAG. Rose et. al., 2007<sup>4</sup> have reported some novel polymorphism in MYOC gene in POAG patients from South Indian population. Association of any of the MTHFR gene polymorphism with Primary Open Angle Glaucoma has not yet been studied in Indian population. Although some evidences are present for association studies on Caucasian population and Pakistani cohorts. Hyperhomocysteinemia (hyperHcy) has been shown to promote

apoptosis of RGCs<sup>5</sup>, which in turn is a result of reduction in the activity of an enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR, EC 1.5.1.20). MTHFR is a fundamental enzyme involved in metabolism of homocysteine (Hcy) and folate. It catalyzes reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which acts as methyl donor for remethylation of Hcy to methionine. It is a crucial enzyme in this pathway that diverts folate content towards Hcyremethylation, rather than its consumption in DNA and RNA biosynthesis<sup>6</sup>. The C677T SNP in the MTHFR gene, known as rs1801133, results in an alanine to valine change at position 222 of the enzyme, which is well known to cause reduction in enzymatic activity of MTHFR and hence elevation in plasma homocysteine levels<sup>7</sup>.

### **Aim of study:**

MTHFR i.e. Methylene Tetra Hydro Folate Reductase gene (NM-005957) has been selected for this study. This study was designed to investigate the role of the MTHFR 677C>T polymorphism in a large cohort of patients with POAG. So the basic aim was to check whether this polymorphism of MTHFR gene shows any association with Primary Open Angle Glaucoma in North Indian Population.

### **Material and Methods:**

In this case-controls study a total of 80 patients with POAG and 80 controls have been investigated at Department of Ophthalmology, Institute of Medical Sciences, Banaras Hindu University, in collaboration with Department of Molecular & Human Genetics, Faculty of Science, Banaras Hindu University, Varanasi. The study was approved by the Institutional ethical committee.

### **[a] Inclusion criteria:**

1. AnIOP before initiation of a pressure lowering therapy of at least 21 mm Hg.
2. An open anterior chamber angle (Grade 3 and above by Shaffer's Classification).
3. Optic disc changes characteristic for glaucoma (notching, thinning of Neuro Retinal Rim, increased C/D ratio) with a C/D ratio of  $\geq 0.8$  in at least one eye.

### **[b] Exclusion criteria:**

1. Age<40
2. Other ocular disorders like uveitis, retinal detachment, retinal vascular disorders etc.
3. Patients with Diabetes, Hypertension or with any other chronic disorders would be excluded from the study.
4. Other forms of glaucoma like pigmentary glaucoma, pseudo-exfoliation syndrome, steroid induced glaucoma etc.

### **Clinical investigation:**

Medical history concerning arterial hypertension, diabetes mellitus, cardiovascular events and recent medication was obtained from all patients. All participants were from same geographical area (north India). All patients were thoroughly examined using slit lamp biomicroscopy using 78D or 90 D lenses. Intra Ocular Pressure (IOP) was measured by Goldman applanation tonometer; angle structures were assessed and graded by using Goldman single mirror gonioscope and visual field examination (automated perimetry by Humphrey field analyser) was done in patients wherever possible. Control subjects consisted of 80 unrelated subjects with no morphological or functional

damage indicative of Primary or Secondary Open Angle Glaucoma. Control subjects were admitted to the department for cataract surgery.

Peripheral blood samples (4-5 ml) of patients and controls were received in heparinized syringes. Samples were immediately stored at 4°C until the extraction of genomic DNA. In order to make the groups maximally comparable controls were matched to cases by age, gender, concurrent diagnosis of hypertension, smoking and diabetes. POAG patients had a mean age of 49.625. Control samples had a mean age of 56.825. There is no significant difference between mean age of two groups.

#### **Protocol for genomic DNA isolation from peripheral Blood:**

Following protocol was used for DNA extraction from peripheral blood leukocytes using 5 ml of whole blood.

##### **Solution A:**

- Add 109.59 of sucrose in 985 ml of H<sub>2</sub>O.
- Autoclave at 15 psi at 121°C for 20 mm.
- Add 5 ml of 1 M MgCl<sub>2</sub> and 10 ml of 100X TritonX.
- Mix well, aliquot and use.
- Store at 4°C.

##### **Solution B :**

- 1M Tris-CI (pH-8.0) 40 ml
- 0.5M EDTA (pH-8.0) 12 ml
- 1M NaCl 15 ml

- Deionized H<sub>2</sub>O 33 ml

##### **Solution C (5M Sodium per chlorate):**

- Add 35.115 g of Sodium per chlorate to 50 ml of H<sub>2</sub>O.
- Autoclave at 15psi, at 121°C, for 20 mm.
- Store at room temperature.

