Molecular Genomics of Primary Open-Angle Glaucoma

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Abstract

Glaucoma is a group of degenerative optic neuropathies and the leading cause of irreversible blindness worldwide. Primary open-angle glaucoma (POAG) is a major type of glaucoma in most populations. It is classified into high-tension glaucoma (HTG) and normal-tension glaucoma (NTG) according to the level of intraocular pressure. POAG has complex etiology. It could be monogenic or caused by multiple risk factors. At least 22 linkage loci have been mapped, with 3 genes (*MYOC*, *OPTN*, and *WDR36*) identified. Also, more than 30 susceptibility genes have been reported, many of which, however, remain unverified.

This thesis describes our work on the identification of new POAG genes by using a 3-tiered strategy: (1) to identify new genetic profiles of variants around the *CAV1*, *CAV2*, *CYP46A1*, *LMX1B*, *NTF4*, *PLXDC2*, *TLR4*, *TMTC2*, *ZP4* genes and the 2p16.3 locus; (2) to evaluate *CNTF* and *SPARC* as disease genes for POAG; and (3) to map the causal gene at the GLC1N locus for juvenile-onset POAG (JOAG).

Totally 1645 unrelated participants were enrolled, including a Hong Kong cohort of 281 HTG, 311 NTG and 248 controls, a Shantou cohort of 102 HTG, 28 NTG and 298 controls and, a Beijing cohort of 177 HTG and 200 controls. Also involved were members of the GLC1M-linked Philippine pedigree and the GLC1N-linked Hong Kong pedigree with JOAG, which have been previously described.

Differential association profiles were found for SNPs in/near *CAV1*, *CAV2*, *CYP46A1*, *LMX1B*, *PLXDC2*, *TLR4*, *TMTC2*, *ZP4* and 2p16.3. SNPs at *CAV1*, *CAV2*, *TLR4* and

2p16.3 were associated with POAG, whilst SNPs around other genes were unlikely to be risk factors for the disease, at least in Chinese. *TLR4* rs7037117 was associated with HTG in southern Chinese (P=0.0016, OR=2.72, recessive model). SNP rs1533428 at 2p16.3 showed an age-specific association of with late-onset POAG (age at diagnosis >60 years; $P=1.14\times10^{-5}$, OR=2.02, dominant model) but not with juvenile- and adult-onset POAG. Moreover, rs1533428 formed a joint effect with rs7037117 to confer stronger risk to HTG ($P=2.8\times10^{-4}$, OR=4.53). Besides, rs4236601 near the *CAV1* and *CAV2* genes was confirmed as a risk factor for POAG and another two protective SNPs rs6975771 and rs959173 were identified; moreover, that the risk and protective alleles were located in different haplotypes suggested multiple roles of the genes.

Apart from associated genes, a candidate causative gene *NTF4* was screened and two novel putative mutations (Gly157Ala and Ala182Val) detected, likely accounting for 0.29% of POAG. In the exploration of new POAG genes, two functional candidates *CNTF* and *SPARC* were screened and excluded.

In the mapping of the causal gene at GLC1N, a truncation mutation c.1090delT in the *MEGF11* gene was found to be cosegregated with glaucoma in the GLC1N-linked pedigree. Subsequent identification of c.1090delT in an unrelated JOAG patient supported that it is a disease-causing mutation. The identification of four splice-site mutations (IVS17+2insT, IVS17-4C>G, IVS17-2A>G and c.2472A>C) exclusively in patients provided further evidence supporting *MEGF11* as a causative gene for POAG. Mutations in this gene likely account for approximately 1% of POAG or 2% of JOAG.

摘要

青光眼是一組退行性視神經病，是世界上首要的致盲疾病。原發性開角型青光眼（POAG）是最常見的青光眼類型。其根據眼內壓水平可進一步分爲高眼壓性青光眼（HTG）和正常眼壓性青光眼（NTG）。POAG 的病因複雜，可由單基因或多因素引致。目前已有 22 個 POAG 連鎖位點被發現，但其中只有 3 個基因獲鑒定：*MYOC*，*OPTN* 與 *WDR36*。另經報道的關聯基因超過 30 個，但多数尚待验证。

本論文描述了我們尋找新 POAG 基因的工作：（1）通過研究 *CAV1*, *CAV2*, *CYP46A1*, *LMX1B*, *NTF4*, *PLXDC2*, *TLR4*, *TMTC2* 與 *ZP4* 基因及 2p16.3 位點附近的序列變異而識別新的基因關聯模式；（2）評估 *CNTF* 與 *SPARC* 基因作為 POAG 疾病基因的可能性；以及（3）在 GLC1N 連鎖位點上鑒定其致病基因。

本研究共納入 1645 名參與者，包括源自香港的 281 名 HTG 患者，311 名 NTG 患者及 248 名對照者；源自汕頭的 102 名 HTG，28 名 NTG 及 298 名對照者；以及源自北京的 177 名 HTG 及 200 名對照者。另被納入的有與 GLC1M 連鎖的菲律賓青少年開角型青光眼（JOAG）家系及與 GLC1N 連鎖的香港 JOAG 家系成員。

通過對已報道基因的研究，我們發現了不同的關聯模式。其中，*CAV1*, *CAV2*, *TLR4* 與 2p16.3 上的 SNPs 與 POAG 有顯著關聯, 而其它基因則與疾病無關聯。*TLR4* rs7037117 与南方人群的 HTG 有關聯（P=0.0016, OR=2.72， 隱性模型）。2p16.3 rs1533428 与老年發病型（診斷年齡 >60 歲）POAG 存在年齡特異性關聯（$P=1.14\times10^{-5}$, OR=2.02, 顯性模型）。另外，rs1533428 與 *TLR4* rs7037117 有疊加效應，使患病風險提高 4.53 倍（$P=2.8\times10^{-4}$）。對于 *CAV1/CAV2* 位點上的 SNPs，我們除證實 rs4236601 作為危險因子外還發現 2 個保護性 SNPs （rs6975771 與

rs959173），並發現不同 SNP 上危險性與保護性等位基因位于不同單體型，提示該基因對 POAG 或有多重作用。

在對致病基因的研究中，我們在 *NTF4* 基因上發現了 2 個新突變（Gly157Ala 與 Ala182Val），約影響中國 POAG 的 0.29%。另外，我們排除了 *CNTF* 與 *SPARC* 作爲 POAG 疾病基因的可能。

在篩查 GLC1N 位點的致病基因時，我們在 *MEGF11* 基因上發現了一個可能的致病突變（c.1090delT）在該與 GLC1N 連鎖的 JOAG 家系中呈共分離。隨後我們在另一 JOAG 患者中發現該突變，證實了 c.1090delT 是一致病突變。另外，我們在其它患者中發現 4 個剪接位點上的突變：IVS17+2insT, IVS17-4C>G, IVS17-2A>G 與 c.2472A>C，進一步支持了 *MEGF11* 是 POAG 的致病基因，約影響 1%的 POAG 或 2%的 JOAG。

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List of Abbreviations

Nucleotides

A	Adenine
C	Cytosine
G	Guanine
T	Thymine

Amino acids

Ala	A	Alanine
Arg	R	Arginine
Asn	N	Asparagine
Asp	D	Aspartic Acid
Cys	C	Cysteine
Gln	Q	Glutamine
Glu	E	Glutamic Acid
Gly	G	Glycine
His	H	Histidine
Ile	I	Isoleucine
Leu	L	Leucine
Lys	K	Lysine
Met	M	Methionine
Phe	F	Phenylalanine
Pro	P	Proline
Ser	S	Serine
Thr	T	Threonine
Trp	W	Tryptophan
Tyr	Y	Tyrosine
Val	V	Valine
Ter	X	Stop codon

General

AAD	Age at diagnosis
ACG	Angle-closure glaucoma
aCGH	Array comparative genomic hybridization
ALS	Amyotrophic lateral sclerosis
AMD	Age-related macular degeneration
ANCOVA	Analysis of covariance
AOAG	Adult-onset open-angle glaucoma
APOE	Apolipoprotein E
ASD	Alternative Splicing Database
ASSA	Automated Splice Site Analyses
bp	Base pair
CAV1 / CAV2	Caveolin 1 / Caveolin 2
CDH1	Cadherin 1, type 1, E-cadherin
CDKN1A	Cyclin-dependent kinase inhibitor 1A
CDR	Cup-to-disc ratio
CED-1	Cell death abnormal 1

CFH	Complement factor H
CI	Confidence interval
CLN6	Ceroid lipofuscinosis, neuronal, 6
cM	Centimorgan
CNTF	Ciliary neurotrophic factor
CNV	Copy number variation
CYP1B1	Cytochrome P4501B1
CYP46A1	Cholesterol 24S-Hydroxylase
ddH2O	Double distilled water
DENND4A	DENN/MADD domain containing 4A
DMXL1	Dmx-like 1
DNA	Deoxyribonucleic acid
DPP8	Dipeptidyl-peptidase 8
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EM	Expectation-Maximization
eNOS	Endothelial nitric oxide synthase
FALS	Familial amyotrophic lateral sclerosis
FDR	False discovery rate
GLC	Glaucoma genes
GLC1A-N	Primay open-angle glaucoma gene A to N (refer to Table 1.1)
GSTM1	Glutathione S-transferase mu 1
GWA	Genome-wide association
HGNC	HUGO Gene Nomenclature Committee
HSPs	Heat shock proteins
HTG	High-tension glaucoma
HTRA1	HtrA serine peptidase 1
HWE	Hardy-Weinberg equilibrium
IOP	Intraocular pressure
IRAK4	Interleukin-1R-associated kinase 4
JOAG	Juvenile-onset open-angle glaucoma
kb	Kilobase
KO	Knockout
LD	Linkage disequilibrium
LMX1B	LIM homeobox transcription factor 1-beta
LOAG	Late-onset open-angle glaucoma
LOD	Logarithm of odds
LOXL1	Lysyl oxidase-like 1
LPS	Lipopolysaccharide
MAF	Minor allele frequency
Mb	Megabase
MDR	Multifactor dimensionality reduction
MEGF10	Multiple epidermal growth factor-like domains protein 10
MEGF11	Multiple epidermal growth factor-like domains protein 11
mg	Milligram
ml	Milliliter
mM	Millimolar
mmHg	Millimeters of mercury
mRNA	Messenger RNA
MS	Multiple sclerosis

MYOC	Myocilin
NGF	Nerve growth factor
NMD	Nonsense-mediated mRNA decay
NR2E3	Nuclear receptor subfamily 2, group E, member 3
NSC	Neural stem cell
NTF4	Neurotrophin-4
NTG	Normal-tension glaucoma
OAG	Open-angle glaucoma
OD	Right eye
OLFM2	Noelin 2
OMIM	Online Mendelian Inheritance in Man
OPA1	Optic atrophy 1
OPTN	Optineurin
OR	Odds ratio
OS	Left eye
PACG	Primary angle-closure glaucoma
PAK7	p21 protein-activated kinase 7
PAR	Population attributable risk
PCG	Primary congenital glaucoma
PCR	Polymerase chain reaction
POAG	Primary open-angle glaucoma
PolyPhen	Polymorphism Phenotyping
r^2	Coefficient of determination (in linkage disequilibrium)
RGC	Retinal ganglion cells
Ri	Information content
RNA	Ribonucleic acid
RP	Retinitis pigmentosa
RPE	Retinal pigment epithelium
rRNA	Ribosomal RNA
SD	Standard deviation
SIFT	Sorting Intolerant From Tolerant
SMAD6	Mothers against decapentaplegic, drosophila, homolog of, 6
SNP	Single-nucleotide polymorphism
SNPSpD	SNP spectral decomposition
SPARC	Secreted protein, acidic and rich in cysteine
TLR4	Toll-like receptor 4
TM	Trabecular meshwork
TMTC2	Transmembrane and tetratricopeptide repeat containing 2
TNF	Tumor necrosis factor
TrkB	Tyrosine kinase receptor B
TULP3	Tubby like protein 3
UTR	Untranslated region
VCDR	Vertical cup-to-disc ratio
WDR36	WD repeat domain 36
XFS/XFG	Exfoliation syndrome/Exfoliation glaucoma
ZP4	Zona pellucida glycoprotein 4
µl	Microliter

Publications

Publications based on the work of this thesis (submitted/in preparation)

1. **Chen LJ**, Tam PO, Leung DY, Fan AH, Zhang M, Tham CC, Zheng Y, Wang J, Liang XY, Chen H, Fan BJ, Wang N, Pang CP. Identification of A Common SNP as Genetic Marker for Primary Open-Angle Glaucoma [under review].
2. **Chen LJ**, Tam PO, Tham CC, Liang XY, Chiang SW, Canlas O, Ritch R, Rhee DJ, Pang CP. Evaluation of *SPARC* as a Candidate Gene of Juvenile-onset Primary Open-Angle Glaucoma by Mutation and Copy Number Analyses [under review].
3. **Chen LJ**, Chiang SW, Tam PO, Leung DY, Fan AH, Zhang M, Zheng Y, Wang J, Tham CC, Lam DS, Thorleisson G, Pang CP. Risk and protective *CAV1/CAV2* alleles associated with primary open-angle glaucoma [under review].
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5. **Chen LJ,** Tam PO, Liang XY, Chiang WY, Leung DYL, Tham CC, Pang CP. Ethnic diversities in the association of *NTF4* with primary open-angle glaucoma [Manuscript submitted].
6. **Chen LJ**, Tam PO, Tham CC, Yam GH, Liu J, Jia LY, Chiang SW, Fan BJ, Zhang MZ, Wang N, Lam DS, Pang CP. Identification of the causal gene at the GLC1N locus for primary open-angle glaucoma [Manuscript in preparation].

Publications related to the work of this thesis

1. *Lai TY, ***Chen LJ**, Yam GH, Tham CC, Pang CP. Development of novel drugs for ocular diseases: possibilities for individualized therapy. Personalized Medicine [2010 in press].

