ASSOCIATION OF *ENOS* POLYMORPHISMS WITH PRIMARY ANGLE-CLOSURE GLAUCOMA

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Abstract

Purpose: Recently, several studies have investigated genetic associations between *Cytochrome P450 (CYP1B1), Endothelial nitric oxide synthase* (*eNOS)* and *Neurotrophin-4 (NTF4*) with primary angle-closure glaucoma (PACG) in various ethnic groups. Here we investigate the association of these candidate genes with PACG in samples from Australia and Nepal.

Method: A total of 235 patients with PACG (106 Nepalese and 129 Australian) and 492 controls (204 Nepalese and 288 Australian) were included. Tag single nucleotide polymorphisms (SNPs) were selected to cover the majority of common variation within the candidate genes and genotyped in DNA extracted from peripheral whole blood. Allele and haplotype analyses were conducted in PLINK. Bonferroni correction was applied for the total number of SNPs in this study (p=0.05/15=0.003)

Results: In the Australian cohort, one *eNOS* SNP rs3793342 shows significance association with PACG in the Australian cohort after Bonferroni correction (p-value 0.003, OR 0.5 95% CI 0.3-0.8). After adjusting the results for sex and age both SNPs rs3793342 and rs7830 showed significance after Bonferroni correction (p-value of 0.001 and 0.003, respectively). The *eNOS* haplotype of all 7 typed SNPs showed significant association with a global p-value of 0.019, with the CGCAATC haplotype giving a specific p-value of 0.008 and odds ratio of 1.5 (95% CI 0.9-2.4). In the Nepalese cohort, SNPs in *CYP1B1* and *NTF4* genes showed borderline association with PACG but did not survive Bonferroni correction.

Conclusions: The present data support the involvement of common variations in *eNOS* with PACG pathogenesis. Differences were observed in the two populations studied, and additional replication studies in other populations are necessary to confirm these associations

Introduction

Glaucoma is the leading cause of irreversible blindness worldwide [1] with primary open angle glaucoma (POAG) being the most prevalent subtype. However primary angle-closure glaucoma (PACG) is responsible for almost half of all glaucoma blindness. [2] Family history and ethnicity are important risk factors for PACG. The disease is more prevalent amongst Asians and Eskimos than in Caucasians and Africans. [3] In addition, first degree relatives of patients with PACG have a higher probability of developing narrow angles. [4, 5] The number of patients with PACG worldwide is expected to rise from around 16 million in 2010 to 21 million by the year 2020 [6] with the majority of bilaterally blind PACG patients expected to be of Asian ethnicity. [7]

Patients with PACG share certain anatomical biometric features including short anterior chamber depth with narrowing in the irido-corneal drainage angle, increased lens thickness and anterior apposition of the lens, short axial length and hyperopic (farsightedness) refractive error. [8]

The leading cause of PACG is from obstruction of the trabecular meshwork, which leads to the accumulation of aqueous humor and subsequent increase in intraocular pressure. This in turn causes progressive destruction of the optic nerve with corresponding loss of the peripheral visual field. [6]

PACG is a complex heterogeneous disease. Recently Vithana and colleagues conducted a large two-staged genome-wide association study and reported three susceptibility loci: rs11024102 in *PLEKHA7;* rs3753841 in *COL11A1*, and rs1015213 located between *PCMTD1* and *ST18*. [9] Prior to this, evaluation of PACG genetics

has been conducted through evaluation of candidate genes chosen for biological plausibility or association with similar phenotypes. Several genes have previously been reported to be associated with other subtypes of glaucoma. For example *Cytochrome P450* (*CYP1B1*) is well known to cause primary congenital glaucoma (PCG), [10, 11] while *Endothelial nitric oxidase synthase* (*eNOS*) [12, 13] and *Neurotrophin-4* (*NTF4*) have been implicated in primary open angle glaucoma (POAG). [14] As all three types of glaucoma (POAG, PCG and PACG) are characterised by destruction of the optic nerve and progressive increase in the cup:disc ratio, generally with elevated intraocular pressure, we hypothesised that variations within these POAG and PCG genes may also be associated with PACG.