##### **Procedure:**

In 50 ml polypropylene tube, 3-5 ml of blood sample taken, added with 15 ml (3-5 vol. of blood) of 0.9% NaCl and mixed well. This was centrifuged at 6000rpm for 6 mm at 25°C. Supernatant was discarded and the pellet (red) was saved. 15 ml of Solution A (3-4 vol of blood) was mixed and pellets were dispersed and mixed. This was centrifuged at 6000rpm for 6mm at 25°C. Again white pellets were saved and supernatant discarded. To the pellets, 2ml of solution B was added and mixed gently. 2 ml of chilled chloroform was added, and mix gently, then centrifuged at 6000rpm for 6mm at 25°C. Aqueous layer was taken out with the sterile cut tip in a 15 ml polypropylene tube and mixed with 2 vol of Iso-propanol or 2.5 vol of chilled Ethanol to precipitate the DNA. Precipitated DNA was taken out with sterile cut tip in a micro centrifuge tube. 1 ml of 70% ethanol was added for washing and then alcohol was discarded and dried. 150 µl of TE buffer added further and kept in 37°C overnight for dissolving the genomic DNA and stored at 4°C.

### Polymerase Chain Reaction:

The desired region (Chr 1: 11778844-11778989) was amplified using following set of primers. The genomic region amplified was of 146 bp in length.

C677TFpS'TGAAGGAGMGGTGTCTGCGGGA3'

C677T Rp 5'CCTCACCTGGATGGGAAAGATCC3'

### Sequence of amplified region-

5'TGAAGGAGAAGGTGTCTGCGGGAGCCGA  
TTTCATCATCACGCAGCTTTTCTTTGAGGCT  
GACACATTCTCCGCTTTGTGAAGGCATGC  
ACCGACATGGGCATCACTTGCCCCATCGTC  
CCCGGATCTTTCCCATCCAGGTGAGG 3'

The PCR amplification was carried out in 25 µl of reaction volume by using 100ng/µl (1µl) genomic DNA for each sample in GeneAmp PCR System 9700 of Applied Biosystem. The PCR mix consisted of 10X Taq buffer (2.5µl), 25mM MgCl<sub>2</sub> (1.5µl), 1.25mM dNTP mix (4µl), 1µl of 10 pmole primers (forward and reverse each

### Observation and Results:

After PCR amplification, amplified product was analysed on 2% agarose gel and PCR product of 146bp as expected was obtained.

and 0.5 Unit of Taq polymerase. PCR was carried out at pre standardized conditions: denaturation at 94°C for 5 min, and 94°C for 1 min, followed by annealing at 62°C for 1 min, and then extension at 72 °C for 1 min, repeated for 35 cycles followed by a final extension step at 72 °C for 3 min.

### Agarose Gel Electrophoresis:

The PCR products (5µl) were checked in 2% Agarose gel containing ethidium bromide 0.5µg/ml. Amplified products were stored at -20°C till further procedure. Agarose gel mixture (2% Agarose, 1X TAE and 4µl/100ml ethidium bromide) was poured in gel casting tray fitted with a comb. It was allowed to set for approximately 30 minutes at room temperature. The comb was removed. The gel was put into running buffer and 5µl DNA samples with loading dye were loaded in each well. The PCR samples were run at 100 Volt for 10-20 minutes. After run, the gel was visualized in Gel DOCTM XR+ Imaging system of BIO-RAD.



Gel picture showing 146 bps amplified PCR product. No amplification in G25. TT +ve control, homozygous for mutant allele.

**RFLP Results:**

The amplified product with mutant allele had unique restriction site for the enzyme Hinf I. As result, after restriction digestion, homozygous for wild allele represented a single fragment of 146 bps, and the homozygous for mutant allele produced 2 fragments of 124 bps, and 22 bps. Consequently the heterozygous state produced 3 bands of 146 bps, 124 bps and 22 bps (which is too short to be visualized by agarose gel electrophoresis).



Gel picture of RFLP showing wild type/ normal(CC), homozygous mutant(TT) and heterozygous (CT) allele of Hinf I polymorphism of MTHFR.

**Demographic Data:**

The demographic data like number of patients mean age, male: female ratio was shown in table 1. The table 2 presents genotype distribution and frequency of the MTHFR 677T allele in patients with POAG and control subjects.