2. *Zhao J, *Lam DS, **Chen LJ**, Wang YX, Zheng C, Lin Q, Rao SK, Fan DS, Zhang M, Leung PC, Ritch R. A Randomized Trial of Patching versus Acupuncture for Anisometropic Amblyopia in Older Children: 6 month results. Archives of Ophthalmology [2010 in press].
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Conference presentation

1. **Chen LJ**, Tam POS, Leung DYL, Tham CCY, Zhang X, Liang X, Chiang SWY, Lam DSC, Pang CP. Association analysis of 14 SNPs in 6 newly identified POAG susceptibility genes/loci. ARVO 2010 Annual Meeting, Fort Lauderdale, Florida, USA. May 2-6, 2010. Abstract A274.
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4. **Chen LJ**, Tam POS, Zhang X, Chiang SWY, Lai TYY, Pang CP. Evaluation of the *PRI* gene in Chinese Patients with Retinitis Pigmentosa. The American Society of Human Genetics 59th Annual Meeting. Honolulu, Hawaii, USA. Oct 20-24, 2009. Abstract 454W.
5. Tam POS, Zhang X, Yang YP, **Chen LJ**, Chiang SWY ,Lai TYY, Pang CP. Evaluation of the *NR2E3* and *NRL* genes in Chinese Patients with Retinitis Pigmentosa. The American Society of Human Genetics 59th Annual Meeting. Honolulu, Hawaii, USA. Oct 20-24, 2009. Abstract 455W.
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Chapter 1

Introduction

1.1 Glaucoma – the leading cause of irreversible blindness

Blindness, an essential health and social problem, affects approximately 45 million people worldwide (World Health Organization Fact Sheet 2009). And the number will probably increase to 76 million by 2020 if no interventions are available to arrest the progress (Pizzarello et al. 2004).

Cataract is the leading cause of blindness, accounting for approximately 50% of cases (Figure 1.1) (Resnikoff et al. 2004). However, as the advent of efficient surgical techniques, cataract-related blindness is considered curable. In contrast, disorders impairing the retina, such as glaucoma, age-related macular degeneration (AMD), diabetic retinopathy, etc., are problematic as no treatments are effective to restore the vision loss in the patients. Among them, glaucoma is the leading cause of irreversible blindness in many countries, accounting for approximately 12% of blindness with increasing trend (Resnikoff et al. 2004).

Glaucoma is a group of degenerative optic neuropathies exhibiting a progressive degeneration of retinal ganglion cells (RGCs) and their axons, resulting in a characteristic appearance of the optic disc (known as optic-nerve cupping) and a concomitant pattern of visual field loss (Figure 1.2) (Kwon et al. 2009; Weinreb and Khaw 2004). The pathological changes of glaucoma are irreversible. It not treated early and appropriately, glaucoma can progress to visual disability and eventually blindness (Figure 1.3).

1.1.1 Intraocular pressure and optic disc in glaucoma

The intraocular pressure (IOP), a measurement of the fluid pressure inside the eye, is a major parameter in glaucoma, typically ranging from 10 to 21 mmHg (Kwon et al. 2009).

The fluid, known as aqueous humor, is a transparent fluid filling the anterior and posterior chambers for nourishing the iris, lens and cornea as well as maintaining the globular shape of the eye. Aqueous humor is secreted by the ciliary body into the posterior chamber. It passes through the pupil into the anterior chamber and then exits the eye via the conventional outflow pathway (also known as trabecular pathway, which is composed of the trabecular meshwork, juxtacanalicular connective tissue, endothelial lining of Schlemm's canal, collecting channels, and aqueous veins) at the anterior chamber angle, and/or independently through the unconventional, i.e., uveoscleral, outflow pathway, which is open to aqueous humor in the region of the anterior insertion of ciliary muscle at the chamber angle (Tamm 2009; Weinreb and Khaw 2004) (Figure 1.4). The IOP is maintained and regulated by a balance between production and drainage of aqueous humor. Impairment of the balance can lead to IOP elevation predisposing to glaucoma.

The human retina is a light sensitive tissue lining the inner surface of the eye. It is a layered structure where the innermost is the retinal nerve fiber layer comprising the axons of RGCs. These axons converge on the optic disc to form the optic nerve. The fibers exit the eye after traversing a series of perforated connective tissue sheets known as the lamina cribrosa, and synapse in the lateral geniculate nucleus of the brain (Weinreb and Khaw 2004). In human, the convergence of the axons forms a central depression in the optic disc, known as the cup. The area that located between the edge of the disc and the physiological cup is called neuroretinal rim, an important structure in diagnosis of glaucoma. In glaucomatous eyes, the degeneration of RGCs along with a loss of retinal nerve fibers and supporting structures may lead to a characteristic excavation of the optic disc and a narrowed neuroretinal rim, appearing as in an increased cup-to-disc ratio (CDR).

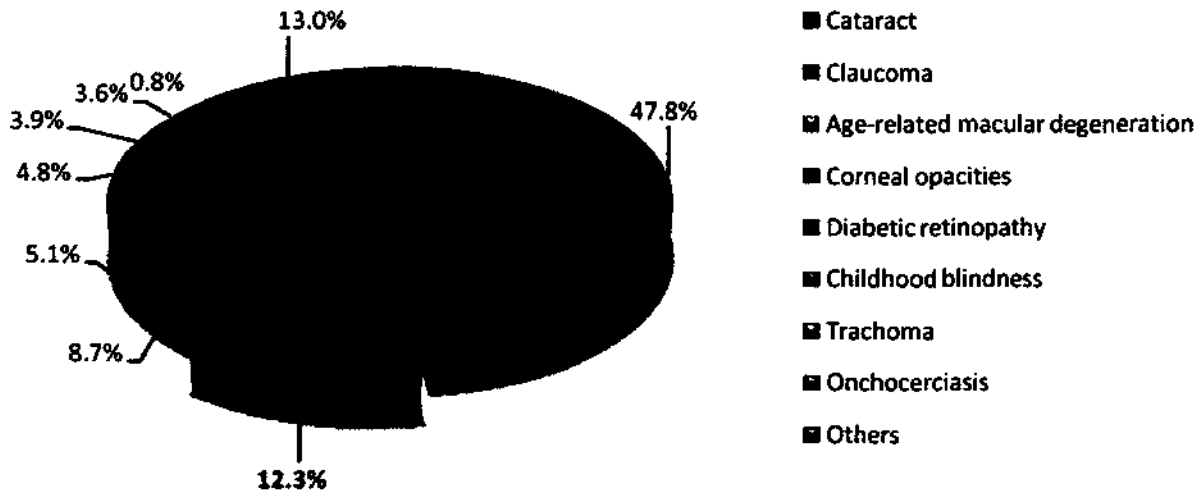


Figure 1.1. Global causes of blindness as a percentage of total blindness

The percentages are referred to the report of World Health Organization on global data of visual impairment in the year 2002 (Resnikoff et al. 2004).

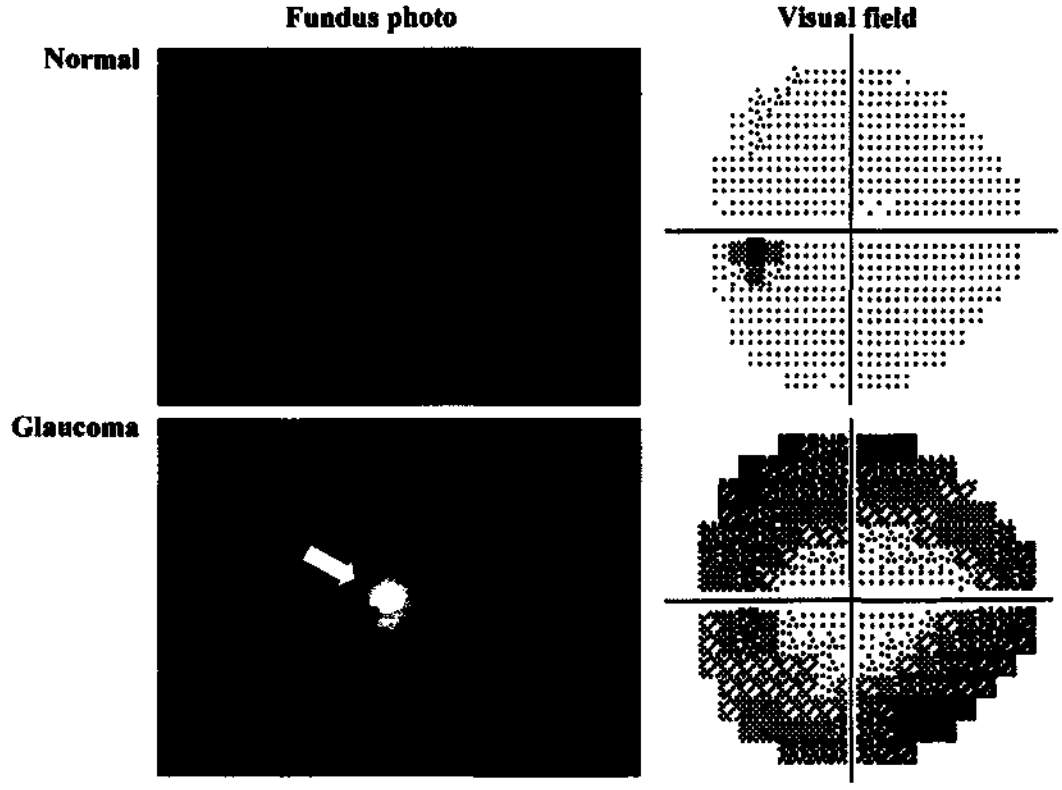


Figure 1.2. Appearances of glaucomatous optic disc and visual field

The figure shows the appearances of the optic disc and visual field in a normal and glaucoma subject. The arrow indicates the optic-nerve cupping at a glaucomatous fundus. (The fundus photos and visual field diagrams were provided by the glaucoma subspecialty of the Department of Ophthalmology and Visual Sciences, the Chinese University of Hong Kong)

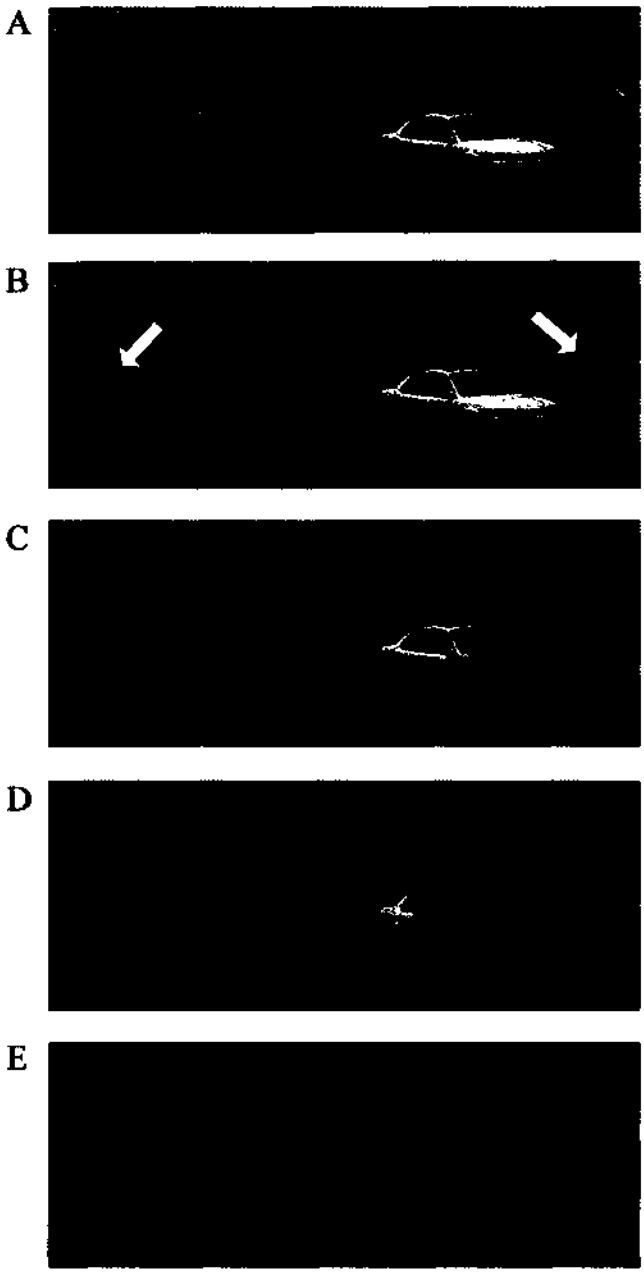


Figure 1.3. Progression of visual field loss in glaucoma

A: Visual field of person with normal vision.

B: Early stage of glaucoma (the yellow arrows indicate the mild visual field loss).

C: As the disease progresses, peripheral vision begins to fail, while the central vision remains.

D: During the advanced stages of glaucoma, only a small central area of vision remains.

E: End stage of glaucoma, where total blindness occurs.