CYP1B1 is the most common gene known to be involved with the pathogenesis of PCG. [15, 16] It accounts for around 20% of PCG cases in Australia. [17] Sarfarazi and colleagues [10] hypothesized that *CYP1B1* [OMIM 601771] is involved in the development of the anterior chamber angle of the eye, making it a gene of interest for PACG. Chakrabarti and colleagues [18] sequenced the coding region of the gene and found association of mutations with PACG in an Indian cohort consisting of 90 cases and 200 controls. A smaller study of 29 PACG patients from the Middle East did not report any known or novel polymorphisms in *CYP1B1*, using direct sequencing. [19]

Nitric oxide (NO) is synthesized in the vascular endothelium by endothelial nitric oxide synthase 3 (also known as *eNOS*), from the substrate L-arginine. [20] *eNOS* [OMIM 163729] over expression is thought to be neuroprotective by causing vasodilation and increased blood flow in human eye tissues. [21] Overexpression of *eNOS* was also reported to lower intraocular pressure in the mouse eye by increasing

the pressure dependent drainage. [22] Other factors such as asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) play an inhibitory role in the production of NO. Our laboratory found that serum levels of both ADMA and SDMA are significantly elevated in patients with advanced POAG suggesting dysregulation of the NO system in this disease. [23]

Furthermore NO enhances the activity of *Matrix metalloprotinase-9* (*MMP9)* [24] which has also been reported to be associated with PACG. [25, 26] Alteration in *MMP9* activity during eye development may lead to hyperopic refractive error which is a risk factor for PACG. [26, 27] It has been proposed that alteration of *eNOS* expression causes impairment of blood flow and subsequent development of angle-closure. The 27-bp variable number of tandem repeat (VNTR) polymorphism in intron 4 of *eNOS* is believed to alter the production of nitric oxide and cause vascular deregulation. [28] This variation was found to be associated with PACG in Pakistani cohorts. [29] A study of Han Chinese used tag SNPs to identify association of common variants in the *eNOS* gene in 88 patients with PACG, but no associations were observed. [30]

Heterozygous mutations in *NTF4* [OMIM 162662] were recently reported to be responsible for 1.7% of POAG cases of European descent. [14] Neurotrophin plays a vital role in the neuronal cell development, survival and differentiation and it was suggested that the pathway may also prevent neuronal damage in retinal ganglion cells. [14] Two studies have looked for association with PACG in Indian [31] and Caucasian European [32] populations respectively by sequencing the coding exons, but failed to detect association.

Thus, although these three genes are plausible candidates, results are inconsistent between published studies and even less so across ethnicities. Thus, in this study, we aimed to investigate the association between tag SNPs in these three genes and PACG in both Australian and Nepalese cohorts in order to determine if common variation in these genes could contribute to disease in these populations.

Methods

Australian Caucasian participants were recruited from Ophthalmology clinics in Australia through the Australian and New Zealand Registry of Advanced Glaucoma. [33] Approval was obtained from the human research ethics committee of the Southern Adelaide Health Service and Flinders University. All participants were self-reported Caucasian. The Nepalese cohort was recruited from the Nepal Glaucoma Eye Clinic, Tilganga Institute of Ophthalmology, Kathmandu Nepal by one of the authors (S.S.T). The study was approved by the Institutional Review Committee of the Tilganga Institute of Ophthalmology (TIO). All participants were from Nepal and detailed ethnic group information was collected. [34, 35] This study has been conducted in accordance with the Declaration of Helsinki and its subsequent revisions. Informed consent was obtained from each individual.

Following the International Society of Geographical and Epidemiological Ophthalmology (ISGEO) [36] diagnosis of PACG was based on the presence of glaucomatous optic neuropathy with cup:disc ratio≥ 0.7, intraocular pressure more than 21 mmHg, peripheral visual loss, presence of at least 180 degrees of closed angle in which the trabecular meshwork is not visible on gonioscopy. In this study 129 Australian and 106 Nepalese affected participants were recruited, they were all identified with PACG.