**Table -1:** Demographic data of cases and controls

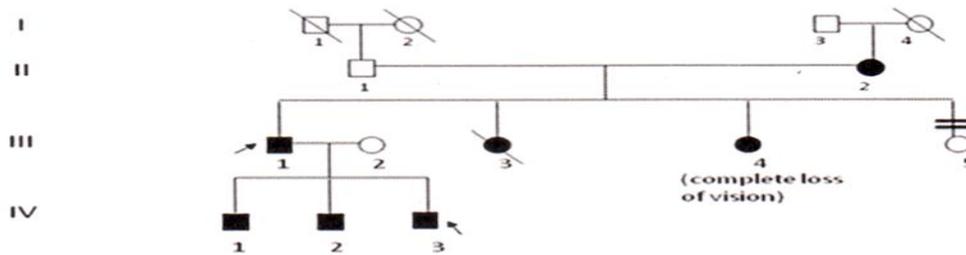
	<b>Patients with POAG</b>	<b>Control Subjects</b>
No of patients	80	80
Age (years)	49.625±17.419	56.825±13.326
Mean ± SD range	80-22	80-30
Gender (Male : Female)	1.05:1	1:1.05

**Table- 2:** MTHFR C677T genotypes in cases and controls

<b>GENOTYPE</b>	<b>CASES N = 80</b>	<b>CONTROLSN = 80</b>
677 CC	30 (37.5%)	49 (61.25%)
677 CT	48 (60.0%)	30 (37.5%)
677TT	02 (2.5%)	01(1.25%)
	$\chi^2= 9.05; df=1; p=0.01$	-----
CT VS CC OR = 95% CI=	2.55(1.37-4.76)	-----

**Pedigree of families with multiple afflicted:** In this study we have also found two families with significant familial history of Primary open angle glaucoma. We have done the mutational analysis of only some members of the family due to the inability to get the blood sample of the whole family.

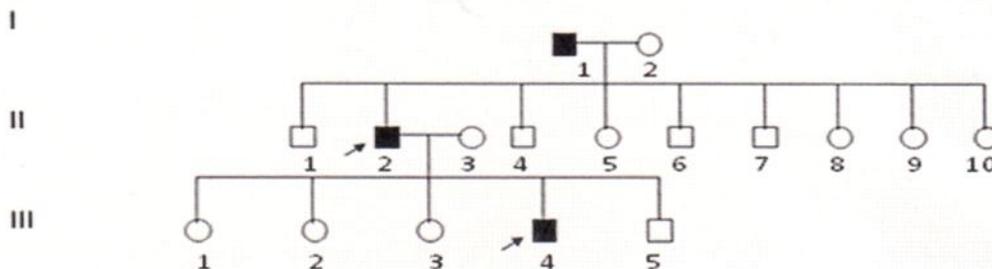
**Pedigree 1:**



III.1  
 IOP R/E-42mm of Hg  
 L/E-42mm of Hg  
 C/D ratio R/E-0.8  
 L/E-0.9

IV.3  
 R/E-42mm of Hg  
 L/E-42mm of Hg  
 R/E-0.6  
 L/E-0.6

**Pedigree 2:**



II.2  
 IOP R/E-24mm of Hg  
 L/E-21mm of Hg  
 C/D ratio R/E-0.9  
 L/E-0.9  
 Other finding Neuro Retinal Rim  
 Very thin

III.4  
 R/E-24mm of Hg  
 L/E-21mm of Hg  
 R/E-0.4  
 L/E-0.5  
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**Discussion:**

In these two familial cases we have got samples of two members only including patient. All 4 probands have CT genotype as shown in pedigree 1 and pedigree 2. This is a strong indication towards the association of T allele with POAG phenotype as our statistical analysis also shows similar result.

MTHFR gene (1p36.3) is an important gene of around 20.329kb in length, encoding for a 656 AA long polypeptide, Methylene Tetra Hydro Folate Reductase which acts as an important enzyme for homocysteine (Hcy) metabolism. Homocysteine is metabolized by two pathways- one is remethylation and second