(Modified from http://www.ahaf.org/glaucoma/about/understanding/progression-of-glaucoma.html)

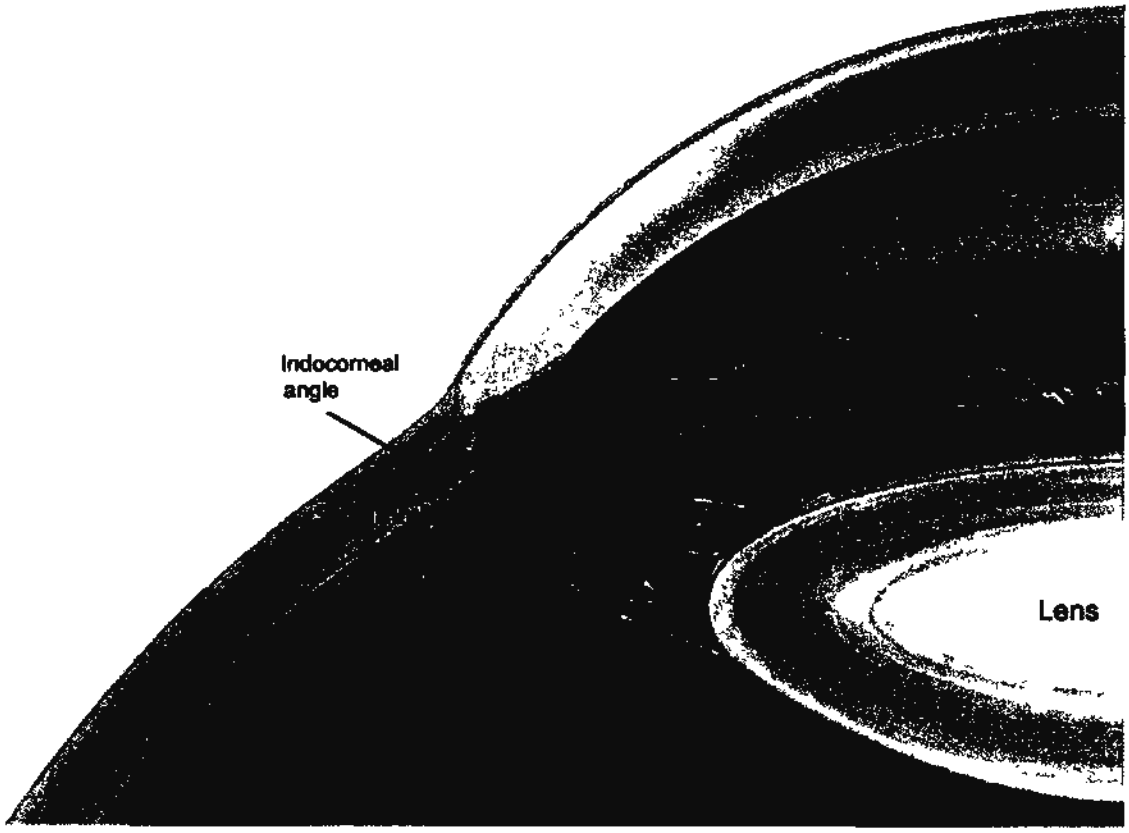


Figure 1.4. Anatomy of anterior segment of the eye and flow of aqueous humor

Aqueous humor is produced by the ciliary body, passes through the posterior chamber and the pupil into the anterior chamber, and exits through the trabecular meshwork at the angle. (Modified from (Kwon et al. 2009))

1.1.2 Clinical characteristics of glaucoma

A typical pattern of damage to the optic disc, referred to as optic-nerve cupping, is a structural characteristic that differentiates glaucoma from other causes of visual morbidity (Kwon et al. 2009). This change is most easily recognized at the superior and inferior poles of the disc. Hence, the vertical cup-to-disc ratio (VCDR) could serve as a simple and relatively robust index of glaucomatous loss of the neuroretinal rim (Foster et al. 2002). Most but not all glaucomatous eyes have increased VCDR and/or asymmetric CDR between eyes. However, some normal subjects can also have large CDR, whereas in some glaucoma patients the CDR could appear normal. Therefore, diagnosis of glaucoma should also include the detection of functional damage, i.e., visual field defects. A "gold standard" of glaucomatous visual field loss has been adopted as the glaucoma hemifield test grading "outside normal limit" and a cluster of three contiguous points at the 5% level on the pattern deviation plot, using the threshold test strategy with the 24-2 test pattern of the Zeiss-Humphrey field analyzer 2, or the equivalents from other devices (Foster et al. 2002).

1.1.3 Classification of glaucoma

According to the appearance of the anterior chamber angle, glaucoma could be classified into open-angle, angle-closure and developmental categories, which are further divided into primary and secondary forms (Figure 1.5).

Primary angle-closure glaucoma (PACG) is diagnosed if the glaucomatous changes are resulted from primary angle-closure, which is diagnosed in an eye with an occludable drainage angle and features indicating that trabecular obstruction by the peripheral iris has occurred, such as peripheral anterior synechiae, elevated IOP, iris whirling (distortion of the radially orientated iris fibers), "glaucomfleken" lens opacities, or excessive pigment deposition on the trabecular surface (Foster et al. 2002), with an absence of other known

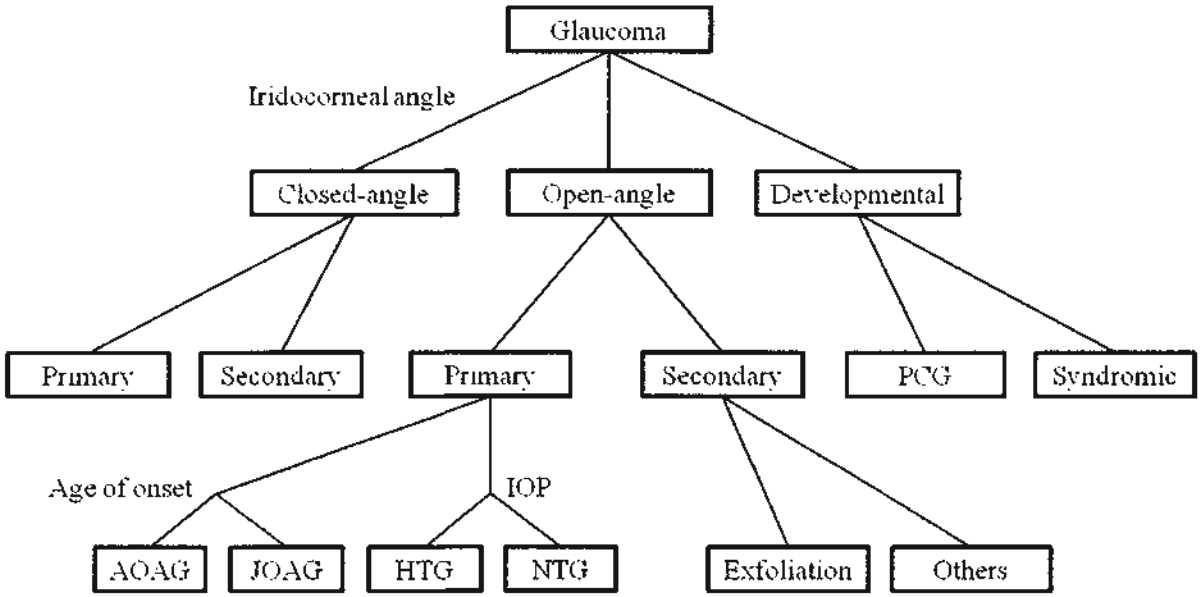


Figure 1.5. Classification of glaucoma

Abbreviation: AOAG: adult-onset open-angle glaucoma; HTG: High-tension glaucoma; IOP: intraocular pressure; JOAG: juvenile-onset open-angle glaucoma; NTG: normal-tension glaucoma; PCG: primary congenital glaucoma.

causes for the changes in the optic nerve. Otherwise, if the glaucoma is secondary to known causes, e.g., inflammatory or neovascular diseases, it is termed secondary angle-closure glaucoma.

Primary open-angle glaucoma (POAG) is diagnosed when the glaucomatous damage is not resulted from angle closure, and where there are no other identifiable explanations for the damage (Foster et al. 2002; Weinreb and Khaw 2004). According to the IOP level, POAG can be classified into high-tension glaucoma (HTG, IOP>21 mmHg) and normal-tension glaucoma (NTG, IOP≤21 mmHg). Regarding the age of onset, POAG could be divided into juvenile-onset POAG (JOAG), which occurs at an earlier age (approximately between 3 and 35 years) and usually but not always presents with high IOP, and adult-onset POAG (AOAG), which has an age of onset >35 years (Fan et al. 2006; Wiggs et al. 1995). If the disease occurs after the age of 60 years, it could be referred to as late-onset POAG (LOAG). However, the adult- and late-onset diseases are usually taken as one category. As such, it could be expected that POAG probably comprises several clinically indistinguishable diseases (Kwon et al. 2009) and is a spectrum of pressure-dependent diseases that reflects different susceptibilities to a given IOP level (Libby et al. 2005). With regard to secondary open-angle glaucoma, it is a group of glaucoma resulted from other forms of ocular pathologies, including exfoliation syndrome, pigment-dispersion syndrome, inflammation, trauma, neovascularization, lens-related and steroid-induced changes (Foster et al. 2002; Kwon et al. 2009). Among them, exfoliation syndrome is the most common identifiable cause of open-angle glaucoma (Ritch 1994).

Developmental glaucoma is a group of glaucoma associated with developmental anomalies that are present at birth, including primary congenital glaucoma (PCG) featured by abnormal development of the iridocorneal angle without other systemic abnormalities,

and secondary glaucoma associated with other ocular (e.g., aniridia) or systemic (e.g., the Axenfeld–Rieger syndrome) anomalies (Kwon et al. 2009; Robert et al. 2009).

1.1.4 Epidemiology of glaucoma

Glaucoma is an important health care issue worldwide. As mentioned above, glaucoma is a leading cause of blindness. It has been estimated that over 8.4 million people are bilaterally blind from primary glaucoma by 2010 (Quigley and Broman 2006). Moreover, glaucoma is also a leading cause of visual impairment. There are approximately 60.5 million people with open-angle glaucoma (OAG) and angle-closure glaucoma (ACG) by 2010. Among all glaucoma patients, approximately 74% are OAG. The mean prevalence of OAG worldwide in 2010 was estimated to be 1.96%, while for ACG 0.69%. OAG is the most prevalent in African derived populations, with a prevalence of 4.16% in people aged >40 years, while ACG prevalence is highest among Chinese people (Quigley and Broman 2006). By 2010, the largest absolute number of OAG and ACG is in China, estimated to be 16 million. Chinese OAG patients accounts for ~18.6% of worldwide OAG and ~47.5% of ACG. Among Chinese people aged >40 years, the prevalence of OAG and ACG is approximately 1.4% and 1.26% respectively (Quigley and Broman 2006). According to a recent survey in southern Chinese, the overall prevalence of glaucoma was approximately 3.8%, with POAG (2.1%) being more common than PACG (1.5%) (He et al. 2006), suggesting that the burden of POAG in China is similar to that of PACG.

1.1.5 Current management of POAG and perspectives for future development

Owing to the high prevalence and significant impact on vision, effective treatments for glaucoma are highly desirable to arrest or slow down disease progression. However, no current treatment could restore the impaired visual function. When a patient is diagnosed

with POAG, he/she is advised to attend for regular follow-up at the ophthalmology clinic. Follow-up is usually life-long. The aim of glaucoma care is to enhance the patient's health and quality of life by preserving visual function and preventing further vision loss without causing untoward effects from the treatment (American Academy of Ophthalmology 2000). At present, treatment of POAG is directed at lowering IOP, which is the only treatable risk factor. Reduction of IOP effectively slows glaucoma progression, whether or not the baseline IOP level is higher than statistical normality (Kass et al. 2002; Leske et al. 2003; The AGIS Investigators 2000). Several modalities of treatment are available for lowering IOP, including drugs, laser surgery, and incisional surgery. Medical treatment, which includes the use of several classes of topical agents, is usually the first line of treatment. The aim of any drug treatment in glaucoma is to achieve a "target IOP", which is best defined as an IOP at which the rate of RGC loss approaches the physiological rate in a particular individual or eye (Weinreb and Khaw 2004). To date, all commercially available glaucoma drugs act by IOP reduction, which is pharmacologically achieved by reducing aqueous production by the ciliary epithelium or increasing aqueous drainage from the anterior chamber (Lai et al. 2010). Currently there are 5 important classes of drugs: (1) parasympathomimetics (e.g. pilocarpine), (2) beta-blockers (e.g. timolol), (3) carbonic anhydrase inhibitors (e.g. dorzolamide), (4) alpha agonists (e.g. brimonidine), and (5) prostaglandin analogues (e.g. latanoprost). Once the initial working target IOP is stably achieved with a personalized drug regimen, the affected eye is closely observed for structural and/or functional signs of glaucoma progression. The combination of two or more topical drug agents can be used if one agent is ineffective. However, if the maximally tolerable drug regimen still fails to stabilize the glaucoma, surgical or laser interventions have to be considered. The most common procedure is trabeculectomy (Weinreb and Khaw 2004). This procedure produces a permanent channel for the drainage of aqueous humor

from the anterior chamber to the subconjunctival space and thereby permits the reduction of IOP. Other procedures for POAG include laser treatments, among which laser trabeculoplasty is the most widely used (Barkana and Belkin 2007; Weinreb and Khaw 2004). During the procedure, laser light is directed at the trabecular meshwork to reduce the resistance to aqueous humor outflow.

IOP lowering treatment has been shown to be beneficial on progression of glaucomatous damage and increase the time period before the onset of blindness (Hattenhauer et al. 1998; Jay and Murdoch 1993; Stewart et al. 2000). However, there is evidence showing that lowering the IOP is not uniformly effective in preventing progression (Stewart et al. 2000). Therefore, it is essential to have other therapies that can act directly to protect the RGCs from further damaging. The most promising glaucoma therapy may be neuroprotection, which is likely the next paradigm shift in glaucoma management (Lai et al. 2010). Since glaucoma can develop and progress in eyes with normal IOP, there should be factors other than IOP contributing to glaucoma development and progression. Identifying and controlling these factors may allow clinicians to arrest glaucoma progression irrespective of IOP. This may include excitotoxicity (Dreyer et al. 1996; Guo et al. 2006; Hare and Wheeler 2009; Hare et al. 2004a; Hare et al. 2004b), mitochondrial dysfunction (McKinnon et al. 2002), protein misfolding (Guo et al. 2007; Ishii et al. 2003; McKinnon et al. 2002), oxidative stress (Cellini et al. 1998; Dilsiz et al. 2006), inflammation (Kipnis et al. 2000), and neurotrophin deprivation (Hempstead et al. 1991; Ip et al. 1992; Klein et al. 1991). Recently, it has been demonstrated that treating patients with advanced glaucoma using topical nerve growth factor (NGF) improved all parameters of visual function (Lambiase et al. 2009). Although the effectiveness of NGF needs to be confirmed by further studies, the neuroprotective therapeutics, which may

activate survival pathways or inhibit death pathways of ganglion cells, could become a treatment option for glaucoma.