Controls were required to have none of the above characteristics, with no known family history of glaucoma. Participants with pseudophakia or secondary angle-closure glaucoma caused by events such as uveitis, trauma or lens subluxation were excluded. The control groups consisted of 288 Australian and 204 Nepalese individuals. The Australian control cohort was ascertained from nursing home

facilities in Adelaide, South Australia. Nepalese controls were participants in a population based study of Kathmandu, Nepal, in which the individuals were chosen specifically to be matched for age, gender and ethnic group to the Nepalese cases [34]. Each participant underwent a complete eye examination including; slit lamp examination of the anterior chamber, gonioscopy, best corrected visual acuity, measurement of intraocular pressure, fundus examination with special attention to optic disc parameters, and visual field assessment. Refraction was carried out using a streak retinoscope (Beta 200, Heine, Germany), which was followed by a subjective refraction [34].

Genomic DNA was extracted from peripheral whole blood using the QiaAmp Blood Midi (Nepalese samples) or Maxi (Australian samples) Kit (Qiagen, Valencia, California).

15 Tag SNPs were selected using the tagger program implemented in Haploview 4.2 (http://www.broadinstitute.org/scientific-community/science/programs/medical-and-populationgenetics/haploview/haploview) to cover the majority of known genetic variation in and around the candidate genes (*CYP1B1* 5 SNPs, *eNOS* 7 SNPs, and *NTF4* 3 SNPs). For the Nepalese cohort, SNPs were selected from the HapMap (http://hapmap.ncbi.nlm.nih.gov/) Han Chinese in Beijing, China (CHB) sample as the most closely related population available at the time of the study. For SNP selection in the Australian cohort we used CEU: CEPH (Utah residents with ancestry from northern and western Europe). Tag SNPs were chosen using pairwise tagging, to have an $r^2>0.8$ with SNPs displaying a minor allele frequency of >5% in the relevant HapMap II population.

Genotyping was conducted using the iPLEX Gold chemistry (Sequenom Inc, San

Diego, California) on an Autoflex mass spectrometer (Sequenom Inc, San Diego, California) at the Australian Genome Research Facility (AGRF), Brisbane. All analyses were conducted using PLINK. [37] SNPs were assessed for compliance with Hardy-Weinberg equilibrium using the χ^2 test. Genetic association was assessed under an allelic model. Analysis is with respect to minor allele of the tag SNPs. P-values were adjusted for sex and age using logistic regression. A Bonferroni correction was applied to each p-value according to the number of SNPs typed in this study (corrected p-value; 0.05/15= 0.0033). Haplotype analyses were conducted in PLINK based on the observed linkage disequilibrium blocks, as visualised using the "solid spine" definition in Haploview. [38]

We further analysed the association between *eNOS* and *MMP9* with PACG in the Australian cohort by comparing the combined risk alleles with the protective one using chi-square test. The data for *MMP9* was that previously published on a subset of the Australian cohort. [25] We selected the significant SNP from each gene *eNOS* rs3793342 and *MMP9* rs17576.

The power of this study at α=0.05 was assessed using the Genetic Power Calculator. [39] The prevalence is similar in both Australian 0.4% [40] and Nepalese cohorts 0.43%. [41] Assuming complete linkage disequilibrium between the disease causing variant and the marker we will have a power of 89% to detect a genotypic relative risk of 1.3 with a risk allele frequency of 0.2 under an additive model.