one is trans-sulfuration. In remethylation, homocysteine acquires a methyl group from N-5-methyltetrahydrofolate or from betaine to form methionine. In the transsulfuration pathway, Hcy is converted to cystathionine by cystathionine  $\beta$ -synthase and finally to cysteine using vitamin- B6 as a cofactor. (Selhub et.al., 1999)<sup>8</sup>. Mutation in MTHFR may lead to reduced activity of Methylene Tetra Hydro Folate Reductase enzyme which ultimately leads to the accumulation of Hcy in blood (hyperhomocysteinemia). Accumulated Hcy has many implications. It is generally held that different mechanisms are responsible for arterial and venous thromboembolic events, and that these mechanisms involve platelet function abnormalities in arterial thrombosis and abnormalities of coagulation and/or fibrinolysis in venous thromboembolism. Increased level of Plasma Hcy leads to neuronal cell death by different apoptotic and excitotoxic mechanism under various conditions. (Lipton et.al.,1997)<sup>9</sup>. It has also been reported that elevated Hcy induces apoptotic cell death in Retinal Ganglion Cells (RGC) by overstimulation of NMDA receptors and caspase-3 activation (Moore et.al. 2001)<sup>10</sup>. Genetic polymorphism in the MTHFR gene is very well established. The most extensively studied are C677T and A1298C single nucleotide polymorphism (SNPs). Kanget.al., 1991<sup>11</sup> identified a thermo labile variant of MTHFR characterized by a C>T substitution at a nucleotide 677 and is associated with elevated plasma homocysteine levels. This SNP results in a missense mutation leading to the substitution of valine for alanine at position 222 of MTHFR enzyme, causing the synthesis of a thermo labile enzyme with a 50% reduction in activity. (Frosst et.al. 1995)<sup>12</sup>. The A1298C polymorphism is located within the COOH terminal regulatory domain of the MTHFR. It results

in the substitution of glutamate for an alanine residue and has also been associated with a mild reduction in enzymatic activity. Bleich and coworkers found a raised plasma homocysteine level and C677T polymorphism in Caucasian glaucoma patients. This was the first study to provide evidence of association of MTHFR C677T polymorphism with Hcy level and open-angle glaucoma. Junemann.et.al, 2002 also reported increased frequency of the C677T polymorphism of MTHFR in patients with Primary Open Angle glaucoma. Michael.S, et.al 2008<sup>13</sup> has checked the association of A1298C polymorphism with POAG.

Previous studies documented in literature have been performed on PACG (Primary Angle Closure Glaucoma), PEX (Pseudo Exfoliation Syndrome), and NTG (Normal Tension Glaucoma). But the actual association still remains controversial. Ethnic differences appear to be the main governing factor behind the varying MTHFR genotype distribution and allele frequencies reported.

However, to date only Junemann et. al. 2002 have reported a positive association of MTHFR with POAG and they investigated a sample size with 76 patients and 71 controls. Study by Bleich and Junemann, 2002 provides the first evidence that homocysteine levels are elevated in patients suffering from POAG. They found that C677T genotype was significantly associated with PACG (CC 69%, CT 21%, TT 10%;  $p=0.001$ ,  $\chi^2=12.6$ ), but not with POAG (CC 71%, CT 28%, TT 1%;  $p=0.98$ ,  $\chi^2=0.02$ ) as compared to the controls (CC 71%, CT 29%, TT 1%). In our study we have investigated a sample size with 80 patients and 80 controls from same geographical area in POAG patients. We have found a very significantly associated with POAG (CC 37.5%, CT 60.0%, & H

2.5%;  $p = 0.01$   $\chi^2 = 9.05$ ) as compared to the controls (CC 61.25 % CT 37.5%, TT 1.25%) in north Indian population.

Recently, Zefterberg et al. also documented no significant differences between the control and the POAG group for the MTHFR 677T allele frequency or for the homozygous MTHFR 677TT genotype. In the study conducted by Mabuchi et al. 2006, genotype distribution of homozygosity (TT) and heterozygosity (CT) was 20.3% and 41.4%, respectively, for Japanese PACG patients. Michael et al., 2007, studied the association of C677T polymorphism with PACG and POAG. They found that prevalence of the MTHFR C/T genotype was 22.2% in POAG, 13.3% in PACG, and 18.6% in controls whereas the MTHFR T/T genotype was present solely in the PACG group (6.9%). The difference in the T/T genotype between PACG and controls was statistically significant ( $p < 0.01$ ). They concluded that MTHFR C677T polymorphism was found to be associated with PACG but not POAG in patients of Pakistani origin.