Another potential agent is the ciliary neurotrophic factor (CNTF), which has been demonstrated to exert protective effect in experimental glaucoma (Pease et al. 2009). Intravitreal CNTF significantly enhanced RGC survival and axonal regeneration in the eye (Cen et al. 2007). Cells isolated from the human optic disc express CNTF and its receptor complex members (Liu et al. 2007), suggesting that CNTF is an endogenous cytokine that may act to protect RGCs. Based on these findings, it could be hypothesized that altered expression or function of CNTF, possibly due to gene mutations, may be associated with glaucoma. Thus, in the work of this thesis we evaluated *CNTF* (OMIM *118945) as a disease gene for POAG.

Currently, most if not all glaucoma patients are treated after development of detectable signs and symptoms. It may already be too late for instituting an effective therapy during the natural course of the disease, and the therapeutic interventions are limited to alleviating symptoms and/or decelerating disease progression. Therefore, to commence treatment prior to the onset of disease will greatly improve the prognosis. This however requires the development of diagnostic equipments, such as Heidelberg Retina Tomograph, GDx Nerve Fiber Analyzer, and optical coherence tomography, for early diagnosis of POAG. As for risk prediction, this will depend on unraveling the mechanism behind the pathogenesis of the disease.

1.1.6 **Etiology and risk factors of POAG**

One characteristic pathological change in POAG is the progressive loss of RGCs (Fechtner and Weinreb 1994), resulting in decreased width of the neuroretinal rim and enlargement of the cup. However, this glaucomatous lesion is not limited to affect RGCs in

the retina; it also involves the death of neurons in the lateral geniculate nucleus and the visual cortex (Weinreb and Khaw 2004). Thus, the glaucomatous changes arise from multiple mechanisms, either individually, additively, or interactively. Among all possible mechanisms, the elevation of IOP is unquestionably related to the death of RGCs and optic nerve fibers in some, if not all, patients with POAG, although factors causing IOP elevation itself remains elusive. When IOP increases above physiological levels, the pressure gradient across the lamina cribrosa also increases. As a result, the lamina cribrosa and the retinal ganglion cell axons undergo deformation and mechanical stress (Bellezza et al. 2003; Weinreb and Khaw 2004). Consequently, there could be a blockade of RGC axonal protein transport due to IOP-induced compression of optic nerve axons at the lamina cribrosa (Fechtner and Weinreb 1994; Quigley et al. 2000). This compression could impair axonal transport of trophic factor leading to trophic insufficiency and subsequent cell-death.

Apart from elevated IOP, other factors can also act individually or collectively to cause the death of RGCs and optic nerve fibers in glaucoma. One of these factors could be local ischemia-hypoxia possibly resulting from dysfunction of blood-flow autoregulation in the retina, which is highly dependent on its blood supply for maintaining high metabolic needs (Weinreb et al. 1997). Another factor contributing to RGC death in glaucoma may be excessive stimulation of the glutamatergic system, specifically the N-methyl-D-aspartate subtypes (Dreyer et al. 1996; Lipton 2003). Other proposed contributors include poorly functioning cellular pumps and glutamate transporters, oxidative stress (formation of free radicals), inflammatory cytokines (tumor necrosis factor and nitric oxide) (Liu and Neufeld 2001; Yan et al. 2000b), and aberrant immunity (Schwartz 2003; Tezel and Wax 2004). In glaucoma patients, the response to an initial optic nerve injury can lead to secondary neurodegeneration of surviving RGCs and their fibers. Thus, even though the primary insult does not directly affect all RGCs and fibers, it may cause alterations in the neuronal

environment that in turn increases the vulnerability of the neurons in proximity (Liu and Neufeld 2001; Schwartz 2003). This may partially explain why some glaucoma progress even if the IOP is well controlled. Further investigations on these aspects are warranted to shed new light on the pathogenesis of the disease.

Besides the above mechanisms, a group of risk factors has also been proposed for POAG. The overall risk of developing a disease increases with the number and strength of risk factors. For glaucoma, the risk increases substantially with the extent of IOP elevation and increasing age. The average IOP, the range of IOP fluctuation, or both may be independent risk factor for POAG (Nouri-Mahdavi et al. 2004). Older age is consistently associated with POAG as prevalence increase steeply with age (Quigley and Vitale 1997). Aging as a natural factor could indicate the deterioration of relevant tissues and potentiating ganglion cell death. Age is also a measure of the duration of exposure to environmental risk factors (Boland and Quigley 2007).

Apart from IOP elevation and older age, states of the individual, including gender, ethnicity and family history are also potential risk factors for glaucoma. These factors are fixed and could be considered, as least partially, as surrogates for the genetic makeup. Regarding gender, although men have been shown to have greater risk for the presence or progression of the disease than women, such correlation has not been proven in most studies (Weih et al. 2001). Ethnicity, although poorly specifiable, is closely related to an individual's general genetic background. African appears to have the highest OAG prevalence. They develop the disease at an earlier age. The damage is usually more severe at the time of detection and surgery is usually less successful (Leske et al. 1994; Tielsch et al. 1991). Chinese has similar rates of OAG to Europeans (Foster and Johnson 2001; He et al. 2006). Finally, family history has long been recognized as a major risk factor for glaucoma. In a population-based study of all available family members of OAG patients,

the relative risk for OAG was more than 10 times higher in first degree relatives (Wolfs et al. 1998). In the Barbados population family study (African-derived persons), 10% of living relatives examined had OAG (Nemesure et al. 2001), whereas in Tasmania nearly 60% of POAG cases were familial (Green et al. 2007). Moreover, siblings of glaucoma patients were found to have increased risk of glaucoma (Sung et al. 2006).

Other potential risk factors for glaucoma include thin cornea (central corneal thickness <556μm), vertical or horizontal CDR greater than 0.4 (as determined from stereoscopic disc photographs), systemic hypertension, cardiovascular disease, myopia, migraine headache, and peripheral vasospasm (Kwon et al. 2009; Weinreb and Khaw 2004). However, evidences for these factors were less consistent and requires further validation.

The identification of different kinds of risk factors indicates that POAG is a complex multifactorial disease probably resulted from additive and/or interactive effects of multiple environmental and genetic risk factors. Unraveling the risk factors for POAG is essential for the understanding of disease mechanisms.

1.2 Genetics of primary open-angle glaucoma

The identification of genes associated with glaucoma may help to define the mechanisms underlying the disease, leading to the development of new DNA-based diagnostic tests and therapeutic approaches. Those individuals at risk who are identified early in the course of the disease and begin therapy prior to significant damage to the optic nerve will have better chance of maintaining useful sight (Wiggs 2007).

However, the identification of glaucoma genes has been difficult because of a general lack of knowledge about the cellular and biochemical events that are necessary for normal IOP regulation and RGC function (Wiggs 2007). Although relatives of glaucoma patients are at higher risk than the general population, the most common forms of glaucoma do not

exhibit Mendelian inheritance (Libby et al. 2005). These multifactorial forms are likely affected by multiple genetic and environmental factors. Even for those glaucoma with autosomal dominant or recessive inheritance, the disease in the patients is still complex and can be affected by many factors (Gould and John 2002). As such, mapping the glaucoma genes remains a challenge.

Currently, approaches to mapping the disease genes for glaucoma generally fall into two categories, namely hypothesis-free and hypothesis-based approaches. Hypothesis-free approach includes mainly the conventional linkage analysis, genome-wide association study, and the new advent of genome sequencing and exome sequencing. Hypothesis-based approach comprises typically candidate gene resequencing and candidate gene polymorphism association analysis. The selection of approach shall depend on the objectives of the study and the resources that are available. But it is a common practice to include different approaches in individual investigation.

1.2.1 **Genetic linkage loci and genes for POAG**

Genes associated with various forms of glaucoma that exhibit autosomal-dominant, autosomal-recessive, or other Mendelian inheritance patterns can be located in the human genome using large affected pedigrees and linkage analysis (Wiggs 2007). Once the chromosomal location of the gene is determined, positional fine-mapping and/or candidate gene resequencing could be used to identify the causal gene and its mutation at the locus. And the gene identified is further screened in other families or unrelated patients to evaluate the mutation spectrum. Such approaches have led to the identification of a majority of disease linkage loci, causative genes and mutations in glaucoma.

To date, at least 22 genetic loci have been linked to POAG in different populations (Allingham et al. 2005; Baird et al. 2005; Jiao et al. 2009; Lin et al. 2008; Monemi et al.

2005; Nemesure et al. 2003; Pang et al. 2006; Sarfarazi et al. 1998; Sheffield et al. 1993; Stoilova et al. 1996; Suriyapperuma et al. 2007; Trifan et al. 1998; Wang et al. 2006b; Wiggs et al. 2000; Wiggs et al. 2004; Wirtz et al. 1997b; Wirtz et al. 1999), including the designated GLC1A to GLC1N and others (Table 1.1). Within these loci, three causal genes, i.e., *myocilin* (*MYOC*; GLC1A) (Stone et al. 1997), *optineurin* (*OPTN*; GLC1E) (Rezaie et al. 2002) and *WD repeat domain 36* (*WDR36*; GLC1G) (Monemi et al. 2005), were identified at respective locus. However, mutations in these genes altogether likely account for less than 10% of POAG and the mutation patterns vary across different populations (Fan et al. 2009; Fan et al. 2005; Fan et al. 2006; Leung et al. 2003). More causal genes and mutations at these known linkage loci and also other unknown regions are yet to be identified.

1.2.1.1 GLC1A and the *myocilin* gene

The fist linkage locus for POAG was mapped to chromosomal region 1q21-q31 (Sheffield et al. 1993), designated as GLC1A (OMIM# 137750). Subsequently, the *myocilin* (*MYOC*; OMIM# 601652) gene was identified as the causal gene at this locus (Stone et al. 1997). And the role of *MYOC* in POAG has been confirmed by mutational testing in large cohorts of patients with juvenile- or adult-onset POAG. A variety of *MYOC* mutations have been detected in up to 20% of patients with JOAG and 1.5 to 5% of patients with AOAG in populations around the world (Fingert et al. 1999; Fingert et al. 2002; Pang et al. 2002; Stone et al. 1997; Wiggs et al. 1998). Some mutations are specifically associated with the juvenile-onset form, while others are more common in adult-onset patients. One major clinical feature of *myocilin*-related glaucoma is the elevation of IOP (Fingert et al. 2002). Mutations causative for JOAG are often correlated with severe IOP elevation, usually greater than 40mmHg, whereas mutations for AOAG typically cause

Table 1.1. Reported linkage loci and genes for primary open-angle glaucoma

Location	Locus	Phenotype	Gene	Population	Reference
1q21-31	GLC1A	JOAG	*MYOC*	Caucasian (UK, US, etc.)	(Sheffield et al. 1993); (Stone et al. 1997)
2p14	–	AOAG	–	Caucasian (north US)	(Wiggs et al. 2000)
2p16.3-15	GLC1H	AOAG	–	Caucasian (UK)	(Suriyapperuma et al. 2007)
2cen-q13	GLC1B	AOAG	–	Caucasian (UK, Australian)	(Stoilova et al. 1996); (Charlesworth et al. 2006)
2q33-34	–	AOAG	–	Afro-Americans (Barbados)	(Nemesure et al. 2003)
3p21-22	GLC1L	AOAG	–	Caucasian (Tasmanian)	(Baird et al. 2005)
3q21-24	GLC1C	AOAG	–	Caucasian (US, Greek)	(Wirtz et al. 1997b); (Kitsos et al. 2001)
5q22.1	GLC1G	AOAG	*WDR36*	Caucasian (US)	(Monemi et al. 2005)
5q22.1-32	GLC1M	JOAG	–	Asian (Philippine)	(Pang et al. 2006)
7q35-36	GLC1F	AOAG	–	Caucasian (US)	(Wirtz et al. 1999)
8q23	GLC1D	AOAG	–	Caucasian (US)	(Trifan et al. 1998)
9q22	GLC1J	JOAG	–	Caucasian (north US)	(Wiggs et al. 2004)
10p12-13	–	AOAG	–	Afro-Americans (Barbados)	(Nemesure et al. 2003)
10p15-14	GLC1E	AOAG (NTG)	*OPTN*	Caucasian (UK)	(Sarfarazi et al. 1998); (Rezaie et al. 2002)
14q11	–	AOAG	–	Caucasian (north US)	(Wiggs et al. 2000)
14q21-22		AOAG	–	Caucasian (north US)	(Wiggs et al. 2000)
15q11-13	GLC1I	AOAG	–	Caucasian, African-American	(Allingham et al. 2005); (Woodroffe et al. 2006)
15q22-24	GLC1N	JOAG	–	Chinese (Hong Kong)	(Wang et al. 2006b)
17p13	–	AOAG	–	Caucasian (north US)	(Wiggs et al. 2000)
17q25	–	AOAG	–	Caucasian (north US)	(Wiggs et al. 2000)
19q12-14	–	AOAG	–	Caucasian (north US)	(Wiggs et al. 2000)
20p12	GLC1K	JOAG	–	Caucasian (north US)	(Wiggs et al. 2004)

Note:

AOAG: adult-onset POAG; JOAG: juvenile-onset POAG; UK: United Kingdom; US: United States.

maximum IOP of 25 to 40mmHg (Alward et al. 1998; Fingert et al. 2002; Pang et al. 2002). In patients with *MYOC* mutations, high IOP affects not only the age of onset but also the progression of glaucoma (Kwon et al. 2009). Moreover, patients with *MYOC* mutations may be less responsive to pharmacological treatment and more likely to have undergone glaucoma drainage surgery (Craig et al. 2001; Fingert et al. 2002; Johnson et al. 1993; Zhuo et al. 2008). Therefore, to identify subjects with *MYOC* mutations, mostly in families with *myocilin* glaucoma, should facilitate early detection and treatment, monitoring the treatment effects and progression of the diseases.