Results

417 Australian and 310 Nepalese participants were enrolled in this study. All cases in the Nepalese cohort presented with PACG, of which 53 cases were reported to have

had an acute attack. In the Australian cohort 129 cases were identified with PACG (35 with previous history of acute attack). **Table 1** displays the demographic characteristics and clinical data of the cases and controls for each cohort. No SNP deviated from Hardy-Weinberg equilibrium in either cohort ($p>0.05$). The physical locations of the tag SNPs are presented in **Figure 1.**

The minor allele frequencies and allelic association p-values of typed SNPs in the Australian cohort are presented in **Table 2.** Three SNPs in *eNOS* were found to show significant association with PACG; rs3793342, T allele, with p-value of 0.003 (OR 0.5, 95%CI 0.3-0.8), rs3918188, A allele, with p-value of 0.014 (OR1.5, 95%CI 1.1-1.9), and rs7830, A allele, p-value=0.007 (OR 0.7, 95%CI 0.5-1.0). Only rs3793342 survived correction for multiple testing of 15 SNP (p-value 0.001). However, after justifying the Australian cohort for age and sex, both rs3793342 and rs7830 were significant with p-value of 0.001 and 0.003, respectively. One haplotype in the *eNOS* gene also showed association with PACG in the Australian cohort, with a global p value of 0.019. The CGCAATC haplotype conferred risk, with significant p-value of 0.009 (OR 1.5, 95%CI 0.9-2.4). This haplotype contains the risk alleles of all the three nominally associated SNPs (**Table 3**).

When we further analysed the association between *eNOS* and *MMP-9* with PACG in the Australian cohort, a significant difference in the rate of PACG was found between individuals carrying the risk alleles of both SNPs rs17576 in *MMP-9* and rs3793342 in *eNOS* (105 individuals) with those who do carry the protective allele only (264 individuals) with p-value 0.048 (OR 2.8).

No statistically significant association was observed across the *CYP1B1* or *NTF4* loci.

The allele frequencies and association p-values of typed SNPs in the Nepalese cohort are presented in **Table 4.** Two SNP from the *CYP1B1* gene were nominally significant; rs10916, G allele, with odds ratio 2.1 (95%CI 1.1-4.0, p=0.02), and rs162561, A allele, with odds ratio 2.2 (95% CI 1.1-4.3, p=0.01). Both SNPs remained significant after adjustment for sex and age, however did not survive Bonferroni correction. Similarly in the *NTF4* gene one SNP, rs11669977 T allele, showed p-value of 0.04 with odds ratio of 1.5 (95% CI 1.0-2.4) but did not survive correction for the 15 SNPs typed. No significant associations were identified with *eNOS* in this cohort.

In the Nepalese cohort, the global p-value for the haplotypes of all five SNP markers tested in *CYP1B1* gene was not significant (0.11). However, one haplotype, AGCAC, showed a nominally significant association with PACG with p-value of 0.02 (odds ratio 2.2, 95% CI 0.7-6.7) (**Table 5**), but did not survive correction for multiple testing of five haplotypes (corrected p-value 0.1). The remaining genes did not show any significant haplotypic association in this cohort (data not shown).

Discussion

Primary angle-closure glaucoma is a complex disease, believed to arise from interactions between genetic variants and environmental effects. Here we chose three genes targeting different function in the pathogenesis of glaucoma; development of anterior chamber (*CYP1B1*), retinal ganglion cell development and survival (*NTF4)*, and regulation of intraocular pressure (*eNOS*). All three genes have previously been implicated in POAG or PCG, and have been proposed as likely candidates for PACG.

CYP1B1 is well known for its association with primary congenital glaucoma (PCG). [11, 42, 43] It is expressed in tissues of the anterior chamber of the eye such as ciliary body, iris and trabecular meshwork. [29, 30] *CYP1B1* was hypothesized to take part in the normal development and function of the eye and is involved in the development of the anterior chamber angle. [10] Association was found with PACG in an Indian population [30], but not in a Middle Eastern cohort using sequencing methodology. [19] Here we genotyped tag SNPs to look for common variations in and around the gene. Our study shows nominal association in the Nepalese cohort under both single SNP and haplotypic analyses, however this association was not considered significant given the number of tests conducted. Thus, a larger cohort will be required to confirm this putative association.