Micheal et al, 2009<sup>13</sup> have studied the methylenetetrahydrofolate reductase (MTHFR) C677T and A1298C genotypes and plasma concentrations of total homocysteine (tHcy) in Pakistani patients with primary open angle glaucoma (POAG) and primary angle closure glaucoma (PACG). This was a prospective case-control study. A total of 295 patients (173 POAG, 122 PACG) and 143 age- and sex matched controls were subdivided into two ethnic groups, Punjabis (Punjab province, central Pakistan) and Pathans (North-West Frontier Province, northern Pakistan). Genotypes of the MTHFR C677T and A1298C polymorphisms were detected by polymerase chain reaction—restriction fragment length polymorphism (PCR-RFLP). An enzyme-linked immunosorbent assay

was used to determine the total serum homocysteine (tHcy) levels. Associations were determined by logistic regression analysis. Frequency distributions of genotypes and combined genotypes as well as homocysteine levels were obtained. The overall distribution of the C677T genotype was found to be significantly associated with PACG (CC 69%, CT 21%, TT 10%;  $p = 0.001$ ,  $\chi^2 = 12.6$ ), but not with POAG (CC 71%, CT 28%, TT 1%;  $p = 0.98$ ,  $\chi^2 = 0.02$ ) as compared to the controls (CC 71%, CT 29%, TT 1%). The Pathan cohorts revealed no association with the disease; however, the Punjabis demonstrated a significant association with PACG (CC 75%, CT 11%, TT 13%;  $p < 0.001$ ,  $\chi^2 = 17.2$ ). PACG in the Punjabi subjects was also significantly associated with the A1298C polymorphism (AA 43%, AC 54%, CC 3%;  $p < 0.001$ ,  $\chi^2 = 33.9$ ) as compared to the controls. Combined genotype data showed no association with POAG; however, a significant association with all combined genotypes was observed in the overall PACG subjects ( $p < 0.05$ ,  $\chi^2 = 20.1$ ). This difference was particularly apparent in the TTAA and TTAC combinations that were completely absent in the control groups ( $p < 0.05$ ,  $\chi^2 = 49.6$ ). Mean serum tHcy levels were found to be significantly increased in the POAG ( $15.2 \pm 1.28$   $\mu\text{mol/l}$ ,  $p < 0.001$ ) and PACG ( $20.8 \pm 4.8$   $\mu\text{mol/l}$ ) groups as compared to the controls ( $10.0 \pm 0.97$   $\mu\text{mol/l}$ ). The tHcy levels in the TT and AC genotype were significantly elevated in the PACG group ( $67 \pm 12.39$   $\mu\text{mol/l}$ ,  $p < 0.001$ ;  $23 \pm 5.94$   $\mu\text{mol/l}$ ,  $p = 0.027$ ) as compared to the controls. The TT and AC genotypes of MTHFR C677T and A1298C polymorphisms and the combined genotype TTAC were associated with PACG in Punjabi subjects of Pakistani origin and correlated with the high serum tHcy levels seen in these patients.

To date largest study on association of this polymorphism with POAG was done by Mossbock et al. 2006. This case-control study included a total of 553 participants comprising 204 patients with POAG, 138 patients with PEXG, and 211 control subjects. Genotyping for the MTHFR 677C>T polymorphism was performed by polymerase chain reaction (PCR). No significant difference in the genotype distribution of the MTHFR 677C>T polymorphism was found between control subjects and patients with POAG or PEXG. The prevalence of the MTHFR 677TT genotype was 6.9% in patients with POAG, 11.6% in patients with PEXG, and 9.5% in control subjects. The present data suggested that the MTHFR 677C>T polymorphism itself is not a major genetic risk factor for POAG and PEXG in a central European population.

In our case control study we have started with 80 cases and 80 age matched controls from Eastern UP and Bihar. A significant association is found in this population. The C677T genotype is found to be very significantly associated with POAG (CC 37.5%, CT 60.0%, & TT 2.5%;  $p=0.01$   $\chi^2=9.05$ ) as compared to the controls (CC 61.25 % CT 37.5%, TT 1.25%). There is a significant difference in the frequency of distribution of genotype. The homozygous mutant genotype (TT) is also 1.25% more in cases as compared to controls. A Measurement of homocysteine level in

patient would have given some satisfactory results in this retrospective study.

### Conclusion:

A number of polymorphisms in different genes such as -1000 G>C in MYOC, the E allele in APOE, and the Arg72Pro polymorphism in p53 have been claimed to have significant association with POAG. This might be dependent on the populations under study. From that perspective we examined the association of the SNP (C677T) with POAG and found a significant association with the disease ( $p=0.01$ , table 2). It is, however possible that the identified SNPs might have potential use in the future for monitoring variable penetrance in familial POAG where MTHFR might act as a modifier gene, and/or efficacy of potential drugs to treat the disease.

This study is the first to report an association of the CT genotype with POAG in northern India. The present investigation also provides evidence of the importance of conducting such studies in different ethnic groups, as even in closely living populations, there is clearly a difference in frequencies of genotype leading to different associations with disease.

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