Mutations in *MYOC* are among the most common causes of inherited eye disease with a known molecular basis (Kwon et al. 2009). So far, however, the mechanism of how mutant myocilin causes glaucoma is not fully understood. Myocilin is produced in many tissues, including the ciliary body (Ortego et al. 1997) and trabecular meshwork (Polansky et al. 1997), the two ocular structures that regulate IOP. It is likely that mutant forms of myocilin protein have an abnormal function that may result in a retention of the protein in the cell, forming heterodimers and heteromultimers with wild-type protein, and these complexes remain sequestered intracellularly (Gobeil et al. 2004; Liu and Vollrath 2004; Zhou and Vollrath 1999). The mutant proteins may be toxic to the trabecular cells and/or may prevent processing and secretion of other proteins that are necessary for the normal function of the trabecular outflow pathways (Wiggs 2007), impairing the pathways, leading to IOP elevation and subsequently glaucoma.

1.2.1.2 GLC1E and the *optineurin* gene

A second locus, in which the gene has been identified, is the GLC1E (OMIM# 137760) locus for adult-onset POAG. It was mapped on chromosomal region 10p14-p15 in a pedigree with NTG (Sarfarazi et al. 1998). Subsequently, a mutation Glu50Lys in the

optineurin (*OPTN*; OMIM *602432) gene was found to be cosegregated with the disease in the pedigree, confirming the role of *OPTN* as the causal gene at GLC1E (Rezaie et al. 2002). In the same study, *OPTN* mutations were found to account for 16.7% of hereditary forms of NTG, with an additional attributable risk factor of 13.6% in both familial and sporadic cases (Rezaie et al. 2002). However, such a large contribution of *OPTN* was not proven by subsequent studies in different cohorts (Alward et al. 2003; Liu et al. 2008b; Tang et al. 2003; Wiggs et al. 2003). A notable example is that, in a study involving 1048 POAG patients, only one mutation Glu50Lys was detected in one NTG patient, suggesting that this mutation likely accounts for less than 0.1% of all cases with POAG (Alward et al. 2003). Moreover, *OPTN* was found to contribute little to POAG in Japanese (Tang et al. 2003), in which NTG is a predominant form. Thus, *OPTN* mutations do not appear to have major contribution to POAG genetics. However, it is likely that *OPTN* mutations do not induce glaucoma unless the genetic context is permissive (Libby et al. 2005). This may explain, at least partially, why *OPTN* mutations do not always show high penetrance in pedigrees with POAG and can even occur in unrelated control subjects. There should be other genetic factors interacting with *OPTN* to determine the development of glaucoma. So far, the role of OPTN in the etiology of POAG remains elusive. Inferred by its role in NTG, OPTN may affect RGC viability. The protein is localized throughout the eye, including the trabecular meshwork, Schlemm's canal, ciliary epithelium, retina, and optic nerve (Rezaie et al. 2002; Sarfarazi and Rezaie 2003). Its expression in neuronal and glial cells in the retina and optic nerve indicates that it could directly affect RGC survival (Rezaie et al. 2002). OPTN also appears to interact with proteins that regulate apoptosis, including TNF-α (Funayama et al. 2004; Li et al. 1998; Sarfarazi and Rezaie 2003), suggesting that it may be involved in the regulation of cell death.

1.2.1.3 GLC1G and the *WD repeat-containing protein 36* gene

The *WD repeat-containing protein 36* (*WDR36*; OMIM *609669) gene is the third putative causative gene for POAG, identified in the AOAG locus GLC1G (OMIM# 609887) at 5q22.1 (Monemi et al. 2005). *WDR36* mutations were identified in approximately 5–7% of unrelated patients with HTG or NTG, indicating that the gene is involved in both types of glaucoma (Monemi et al. 2005). However, no proven causative mutation in *WDR36* was detected in subsequent studies in other study populations, including American, Australian, German, Indian, Japanese or Chinese (Fan et al. 2009; Hauser et al. 2006; Hewitt et al. 2006b; Jia et al. 2009; Kramer et al. 2006; Kumar et al. 2007; Miyazawa et al. 2007; Pasutto et al. 2008). In contrast, certain missense and/or noncoding variants, either individually or additively, were found to be present at higher frequencies in patients than in controls (Fan et al. 2009; Hauser et al. 2006; Miyazawa et al. 2007; Pasutto et al. 2008), suggesting that *WDR36* could be a susceptibility gene for POAG. Moreover, POAG patients with a *WDR36* variant, such as Leu25Pro, Asp33Glu, His212Pro, Ala353Ser, Ile361Val, Ala449Thr, Arg529Gln, Ile604Val, Asp658Gly and Met671Val, were found to have more severe disease than those without a variant, suggesting that *WDR36* likely acts as a glaucoma modifier gene (Hauser et al. 2006). Further functional studies also supported this point. WDR36 functions in ribosomal RNA processing and interacts with the p53 stress-response pathway. Loss of Wdr36 function may lead to an activation of the p53 stress-response pathway, suggesting that co-inheritance of defects in p53 pathway genes may influence the impact of WDR36 variants on POAG (Skarie and Link 2008). Furthermore, it has been demonstrated that WDR36 mutations alone did not produce any significant defects in cell viability or rRNA processing. However, when combined with a disruption of the co-chaperone STI1, nearly 50% of the tested variants had increased or decreased cell viability which corresponded to reduced or elevated levels of pre-rRNA

respectively (Footz et al. 2009). These findings suggest that, in the permissive genetic background WDR36 variants can lead to an altered cellular phenotype, supporting that WDR36 may participate in polygenic forms of glaucoma.

1.2.1.4 GLC1H and the 2p16.3 region

Besides *MYOC*, *OPTN* and *WDR36*, causal genes at other linkage loci remain unidentified. Recently, a linkage study using 146 multiplex families with adult-onset POAG has identified a strong linkage signal on chromosomal region 2p16.3 in the Afro-Caribbean population of Barbados (Jiao et al. 2009). This linkage region overlaps partially with the GLC1H (OMIM %611276) locus mapped to 2p15-p16 (Suriyapperuma et al. 2007). The 2p15-16 region has also been linked to autosomal dominant JOAG in Chinese (Lin et al. 2008). Hence, the 2p16 region may harbor a gene for POAG in different populations.

Apart from linkage analysis, Jiao and coworkers also performed a fine-mapping to identify the causal variants at 2p16. A set of single nucleotide polymorphisms (SNPs) on chromosome 2p were evaluated in two independent groups of the Barbados Family Study of Glaucoma participants, including a discovery group (130 POAG, 65 controls) and a replication group (122 POAG, 65 controls). Strong associations were identified with SNPs rs12994401 ($P<3.34\times10^{-9}$ and $P<1.21\times10^{-12}$ respectively) and rs1533428 ($P<1.55\times10^{-7}$ and $P<6.75\times10^{-6}$ respectively) and POAG in both groups. A common disease haplotype defined by these two SNPs, which were in strong linkage disequilibrium (LD, D'=0.72), were found to be strongly associated with POAG (Jiao et al. 2009).

Not until very recently, association of these two SNPs at 2p16.3 with POAG has been evaluated in another two African-derived populations, one African-American and the other Ghanaian (West African). Conflicting association patterns were found. Rs12994401 but not rs1533428 was detected to be associated with POAG in the African-American sample,

while no association was found for the two SNPs in the Ghanaian sample. And these two SNPs were in weak LD (r^2<0.35) (Liu et al. 2010b).

In the work of this thesis, we investigated the associations of SNPs rs1533428 and rs12994401 with POAG.

1.2.1.5 The *neurotrophin-4* gene and the linkage locus at 19q

Impaired neurotrophin signaling in retinal cells or lack of trophic support in the retina by neurotrophins has been postulated to contribute to the development of glaucoma. Within the neurotrophin protein family, the neurotrophin 4 (NT-4) may activate its receptor tyrosine kinase receptor B (TrkB) on RGCs, transducing its trophic effects in neurons and preventing their death *in vitro* and in animal models with axotomy (Cheng et al. 2002; Clarke et al. 1998; Cohen et al. 1994; Peinado-Ramon et al. 1996). NT-4 expressions are upregulated in the retina after ischemic lesion. Furthermore, in animals lacking NT-4, the retinal damage is more severe than in control animals (Harada et al. 2005). Based on these findings, the *neurotrophin-4* (*NTF4*; OMIM *162662) gene was recently proposed as a candidate gene for POAG through screening of 4 groups of European patients from Germany and Netherlands (Pasutto et al. 2009). Seven heterozygous mutations were found to account for approximately 1.7% of patients. Moreover, expression of recombinant NT-4 with the most frequent mutation Arg206Trp led to decreased activation of TrkB, suggesting that the mutant proteins may act in the pathophysiology of glaucoma through a loss of neurotrophic function (Pasutto et al. 2009). In an earlier linkage study, chromosomal region 19q12-14 was identified as a putative POAG locus (Wiggs et al. 2000), but the causal gene remained unidentified. The *NTF4* gene, which is located at 19q13.33, may be a candidate causal gene at this locus. Further confirmation can be obtained from mutation screening in the POAG pedigree linked to the 19q locus.

Not until very recently, lack of association between *NTF4* variants and POAG has been reported in another population of European ancestry in the southeastern United States (Liu et al. 2010a) and an Indian population (Rao et al. 2010). As such, the role of *NTF4* in POAG remains inconclusive. In the work of this thesis, we investigated the mutation profile of *NTF4* to verify its role as a disease gene for POAG.

1.2.1.6 GLC1M linked with juvenile-onset POAG

Our group has identified a novel linkage locus in a Philippine pedigree with JOAG (Pang et al. 2006). This pedigree had 95 members, among which 27 have been recruited for our studies. Nine of the 27 participants were diagnosed with JOAG. After excluding *MYOC*, *OPTN* and *WDR36* as the causal gene, a genome-wide linkage scan was conducted. A maximum LOD (logarithm of odds) score of 4.82 at θ=0.00 was obtained for marker D5S2011. Markers D5S2065, D5S1384, D5S471, D5S503, D5S2098, and D5S638 had LOD score values over 4.0 at θ=0.00. Haplotype analysis and recombination mapping further confined this region to 5q22.1-q32 flanked by D5S2051 and D5S2090 spanning 36 Mb. This locus has been designated as GLC1M (OMIM %610535) by HUGO Gene Nomenclature Committee (HGNC).

So far, the causal gene at GLC1M is unknown. To identify the gene, we prioritized the candidate genes by at least six criteria, including genes that are 1) expressed in eye tissues; 2) involved in IOP regulation; 3) affecting ganglion cell viability; 4) associated with other eye or retinal diseases; 5) associated with other neurodegenerative diseases; and 6) located at reported linkage loci. In the work for this thesis, we evaluated the *SPARC* (*secreted protein, acidic and rich in cysteine*; OMIM# 182120) gene as a disease gene for JOAG. This gene is located at chromosomal region 5q31.3-q32 within the GLC1M locus (5q22.1-32). SPARC (also known as osteonectin or BM-40) is a matricellular glycoprotein that

functions primarily to promote extracellular matrix deposition (Rhee et al. 2009). It is expressed at high levels in bone tissue, and is distributed widely in many other tissues and cell types (Maillard et al. 1992). In human eyes, it is found in lens (Yan et al. 2000a), corneal epithelium (Yan et al. 2000a), TM cells (Rhee et al. 2003; Wirtz et al. 1997a) and retinal pigment epithelium (RPE) (Magee et al. 2000; Rodriguez et al. 2000; Yan et al. 2000a). It distributes throughout the trabecular meshwork and is prominent in the juxtacanalicular region (Rhee et al. 2003). In the trabecular meshwork of postmortem human eyes, *SPARC* and another glaucoma gene *MYOC* responded significantly to elevated-IOP (Comes and Borras 2009), and it is one of the most highly upregulated genes in porcine TM cells in response to mechanical stretching (Vittal et al. 2005), supporting an important role of SPARC in IOP regulation. Furthermore, elevated *SPARC* expression has been detected in the iris of POAG patients (Chua et al. 2008), although whether such change was a cause or consequence of glaucoma, or just a phenomenon secondary to the use of topical medications for glaucoma remained unverified. Recently, the SPARC-null mouse has been shown to have lower IOP than the wild-type, likely due to decreased outflow resistance (Haddadin et al. 2009). Moreover, heterozygous mice expressed an intermediate phenotype (Haddadin et al. 2009), indicating a correlation between the dosage of SPARC and the regulation of IOP. These findings suggest that SPARC could be implicated in POAG, probably through the regulation of IOP. To date however, no study has evaluated the involvement of *SPARC* variants in human glaucoma. If any *SPARC* variations are associated with POAG, at least 5 possibilities should be considered: (1) promoter polymorphisms that affect the expression level of the gene; (2) missense variants with gain (or loss)-of-function; (3) nonsense mutations leading to loss-of-function; (4) variants at the exon-intron boundaries altering gene splicing; and (5) copy number variants that may alter gene dosage. In view of the finding that SPARC-null mice have lower IOPs

(Haddadin et al. 2009), we hypothesized that additional copies of *SPARC* may correlate with higher IOP. Moreover, as the GLC1M locus was identified in a pedigree of JOAG (Pang et al. 2006), we in the work of this thesis evaluated *SPARC* as a disease gene for JOAG by mutation screening and copy number analysis.

1.2.1.7 GLC1N linked with juvenile-onset POAG

In a Hong Kong Chinese family with JOAG, our group has identified a novel glaucoma locus (Wang et al. 2006b). The Chinese family comprised 25 members, of which eight were affected. After ruling out *MYOC*, *OPTN* and *WDR36* as the causal gene, a genome-wide linkage scan was conducted (Wang et al. 2006b). A maximum two-point LOD score of 3.31 at θ=0.0 was obtained for marker D15S125. Four adjacent markers, rs2030040, rs169169963, D15S153 and D15S131, gave two-point LOD scores of 2.41, 2.90, 3.02 and 2.68 respectively at θ=0.0. Haplotype analysis and recombination mapping further confined the region to 15q22-q24 spanning 16.6 Mb, flanked by D15S1036 and rs922693. This JOAG locus has now been designated as GLC1N (OMIM %611274) by HGNC.

In the GLC1N locus several candidate genes, i.e., *NR2E3* (*nuclear receptor subfamily 2, group E, member 3*; OMIM *604485), *SMAD6* (*mothers against decapentaplegic, drosophila, homolog of, 6*; OMIM *602931), and *CLN6* (*ceroid lipofuscinosis, neuronal, 6*; OMIM *606725), have been excluded as the causal genes (Wang et al. 2006b). In the work for this thesis, we sought to identify the causal gene at GLC1N for JOAG.