When ocular tissues are subjected to stress, nitric oxide (NO) is released from the ocular vascular endothelium causing an increase in the ocular blood flow and oxygen delivery to the retina. [44] NO is produced from the endothelial nitric oxide synthase

(eNOS or NOS3) enzyme. NO synthesis sites are abundantly located in the ciliary muscle and the outflow pathway of normal human eye. It has been hypothesized to be involved in IOP regulation, either directly through affecting the outflow resistance at the level of the trabecular meshwork, or indirectly through affecting the tone of the ciliary muscle. [45] In addition to a role in IOP modulation, *eNOS* also affects the blood flow to the optic nerve via altering the dilation of the ocular vasculature,. [46] This could be important in determining the nerve's response to stress and dysfunction could lead to retinal ganglion cell death. *eNOS* was shown to be significantly associated with PACG in our Australian cohort at both the allelic and haplotypic level, suggesting that NO regulation plays a pathogenic role in PACG.

NO enhances expression of *MMP9*. [24] As we have previously reported an association of the *MMP9* gene with PACG in this Australian cohort [25] we evaluated the data at both genes combined and show that patients carrying PACG risk alleles at both *MMP9* and *eNOS* have double the risk of developing the disease compared to carrying no risk alleles. As the genetic architecture of PACG is unravelled, more detailed genetic risk matrices could be developed, which will better predict which patients with primary angle-closure suspect are likely to progress to PACG and thus require close monitoring.

NTF4 variants were reported to have a minor contribution in the pathogenesis of POAG. [47] Variations in *NTF4* were not associated with PACG in an Indian population. [31] This study reported that the most prevalent variant, A88V, was present in controls (4.91%) at a higher frequency than in cases (2.85%) [48] which is opposite to the findings in a large cohort of POAG. [14] Neither of our cohorts

showed robust association at this locus. Thus *NTF4* is unlikely to be a risk locus for PACG.

In conclusion, we find no evidence for association of *NTF4* or *CYP1B1* with PACG in either the Australian or Nepalese cohort. SNPs and haplotypes in the *eNOS* gene are associated with PACG in the Australian cohort. The lack of association of this gene in the Nepalese may be due to a true negative finding, or insufficient power in this cohort to detect an effect and larger cohorts will be required to determine this. The NO pathway has long been implicated in glaucoma. The findings here may indicate common molecular pathways leading to optic nerve susceptibility to glaucoma relevant to both POAG and PACG.

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Table 1. Characteristics of the Nepalese and Australian cohorts.

Variables	Australian			Nepalese		
	Case	Control	p-value	Case	Control	p-value
Number	129	288	-	106	204	-
Sex (% female)	62%	53%	0.20	76%	75%	0.85
Mean age in years (SD)	72(11.7)	69(11.2)	0.01	57.3(12.30)	60.3(13.71)	0.07
Mean SE in dioptres (SD)	2.2 (2.8)	0.12 (0.37)	<0.01	-0.30 (1.64)	0.10 (0.31)	0.16
IOP in mmHg	24.5 (14)	14.6 (3.4)	<0.01	21.3 (17.7)	12.8 (2.3)	<0.01
Cup/disc ratio	0.5 (0.25)	0.2 (0.25)	<0.01	0.8 (0.11)	0.2 (0.12)	<0.01

SD, standard deviation; SE, spherical equivalent; IOP intra ocular pressure.

Table 2 Allele frequencies (%) of the SNPs in Australian Cohort and unadjusted p-value for association under the allelic model with odds ratio (95% CI). Values in bold considered significant (p<0.05).