1.2.1.8 Genetic heterogeneity in POAG

Besides GLC1A, GLC1M and GLC1N, another two loci, i.e. GLC1J and GLC1K (Wiggs et al. 2004), were also mapped in pedigrees with JOAG, while the rest loci were mapped in families with AOAG (Table 1.1). These findings suggest that different gene

defects are underlying JOAG and AOAG, although clinical manifestations of the two are similar. Likewise, except for *OPTN* at GLC1E, which is implicated mainly in NTG, other genes or linkage loci are mainly linked to HTG, suggesting that genetic factors underlying these two forms of POAG are at least partially different. In contrast, the spectrum of myocilin glaucoma can range from juvenile glaucoma to typical late-onset POAG (Alward et al. 1998). Also, *WDR36* mutations are found in both HTG and NTG patients (Monemi et al. 2005). Moreover, incomplete penetrance was often observed with mutations in *OPTN* (Alward et al. 2003; Tang et al. 2003), *WDR36* (Hauser et al. 2006; Hewitt et al. 2006b), and even *MYOC* (Angius et al. 2000; Hewitt et al. 2006a; Iliev et al. 2008). These findings altogether may imply that, JOAG and AOAG, as well as HTG and NTG, on one hand are possibly the two sides of a continuum phenotypic spectrum due to common genetic defects, whereas on the other hand, each phenotype could have specific genetic determinants. Moreover, other factors, including susceptibility genes, gene-gene interactions, gene-environment interactions, and ethnic diversities, etc., may have modified the penetrance of the mutations or the manifestation of disease. As such, the identification of causal genes at the other linkage loci, disease-susceptibility genes, population genetic patterns, and gene-gene/gene-environment interactions will help unraveling the genetic architecture of POAG.

1.2.2 **Susceptibility genes for POAG**

Susceptibility genes, also referred to as associated genes, are a group of genes whose sequence variations are not sufficient to cause the disease individually, but can act concomitantly with other genetic, environmental and/or systemic risk factors to determine the development of disease or modify the severity of disease phenotype. The identification of susceptibility genes for POAG is largely attributed to genetic association studies, in which the frequencies of one or more variants are compared between a group of patients

with the disease of interest and a group of controls without the disease. So far the most commonly used marker in association study is single-nucleotide polymorphism, a single base pair change in DNA sequence at a particular point compared with the "common" or "wild-type" sequence (Attia et al. 2009).

To date, more than 30 genes have been reported to be associated with POAG (Table 1.2). However, most of these genes failed to be replicated across different populations. A notable example is the *apolipoprotein E* (*APOE*; OMIM +107741) gene, which has been associated with POAG in Chinese (Fan et al. 2005; Lam et al. 2006), French (Copin et al. 2002), and Saudis (Al-Dabbagh et al. 2009), but not the British (Ressiniotis et al. 2004a; Ressiniotis et al. 2004b), Estonian (Zetterberg et al. 2007) or Turkish (Saglar et al. 2009) populations. Such inconsistency may be due to many reasons, e.g., discrepancies in the definition of phenotypes, heterogeneous genetic makeup for the phenotype, population stratification, population-specific LD, heterogeneous genetic and epigenetic backgrounds, or heterogeneous environmental influences (Kingsmore et al. 2008). Moreover, some associations might have been due to chance, especially for those detected in studies with sample sizes. Therefore, verification of newly identified genetic associations in different populations will help confirming the involvement of the gene in the disorder.

Apart from ethnic diversities, the complexity of POAG genetics also includes phenotypic diversities. As described in the preceding sections, different causative genes are implicated predominantly in specific types of POAG. For example, *MYOC* is primarily mutated in juvenile-onset patients (Stone et al. 1997), whereas *OPTN* mainly in patients with normal IOP (Rezaie et al. 2002), and *WDR36* in adult-onset form of disease with both high and low IOP (Monemi et al. 2005). Likewise, some disease-associated genes, such as the *optic atrophy 1* (*OPA1*; OMIM *605290) gene for NTG (Aung et al. 2002a; Aung et al. 2002b; Powell et al. 2003; Yu-Wai-Man et al. 2009), may also confer susceptibility to a

Table 1.2. Reported susceptibility genes for primary open-angle glaucoma

Gene/locus	Protein	Positive evidence			Negative evidence		
		Population	Sample size*	Reference	Population	Sample size*	Reference
2p16.3	N/A	Afro-Caribbean (Barbados)	130P/65C	(Jiao et al. 2009)	–	–	–
CAV1/CAV2	Caveolin-1/Caveolin-2	Caucasian (Icelandic)	1263P/34877C	(Thorleifsson et al. 2010)	–	–	–
CYP46A1	Cholesterol 24S-hydroxylase	Caucasian (French)	150P/118C	(Fourgeux et al. 2009)	–	–	–
LMX1B	LIM homeobox transcription factor 1-beta	Caucasian (UK)	272H/37N/276C	(Park et al. 2009)	–	–	–
PLXDC2	Plexin domain containing 2	Japanese	827P/748C	(Nakano et al. 2009)	–	–	–
TLR4	Toll-like receptor-4	Japanese	250 N/318C	(Shibuya et al. 2008)	–	–	–
TMTC2	Transmembrane and tetratri-copeptide repeat containing 2	Japanese	827P/748C	(Nakano et al. 2009)	–	–	–
ZP4	Zona pellucida glycoprotein 4	Japanese	827P/748C	(Nakano et al. 2009)	–	–	–
APOE	Apolipoprotein E	Japanese	310 P/179C	(Mabuchi et al. 2005)	Caucasian (UK)	137P/75C	(Ressiniotis et al. 2004b)
ADRB1	Beta-adrenergic receptors	Japanese	211P/294N/240C	(Inagaki et al. 2006)	–	–	–
AGTR2	Angiotensin II receptor	Japanese	190P /268N/240C	(Hashizume et al. 2005)	–	–	–
CYP1B1	Cytochrome P450 1B1	Indian	264P/95C	(Bhattacharjee et al. 2008)	Caucasian (German)	285N/282C	(Wolf et al. 2009)
E-CDH	E-cadherin	Chinese (Taiwan)	60P/103C	(Lin et al. 2006)	Chinese (HK)	405P/201C	(Fan et al. 2010)
EDNRA	Endothelin receptor type A	Korean	67N/100C	(Kim et al. 2006b)	–	–	–
ELOVL5	Elongation of long-chain fatty acids family member 5	Japanese	305N/355C	(Meguro et al. 2010)	–	–	–
eNOS	Endothelium-derived nitric oxide synthase	Caucasian (Australian)	56P/100C	(Tunny et al. 1998)	Chinese (Taiwan)	66P/100C	(Lin et al. 2005)
GSTM1	Glutathione S transferase M1	Caucasian (Estonians)	250P /202C	(Juronen et al. 2000)	Caucasian (Swedish)	200P/200C	(Jansson et al. 2003)

Table 1.2. Continued

Gene/locus	Protein	Positive evidence			Negative evidence		
		Population	Sample size*	Reference	Population	Sample size*	Reference
GSTT1	Glutathione S transferase T1	Caucasian (Turkish)	114P/121C	(Unal et al. 2007)	Chinese (HK)	405P/201C	(Fan et al. 2010)
HSP70-1	Heat-shock proteins	Japanese	211H/290N/241C	(Tosaka et al. 2007)	–	–	–
IGF-II	Insulin-like growth factors-II	Chinese (Taiwan)	60P/104C	(Tsai et al. 2003)	–	–	–
IL1A	Interleukin-1 alpha	Chinese (Taiwan)	156P /167C	(Wang et al. 2006a)	Chinese (Singapore)	100H/94N/79C	(How et al. 2007)
IL1B	Interleukin (IL) 1beta	Chinese (Taiwan)	58P/105C	(Lin et al. 2003b)	Chinese (Singapore)	100H/94N/79C	(How et al. 2007)
iNOS	Inducible form of Nitric Oxide Synthase	Caucasian (Swedish)	200P/ 200C	(Motallebipour et al. 2005)	–	–	–
MFN1	Mitofusin 1	Caucasian (German)	285N/282C	(Wolf et al. 2009)	–	–	–
MFN2	Mitofusin 2	Caucasian (German)	285N/282C	(Wolf et al. 2009)	–	–	–
MTHFR	Methylentetrahydrofolate reductase	Caucasian (Germany)	76 P/71C	(Junemann et al. 2005)	Caucasian (European)	204P/211C	(Mossbock et al. 2006)
NOS3	Nitric oxide synthase gene	Caucasian (US)	527P /1543C	(Kang et al. 2010)	Chinese (HK)	405P/201C	(Fan et al. 2010)
OLFM2	Noelin 2	Japanese	215H/277N /240C	(Funayama et al. 2006)	–	–	–
OPA1	Optic atrophy 1	Caucasian (UK)	83N/100C	(Aung et al. 2002b)	Korean	65N/101C	(Woo et al. 2004)
p21	Effector protein of p53	Chinese (Taiwan)	58P/59C	(Tsai et al. 2004)	Caucasian (UK)	140P/73C	(Ressiniotis et al. 2005)
p53	Tumor protein p53 gene	Chinese (Taiwan)	58P/59C	(Lin et al. 2002)	Japanese	212H/213N /189C	(Mabuchi et al. 2009)
PARL	Presenilin associated, rhomboid-like	Caucasian (German)	285N/282C	(Wolf et al. 2009)	–	–	–
SRBD1	S1 RNA binding domain 1	Japanese	305N/355C	(Meguro et al. 2010)	–	–	–
TAP	Transporter associated with antigen processing	Chinese (Taiwan)	66P/105C	(Lin et al. 2004)	–	–	–
TNFA	TNF-alpha	Chinese (Taiwan)	60P/103C	(Lin et al. 2003a)	Caucasian (AUS)	114 P/228C	(Mossböck et al. 2006)

Note: The genes investigated in this study are listed on the top of the table, while the others are listed in alphabetical order.

* The sample size (the number in the cell) and the phenotypes (P: POAG, H: HTG, N: NTG, C: Control) in each study were shown.

specific form of POAG. Therefore, to involve different disease subtypes during study design or to stratify the phenotypes, e.g., IOP level and age of onset, during data analyses in association studies will help to dissect the contribution of a gene to the disease.

In the next sections, the POAG-associated genes that were investigated in the work of this thesis are described in detail. Other associated genes are summarized in Table 1.2.

1.2.2.1 The *toll-like receptor 4* gene and POAG

Aberrant immunity has been implicated in the pathogenesis of glaucoma, possibly through autoimmune-mediated injury to the optic nerve (Grus et al. 2008; Schwartz 2003; Tezel and Wax 2004; Wax 2000). Autoimmune damage to the optic nerve may occur directly by autoantibodies, or indirectly by way of a "mimicked" autoimmune response to a sensitizing antigen, which in turn injures RGCs (Tezel et al. 2004). Autoimmune-mediated injury may occur mostly in patients with NTG. In these patients, the glaucoma may be an organ-specific autoimmune disease characterized by immune-mediated tissue destruction which occurs in RGCs or their axons (Tezel et al. 2004; Wax 2000).

Related with immunity, the heat shock proteins (hsps), which belong to a diverse family of cellular chaperonins, function as endogenous protectants of eucaryotic cells in response to stress, and also act to elicit immune responses due to their antigenicity on the cell surface of pathogens, have been proposed to have an essential role in either initiation or sustainment of glaucomatous damage (Tezel et al. 2004). As such, genes encoding hsps or proteins interact with hsps can be excellent candidates for glaucoma. The toll-like receptor proteins are a family of phylogenetically conserved receptors that recognize self and nonself molecules, playing important roles in both innate and adaptive immunity (Akira et al. 2001; Andreakos et al. 2004; Kang et al. 2006). TLR4 is a principal lipopolysaccharide-recognition receptor, interacting with exogenous ligands and endogenous HSPs such as

HSP60, HSP70, and HSP96 (Andreakos et al. 2004; Kang et al. 2006). Recently, polymorphisms in the *toll-like receptor 4* (*TLR4*; OMIM +603030) gene were found to be associated with NTG in Japanese (Shibuya et al. 2008). In the study, 8 haplotype-tag SNPs were investigated for disease association. Six SNPs (rs10759930, rs1927914, rs1927911, rs12377632, rs2149356 and rs7037117) showed significant allele and genotype associations in a dominant pattern. SNP rs7037117, located in the 3'-untranslated region (UTR), showed the most significant association, with the minor allele A and the genotypes AA+AG, respectively, conferring a 1.51- and 1.65-fold of increased risk to NTG. LD and haplotype-based association analyses revealed that the haplotype C-G defined by the minor alleles of rs10759930 and rs7037117 was the major associated haplotype, and that the significant associations of SNPs rs10759930, rs1927914, rs1927911, rs12377632, and rs2149356 with NTG might arise from a strong LD with rs7037117 (Shibuya et al. 2008).

To date, association between *TLR4* and NTG has not been reported in other populations. Moreover, *TLR4* has not been implicated in HTG. In the work of this thesis we studied the associations of *TLR4* SNPs with both NTG and HTG.

1.2.2.2 The *cholesterol 24S-Hydroxylase* gene and POAG

Cholesterol and cholesterol metabolism have been implicated in the pathogenesis of glaucoma (McGwin et al. 2004; Song et al. 2005; Yucel et al. 2005). Variants in genes involved in metabolic pathways of cholesterol can be associated with disease susceptibility. Cholesterol-24S-hydroxylase (CYP46A1; OMIM *604087) is a cholesterol-metabolizing enzyme expressed specifically in the neuronal part of the retina, most particularly in RGCs (Bretillon et al. 2007). CYP46A1 and its metabolic product, 24S-hydroxycholesterol, have been linked to neurodegeneration (Bretillon et al. 2000; Kolsch et al. 1999; Lutjohann et al.

2000). An intronic SNP rs754203 in the *CYP46A1* gene has been associated with Alzheimer disease (Li et al. 2006; Wang et al. 2004).

Recently, *CYP46A1* rs754203 was analyzed in 150 POAG patients and 118 control subjects. The TT genotype was found in 61.3% of patients and 48.3% of controls, conferring a 1.26-fold of significantly increased risk of POAG. Interestingly, the TT genotype was at an even higher frequency in patients with NTG (72.2%; 13/18) and may confer stronger risk to this phenotype (Fourgeux et al. 2009). However, due to the small number of NTG patients, such interpretation is inconclusive.

So far, there are no other reports about *CYP46A1* rs754203 and POAG. In the work of this thesis, we investigated the associations of this variant with both HTG and NTG.

1.2.2.3 The *LIM homeobox transcription factor 1-beta* gene and POAG

Recently, SNPs in the *LIM homeobox transcription factor 1-beta* (*LMX1B*; OMIM *602575) gene has been shown to be associated with HTG and NTG in a Caucasian cohort from the United Kingdom (Park et al. 2009). The *LMX1B* gene was selected as a candidate gene for POAG because of its implication in Nail-Patella Syndrome (NPS; OMIM #161200), an autosomal dominant condition in which approximately 1/3 of patients over 40 years of age develop open-angle glaucoma (Mimiwati et al. 2006) and ~26.7% of individuals older than 50 years develop NTG (Bongers et al. 2005). In murine, *Lmx1b* encodes for a LIM homeobox transcription factor that regulates gene expression essential for normal morphogenesis of the anterior segment (Pressman et al. 2000). In human, mutations in *LMX1B* cause a spectrum of ocular phenotypes, including various anterior segment anomalies. However, developmental abnormalities of the anterior segment are not clinically detectable in most patients with *LMX1B* mutations (Park et al. 2009). Therefore, it

is likely that subclinical polymorphisms in this gene may produce subtle and undetected abnormalities in anterior segment structure and/or function, predisposing to glaucoma.

In the study by Park et al., 23 tag-SNPs in *LMX1B* were tested for associations with HTG (n=272) and NTG (n=37). Four SNPs, namely rs7859156, rs6478750, rs10987385 and rs7854658, were nominally associated with HTG, while only rs7859156 remained significant after correction for multiple comparisons (P_{corr}=0.0376, odds ratio (OR) =0.64). Five SNPs, rs944103, rs7854658, rs16929236, rs10733682 and rs867559, were associated with NTG. Among them, rs7854658 conferred a reduced risk while the other four conferred increased risk to NTG. However, none of these SNPs could withstand correction for multiple comparisons. In pooled HTG and NTG, rs7859156 and rs7854658 were significantly associated with the disease after correction, with rs7854658 showing the strongest association (P_{corr}=0.027; OR=0.62) (Park et al. 2009).

So far, the association of *LMX1B* SNPs with POAG has not been reported in other populations. According to the International HapMap Project, the minor allele frequencies (MAF) of *LMX1B* SNPs in Chinese are highly different from that in Caucasians. For example, SNP rs7854658, which showed the strongest association with pooled POAG in the study of Park et al. (Park et al. 2009), is rare in Chinese (MAF=0; HapMap). Thus, in the work of this thesis we investigated the involvement of *LMX1B* SNPs in Chinese POAG.

1.2.2.4 POAG-associated SNPs detected by genome-wide association studies

A majority of reported POAG-associated genes were identified by the candidate gene approach. With the completion of the human genome sequence (Venter et al. 2001), initiation of international HapMap project (International HapMap Consortium 2003), and rapid improvement of SNP genotyping technology (Hirschhorn and Daly 2005), genome-wide association (GWA) study has been widely used for detecting susceptibility

polymorphisms for complex diseases. In a GWA study, the association between SNPs across the genome and disease was tested, usually involving 300,000 or more markers that are reasonably polymorphic and are spread across the genome fairly evenly. This approach is hypothesis free, and is useful in searching for common variants (MAF >5%) that affect a disease (Bodmer and Bonilla 2008). So far, over 587 GWA studies for different phenotypes have been published, involving more than 2865 associated SNPs (A Catalog of Published Genome-Wide Association Studies. Available at: www.genome.gov/gwastudies. [Accessed 18 June 2010]) (Hindorff et al. 2009; Hindorff et al. 2010). For eye disorders, the first success is the identification of the *complement factor H* (*CFH*; OMIM +134370) gene associated with AMD (Klein et al. 2005). For glaucoma, in a large-scale GWA study the *Lysyl oxidase-like 1* (*LOXL1*; OMIM *153456) gene was identified to be strongly associated with exfoliation syndrome (XFS) and exfoliation glaucoma (XFG), a major type of secondary open-angle glaucoma (Thorleifsson et al. 2007). However, the 3 major SNPs of *LOXL1*, i.e., rs2165241, rs1048661 and rs3825942, were not associated with POAG in different populations (Chen et al. 2010), suggesting that *LOXL1* is specific for XFS/XFG. Notably, in the same study the patient group contained 90 POAG cases but no SNP achieved significance at a GWA level (Thorleifsson et al. 2007), suggesting that the risk alleles for POAG have a much smaller effect size and could only be detected by larger-scale GWA studies.

Recently in a GWA study 6 SNPs in three chromosomal loci were identified to be significantly associated with POAG in a Japanese cohort (Nakano et al. 2009). In stage 1 of the study, a genome-wide SNP association was performed using 418 POAG (including HTG and NTG) and 300 control subjects. After data filtering, 331,838 autosomal SNPs were analyzed for disease association. A total of 255 well-clustered SNPs showed significant association with the disease ($P<0.001$). In stage 2, the 255 SNPs were tested in a

separate group of 409 patients and 448 controls, and the combined P value of stages 1 and 2 was calculated. This yielded 6 SNPs with P values <0.0001, including rs547984, rs540782, rs693421 and rs2499601 at 1q43 (nearest gene: *zona pellucida glycoprotein 4*, *ZP4*), rs7081455 at 10p12.31 (nearest gene: *Plexin domain containing 2*, *PLXDC2*), and rs7961953 at 12q21.31 (*transmembrane and tetratricopeptide repeat containing 2*, *TMTC2*). Moreover, the 6 SNPs showed significant association in stage 2 (Nakano et al. 2009).

Recently, lack of association between these 6 SNPs and POAG was reported in an Indian population (Rao et al. 2009), suggesting that the associations could be population specific. In the work for this thesis, associations of three SNPs, i.e., rs693421, rs7081455 and rs7961953, with POAG were investigated.

1.2.2.5 The *caveolin 1* and *caveolin 2* genes and POAG

Recently, in a large-scale GWA study involving 1,263 POAG patients and 34,877 controls from Iceland, a common variant rs4236601[A] was identified to be significantly associated with POAG ($P = 5.0\times10^{-10}$, OR=1.36) (Thorleifsson et al. 2010). The association was replicated in a sample set of 1,708 POAG cases and 1,860 controls from Sweden, United Kingdom and Australia (OR=1.21, P=0.0008), and in 299 POAG cases and 1,607 controls from Hong Kong and Shantou, China (OR=3.33, P=0.003). Thus, rs4236601 appears to be a global marker for POAG. It is located next to the *Caveolin 1* (*CAV1*; OMIM *601047) and *Caveolin 2* (*CAV2*; OMIM *601048) genes at 7q31. However, screening the two genes in Icelandic samples revealed that no other coding variant was associated with the disease after adjusting for rs4236601, suggesting that rs4236601 does not tag a structural mutation in *CAV1* or *CAV2*.

Interestingly, while being a common SNP in Caucasians, with a MAF of 20.7-28.1% in control groups, rs4236601 presents at very low frequencies in Chinese (MAF 0.3-0.6% in

controls) but confers a stronger risk (OR between 3.3 and 5.47). Moreover, rs4236601 is associated mainly with, but not limited to, NTG (Thorleifsson et al. 2010). However, in the study of Thorleifsson et al. the Chinese patients, provided by our group, were mainly HTG subjects. This does not allow an evaluation of rs4236601 associated with NTG.

Our group has performed a GWA study in Chinese NTG (the GWA data are not included in this thesis). This allows us to evaluate the tag-SNPs at the *CAV1/CAV2* locus in NTG. We found that rs4236601 and two SNPs (rs4730742 and rs6466587) in LD conferred a trend towards an increased risk of NTG (OR>1.5, P>0.05). Moreover, three other SNPs (rs926201, rs6975771 and rs959173), which were also in LD, were nominally associated with a reduced NTG risk (OR=0.50-0.55, P<0.05, Figure 1.6). In the work of this thesis, we verified the associations of these six SNPs with HTG and NTG and evaluated the role of *CAV1* and *CAV2* in the genetics of POAG.

1.2.3 **Gene-gene / gene-environment interactions**

As mentioned above, interactions between multiple genetic and environmental risk factors play an important role in the pathogenesis of POAG. However, reports about gene-gene interactions in POAG are thus far limited, while those about gene-environment interactions are even sparser. The *APOE* SNP -491A>T was found to interact with the *MYOC* variant *MYOC*.mt1 to increase IOP in patients with POAG (Copin et al. 2002), although the effect of *MYOC*.mt1 on POAG is still unclear (Fan et al. 2004). It has been found that variants in the *cytochrome P4501B1* (*CYP1B1*; OMIM *601771) gene, a gene for PCG, interacted with *MYOC* to modify the manifestations of JOAG in familial subjects with mutations in both genes, suggesting that *MYOC* and *CYP1B1* may interact through a common pathway and that the inheritance of glaucoma may be multiallelic in some cases (Vincent et al. 2002). Interactions of the *OPTN* and tumor necrosis factor (*TNF*; OMIM

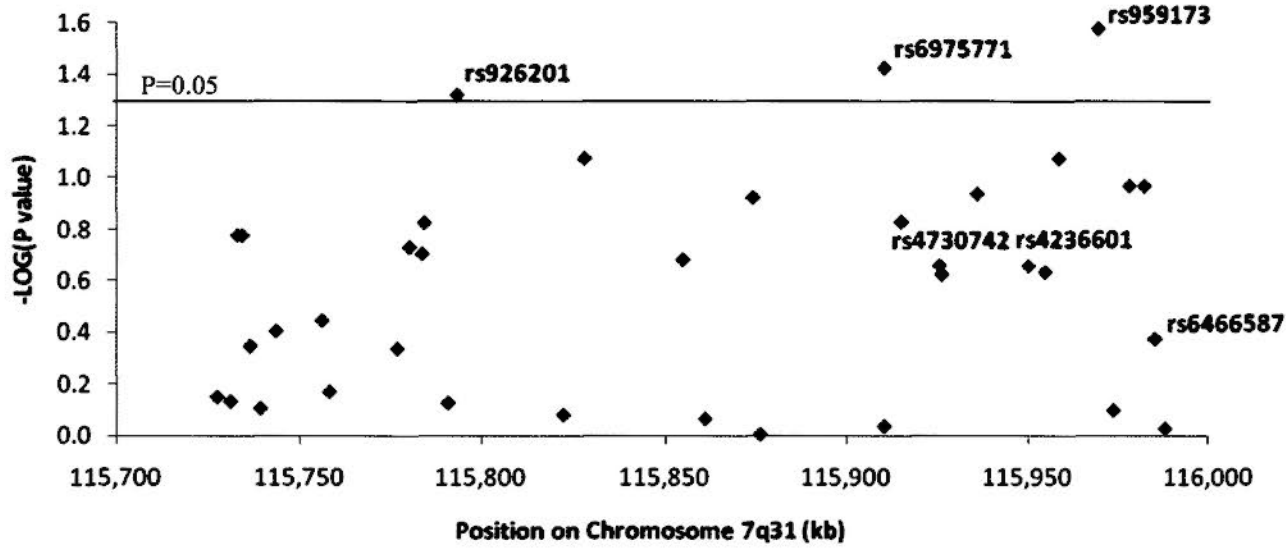


Figure 1.6. Schematic view of the associations between NTG and the 36 *CAV1/CAV2* SNPs involved in our genome-wide association study

The 6 SNPs investigated in this thesis are shown in red color. The 3 SNPs above the horizontal line for P=0.05 (-logarithm=1.3) showed significant association with the disease.

*191160) genes, and of *OPTN* and noelin 2 (*OLFM2*) genes had been identified to contribute to development of POAG (Funayama et al. 2004; Funayama et al. 2006). In our previous studies, putative gene-gene interactions were found among *MYOC*, *OPTN* and *APOE* (Fan et al. 2005); and a high-order interaction model involving *MYOC* IVS2+35A>G, *OPTN* Met98Lys, *OPTN* IVS5+38T>G, *OPTN* IVS8-53T>C, *WDR36* IVS5+30C>T and *APOE* -491A>T in POAG (Jia et al. 2009). These findings altogether support a polygenic etiology of POAG, and exemplify the importance of interaction analysis in association studies with multiple SNPs.

1.2.4 **Copy number variation and POAG**

Apart from SNPs, copy number variations (CNVs), which are DNA segments that present at variable copy number in comparison to a reference genome with the usual copy number of two (Feuk et al. 2006), are also widespread in human genomes and represent a significant source of genetic variation. Numerous CNVs are being identified with various genome analysis platforms, including array comparative genomic hybridization (aCGH) (Pinkel et al. 1998), SNP genotyping platforms (Redon et al. 2006), and next-generation sequencing (Korbel et al. 2007). CNVs can represent benign polymorphic variants. However, CNVs can also be causative for Mendelian or sporadic traits, or associated with complex diseases, possibly through various molecular mechanisms, including gene dosage, gene disruption, gene fusion, position effects, etc (Zhang et al. 2009).

For common eye diseases, reports on genome-wide CNV analyses are currently limited. Recently, a whole-genome copy number screening in a cohort of 400 individuals with POAG and 100 controls showed that rare CNVs involving the *DMXL1* (*dmx-like 1*), *TULP3* (*tubby like protein 3*) and *PAK7* (*p21 protein-activated kinase 7*) genes may affect the development of POAG (Davis et al. 2009). Another attempt of genome-wide CNV analysis

for POAG was conducted in 27 Caucasian and African-American POAG patients and 12 ethnically matched controls to screen for chromosomal CNVs by using high resolution aCGH. No chromosomal deletions or duplications were detected in POAG patients as compared to controls (Abu-Amero et al. 2009). As such, knowledge about CNVs in POAG remains currently limited. Further studies are warranted to discover more CNVs involved in POAG, either by analysis of candidate regions or genome-wide screening.