Gene	Chr	SNP position	SNP	Minor Allele	MAF	p-value	OR (95% CI)	p*
CYP1B1	2	1	rs2855658	A	0.42	0.289	0.9 (0.7-1.2)	0.337
		2	rs10916	G	0.22	0.821	1.0 (0.7-1.4)	0.762
		3	rs162562	C	0.22	0.821	1.0 (0.7-1.4)	0.762
		4	rs162561	A	0.14	0.343	1.0 (0.7-1.4)	0.437
		5	rs2551188	T	0.30	0.675	0.9 (0.7-1.2)	0.619
eNOS	7	1	rs3793342	T	0.08	**0.003**	0.5 (0.3-0.8)	**0.001**
		2	rs1799983	T	0.32	0.776	1.1 (0.8-1.4)	0.671
		3	rs3918227	A	0.08	0.745	1.2 (0.7-2.0)	0.743
		4	rs3918186	T	0.06	0.492	0.6 (0.3-1.2)	0.268
		5	rs3918188	A	0.42	**0.014**	1.5 (1.1-1.9)	**0.033**
		6	rs1808593	G	0.27	0.186	1.1 (0.8-1.6)	0.164
		7	rs7830	A	0.29	**0.007**	0.7 (0.5-1.0)	**0.003**
NTF4	19	1	rs12973356	G	0.10	0.185	0.8 (0.5-1.2)	0.119
		2	rs11669977	G	0.36	0.739	0.9 (0.6-1.2)	0.961
		3	rs4802546	T	0.17	0.554	1.1 (0.8-1.7)	0.714

p*= p-value adjusted for sex and age. OR (95% CI)= odds ratio (95% confidence interval). Chr= chromosome. MAF= minor allele frequency

Table 3. Haplotypes of *eNOS* gene in the Australian population (>1% frequency) and its association with PACG. Bold values are considered significant at the p<0.05 level. The order of the SNPs follow table 2

Haplotype 1 2 3 4 5 6 7	Cases	Controls	OR(95% CI)	p-value
C G C A A T A	0.18	0.19	0.9 (0.6-1.5)	0.844
T G C T C T A	0.04	0.07	0.5 (0.2-1.2)	0.093
C G C A A T C	0.25	0.15	1.5 (0.9-2.4)	0.009
T G C A C T C	0.02	0.05	0.4 (0.1-1.4)	0.083
C T A A C T C	0.08	0.07	1.1 (0.5-2.3)	0.692
C G C A C T A	0.10	0.13	0.8 (0.5-1.5)	0.327
T G C A C T A	0.02	0.05	0.4 (0.1-1.2)	0.055
C T C A C G C	0.22	0.18	1.4 (0.9-2.3)	0.228
C G C A C T C	0.09	0.11	0.9 (0.4-1.9)	0.688

Table 4. Allele frequencies (%) of the SNPs in Nepalese Cohort and unadjusted p-value for association under the allelic model, with odds ratio (95% CI). Values in bold considered significant (p<0.05).

Gene	Chr	SNP position	SNP	Minor Allele	MAF	p-value	OR (95% CI)	p*
CYP1B1	2	1	rs2855658	A	0.19	0.46	1.1 (0.7-1.8)	0.31
		2	rs10916	G	0.09	**0.02**	**2.1 (1.1-4.0)**	**0.02**
		3	rs162562	C	0.13	0.09	1.5 (0.9-2.6)	0.07
		4	rs162561	A	0.1	**0.01**	**2.2 (1.1-4.3)**	**0.02**
		5	rs2551188	T	0.38	0.54	1.1 (0.7-1.5)	0.62
eNOS	7	1	rs3793342	T	0.1	0.99	1.0 (0.5-1.7)	0.95
		2	rs1799983	T	0.19	0.73	1.0 (0.7-1.6)	0.76
		3	rs3918227	A	0.04	0.17	0.5 (0.2-1.2)	0.22
		4	rs3918186	T	0.11	0.92	1.0 (0.6-1.7)	0.9
		5	rs3918188	A	0.33	0.87	0.9 (0.6-1.3)	0.99
		6	rs1808593	G	0.28	0.42	1.1 (0.8-1.7)	0.46
		7	rs7830	A	0.38	0.39	0.8 (0.6-1.2)	0.41
NTF4	19	1	rs12973356	G	0.49	0.67	1.1 (0.7-1.5)	0.77
		2	rs11669977	G	0.22	**0.04**	**1.5 (1.0-2.4)**	0.17
		3	rs4802546	T	0.14	0.46	1.1 (0.8-1.5)	0.36

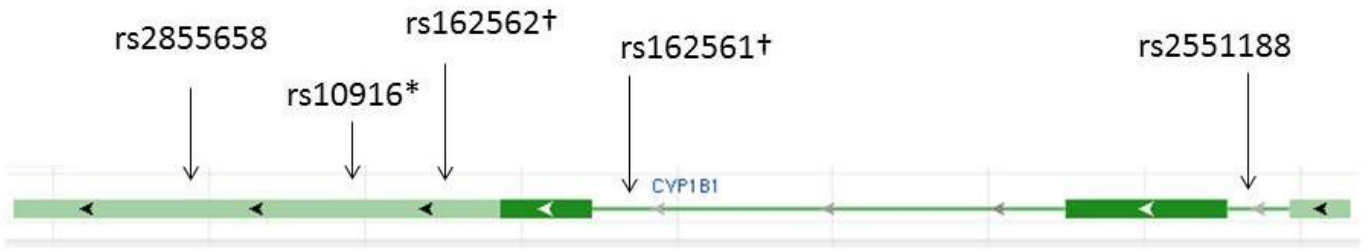
p*= p-value adjusted for sex and age. OR (95% CI)= odds ratio (95% confidence interval). Chr= chromosome. MAF= minor allele frequency.

Table 5. Haplotypes of *CYP1B1* and *NTF4* genes in the Nepalese population (>1% frequency) and its association with PACG. Bold values are considered significant at the p<0.05 level. The order of SNPs follow table 3

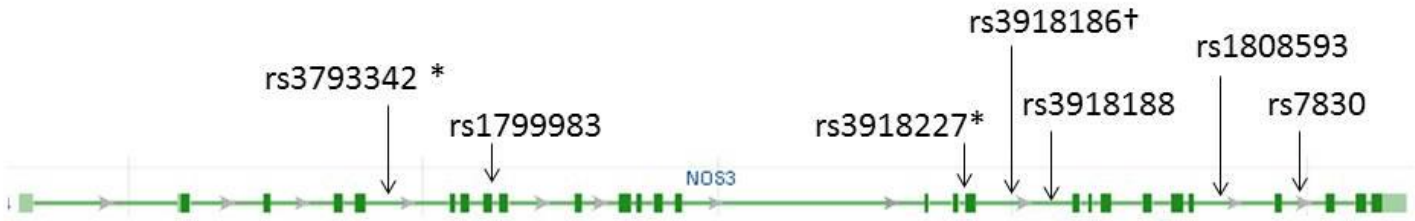
Gene	Haplotype 1 2 3 4 5	Frequency cases	Frequency controls	p-value
CYP1B1	A T C C T	0.03	0.04	0.76
	G T A C T	0.34	0.31	0.53
	A G C A C	**0.10**	**0.05**	**0.02**
	A T A C C	0.05	0.08	0.26
	G T A C C	0.48	0.52	0.24
NTF4	C A C	0.30	0.33	0.57
	G G C	0.22	0.16	0.18
	C G T	0.22	0.22	0.94
	C G C	0.26	0.29	0.38

Figure 1: **Gene ideograms depicting the location of tag SNPs genotyped for each candidate gene.** Exons are indicated by solid boxes and joined by introns indicated by lines. The direction of transcription is indicated by arrows. Translated regions of exons are coloured dark green and untranslated regions are light green. "* and †" indicate the tag SNP selected to represent variation in the Australian and Nepalese cohorts, respectively. Unmarked SNPs are tagging SNPs in both populations. Figure adapted from NCBI website (http://www.ncbi.nlm.nih.gov/gene). (A) *CYP1B1* (B) *eNOS* (C) *NTF4*.

(A)



(B)



(C)