1.2.5 **Current understanding of POAG genetics in Chinese**

Ethnic differences and geographic variations affect the frequencies and nature of human mutations. Investigations of the genetics of eye diseases have revealed discrepant genetic profiles, including mutation spectrums and association patterns, between Chinese and other ethnic populations. Therefore, establishment of a mutation database specific for Chinese is essential for the identification of genetic markers with diagnostic, prognostic, or pharmacological values (Pang and Lam 2002).

Ethnic diversities in the genetic architecture of POAG are well recognized. However, it is still difficult to quantify the ethnic difference, because a large proportion of the genetic components remain undiscovered, and the studies conducted in different populations are imbalanced, with a majority of studies having been conducted in Caucasian populations. So far, it is known that the contributions of *MYOC* mutations causing POAG in Chinese (0.6-1.75%) (Fan et al. 2005; Jia et al. 2009; Pang et al. 2002) is lower than in Caucasians (2-4%) (Fingert et al. 1999) and even Japanese (~2.9%) (Ishikawa et al. 2004; Kubota et al. 2000). Regarding the other two causal genes, i.e., *OPTN* and *WDR36*, their roles in Chinese remain inconclusive. Although putative mutations in *OPTN* (Ile88Thr, Glu103Asp and His486Arg) and *WDR36* (Ile713Val) have been found in 1-1.6% (Fan et al. 2005; Leung et al. 2003) and 3.7% (Fan et al. 2009) of POAG patients, there is still a lack of convincing

evidence to support their roles as causative mutations. Among 176 HTG patients from northern Chinese, no mutation in *OPTN* and *WDR36* was identified (Jia et al. 2009). Recently, however, a missense mutation Lys322Glu in *OPTN* was found to be cosegregated with POAG in a family from the Harbin City in northeastern China. And this mutation was absent in 87 controls, suggesting that it is a causative mutation (Xiao et al. 2009). Therefore, *OPTN* is likely to contribute to a minor portion of POAG cases in China, but the mutation spectrum remains to be studied in larger samples from different areas of China.

In contrast to the disease-causing genes, information of POAG linkage loci in Chinese is sparse. So far, only one novel locus *GLC1N* has been identified in a Hong Kong family with JOAG (Wang et al. 2006b). And a known region 2p15-16 (Suriyapperuma et al. 2007) has been linked to JOAG in two Chinese families from Sichuan, China (Lin et al. 2008). Thus, more linkage loci are yet to be identified.

Regarding susceptibility genes, the *APOE epsilon 4* allele was found to confer a decreased risk to NTG (Fan et al. 2005; Lam et al. 2006) but not HTG (Jia et al. 2009). Recently, SNPs *TNF* rs1800629 (-308G>A) and *TP53 (tumor protein p53)* rs1042522 (Arg72Pro) have been found to be associated with HTG and NTG respectively, while SNPs in other candidate genes, namely *CDH1 (cadherin 1, type 1, E-cadherin), CDKN1A (cyclin-dependent kinase inhibitor 1A), CYP1B1, GSTM1 (glutathione S-transferase mu 1), GSTT1 (glutathione S-transferase theta 1), MTHFR (5,10-methylenetetrahydrofolate reductase), NOS3 (nitric oxide synthase 3),* and *OPA1*, did not show significant association (Fan et al. 2010). These findings reveal that the mutation and association profiles of POAG are fairly different between Chinese and other ethnic groups. Therefore, replication of known susceptibility genes and identification of novel disease genes for POAG in Chinese should be required. And these should depend on the methodologies described in the next sections.

1.3 Gene mapping for POAG

The identification of genes for POAG has been hindered by factors like 1) complexity in glaucoma phenotypes; 2) late age of disease onset in a large portion of patients; 3) unclear pattern of inheritance and reduced penetrance in families; 4) insidious development and progression that result in nearly half of the cases remaining undiagnosed; 5) potential intra-pedigree genetic heterogeneity; and 6) modification of environmental factors.

In the past two decades, the identification of genes underlying Mendelian forms of POAG has been enhanced by the remarkable development of rigorous position cloning, gene mapping and statistical methods. By using linkage analyses and candidate genes resequencing methods, a group of disease loci and genes had been identified. For complex form of disease, whose inheritance pattern is obscure, candidate gene and genome-wide association studies have led to the identification of a group of susceptibility genes. However, large gap remains between current knowledge and a thorough understanding of POAG genetics. Approaches such as linkage and association analyses and integrated methods will continue to play an active role in the gene mapping for the disease.

1.3.1 Genetic linkage study

For Mendelian forms of POAG, linkage analyses in one or more families with certain amount of affected and unaffected members have been used to identify the disease loci. Linkage analysis attempts to identify one (or more) chromosomal locus (loci) in the genome that is associated with the disease by determining which alleles in the locus are segregating with the disease in the families. Genetic markers evenly distributed throughout the genome are used to reduce the number of chromosomal regions. If a set of marker alleles at a certain region are co-segregated with the disease, these markers are assumed to locate near the disease gene (Ott 1999). Using linkage analysis, the likelihood that the loci

are linked could be determined by calculating the LOD score, which is a ratio of two likelihoods: the odds that the loci are linked and the odds that the loci are not linked. A LOD score of 3 or above is usually used as an indication of statistically significant linkage with a 5% chance of error (Mayeux 2005; Ott 1999). Once a location or a set of locations suggestive of linkage are identified, a finer-scale mapping strategy can be used to narrow the critical region, using either a denser set of additional microsatellites or SNPs within a smaller region underlying the highest LOD scores. With the linkage loci, candidate gene resequencing can then be used to identify the causal mutations in the pedigrees linked with the loci. These approaches had led to the identification of the *MYOC*, *OPTN* and *WDR36* genes for POAG (Monemi et al. 2005; Rezaie et al. 2002; Stone et al. 1997).

1.3.2 **Genetic association study**

While linkage analysis is a powerful method for identifying rare, high-risk alleles in Mendelian forms of POAG, genetic association analysis is a better method for identifying genetic variants associated with complex form of the disorder (Hirschhorn and Daly 2005; Neale and Sham 2004). Association study determines whether or not the allele of a specific marker (or a set of markers) is associated with an increased or reduced susceptibility of the disease. It can be conducted in a group of unrelated patients and controls, or in small families or affected sibling pairs. In a simplest form, the data used to test for association are often presented in contingency tables, in which rows usually describe an affection status and columns refer to specific alleles, genotypes or haplotypes. And a commonly used statistical test for association is the χ^2 test of independence (Gordon and Finch 2005). If the χ^2 statistic is greater than the value corresponding with a P value of 0.05, the null hypothesis of no association could be rejected at the 5% type I error rate, and this marker is said to be statistically associated with the disease. However, the conventional threshold of P

<0.05 for a genetic association is in general too loose, especially when multiple markers are involved, which can increase the chance of false-positive findings. Therefore, correction of the P values either by computer simulation or other correction methods for multiple comparisons, and replication of the initial association using independent cohorts from the same or different ethnic populations should be required (Neale and Sham 2004).

1.3.3 **Gene resequencing study**

Although a group of disease loci and genes for POAG have been identified by linkage analysis and association analysis (Tables 1.1 and 1.2), the causal genes at the rest loci and causal variants responsible for many of the initial association signals remain undetermined. Further linkage and association analyses are less capable to fill these gaps. Moreover, some common diseases are likely attributed to rare variants (MAF between 0.1% and 3%) that have greater effect sizes (odds ratio mostly greater than 2) (Bodmer and Bonilla 2008). However, current GWA approach is less capable to detect these rare genetic determinants. Thus, a resequencing approach will be helpful. First, sequencing of the candidate genes in known loci facilitates the identification of causal genes and mutations. Second, sequencing of a known causal gene will help to depict the mutation spectrum. Third, sequencing of the gene tagged by the initial associated-SNP will help to detect the causal variant in the coding or functional regions and to depict the allelic spectrum. Fourth, sequencing of a novel candidate gene will be helpful to evaluate its involvement in the disease. Finally, current massive parallel methods, e.g., next-generation exome sequencing, provide large amount of information at high speed and have successfully been used to identify the causative genes for some Mendelian diseases without prior linkage information, such as Miller syndrome (Ng et al. 2010) and Schinzel-Giedion syndrome (Hoischen et al. 2010). Thus, it could be

expected that some genes for the Mendelian forms of POAG will be discovered in the near future through these high-throughput sequencing platforms.

1.4 Statistical genetics for POAG

Statistical genetics is a combination of statistics and genetics, used for the analysis of genetic data. The principal statistics for linkage and association studies are different. In this thesis, as no linkage study is included, statistical methods for association are focused.

1.4.1 Hardy-Weinberg equilibrium test

Hardy-Weinberg equilibrium (HWE) is a term describing that, in the absence of migration, mutation, natural selection and assortative mating, genotype frequencies at any locus can be completely described by the allele frequencies (Gordon and Finch 2005). In genetic association studies, it is a common practice to check whether the observed genotypes conform to HWE, which appears to hold for most human populations. Deviations from HWE at a marker may suggest problems with genotyping or population substructure or, in the case group, a disease association with the marker (Wigginton et al. 2005). Test of HWE is commonly performed with a simple χ^2 goodness-of-fit test, or an exact test (Wigginton et al. 2005). A marker deviated from HWE in the control group is usually excluded for further association analysis.

1.4.2 Univariate analysis

In genetic association study, a case-control design is the most commonly used. After test for HWE, the association of individual marker with the disease is analyzed by using univariate analysis, which includes allele, genotype and linear trend tests. Genotype test is for both dominant or recessive genotypic effects and additive allelic effects. Allele test is

for additive allelic effect only and is invalid when HWE does not hold. Trend test is also only for additive allelic effects but remains valid when HWE violates (Clayton and McKeigue 2001). All the three types of case-control tests can be performed by using χ^2 test or permutation test. The odds ratios and confidence interval (CIs, usually at a 95% level) should also be estimated and reported. In study with small sample size, Fisher's exact test should be applied to evaluate the association (Clayton 2001; Clayton and Hills 1993).

1.4.3 **Multivariable analysis**

For multiple markers studied in a same study, multivariable analysis could be used after univariate analysis for individual marker. This analysis investigates the markers jointly, providing more information and aiming to find a set of markers that are significantly associated with the disease and to examine potential interactions among the markers. For this, a 2-stage strategy was proposed (Hoh et al. 2000): first, selection of a subset of markers that are "important" for the trait studied and, second, modelling maker-marker (i.e., gene-gene) interactions underlying the trait. For selection of markers, two approaches could be used (Ott and Hoh 2001): one is to individually select markers by using χ^2 test or logistic regression to test each marker, another is to use multivariable selection with methods such as logistic regression or set-association (Ott and Hoh 2001, 2003).

1.4.4 **Linkage disequilibrium**

Linkage disequilibrium refers to a nonrandom relationship between two alleles that typically arises because those alleles are closely linked on a chromosome and therefore infrequently separated from one another by recombination (Gordon and Finch 2005), which is the exchange of chromosomal material among the pair of chromosomes. When a new mutation, probably disease associated, occurs at a particular locus, the variant is physically

connected to all other alleles on the chromosome until it is subjected to recombination. However, association between the mutation and other alleles, especially for those located close to each other, can happen to be present on the same chromosome, even over subsequent generations. Such association gives rise to the observed LD. As the chromosome is passed from one generation to another, recombination breaks down the association, more so for those further apart. As such, LD decays over time or generations, rendering the region of strong disequilibrium around the mutation to be quite small after many generations (Wang et al. 2005). The LD method is important in mapping disease loci. SNPs can be a surrogate for the causal loci, providing a tool for the identification of causal genes in indirect association studies. In practice, various measures of pairwise LD have been proposed, with most capturing the strength of association between pairs of biallelic markers (Devlin and Risch 1995). The most frequently used LD coefficients are D' and r^2, with both ranging from 0 to 1. D' and its confidence bounds is useful to assess the probability for historical recombination between two loci in a given population. When two loci are completely unlinked, D'= 0. Whereas if the two loci are identical, then D'= 1. A D' value closer to 1 suggests that the marker locus is closer to the disease locus (Gordon and Finch 2005). Another common measure for LD is the squared coefficient of determination r^2, which is the most relevant measure for association mapping because there is a simple relationship between r^2 and the sample size required to detect the association between marker loci and the phenotype. Given that a LD of r^2 was measured between a causal locus and a nearby marker locus, to achieve the same power to detect association at the marker locus as that would have at the causal locus, the sample size should be increased by a factor of $1/r^2$ (Mueller 2004).

1.4.5 **Haplotype analysis**

Haplotype refers to a set of closely linked genetic markers present on one chromosome which tend to be inherited together (Gordon and Finch 2005). With the availability of high density SNP markers, haplotypes play an important role in association studies (Liu et al. 2008a). First, haplotypes are critical to understanding the LD pattern across human genome, which is essential for association studies (Ardlie et al. 2002). Second, methods based on haplotypes can be more powerful than those based on individual markers in association studies of mapping genes for complex disease (Akey et al. 2001). Third, haplotype-based methods can potentially capture cis-interactions between two or more causal variants. Biologically, several mutations on a haplotype may cause a series of changes in amino acid coding and therefore cause a larger joint effect on the phenotype (Schaid et al. 2002). Thus, haplotypes are more informative than individual genotypes for revealing disease mechanism at a candidate gene (Liu et al. 2008a).

In practice, it is difficult to obtain the haplotypes of each individual. Most current association studies use unphased genotype data, coupled with statistical and computational methods, to infer haplotypes through estimation of haplotype frequencies and resolution of haplotype pairs within individuals (Liu et al. 2008a). Two major approaches for parameter estimation, i.e., the frequentist's approach, which is usually based on the Expectation-Maximization (EM) algorithm (Long et al. 1995), and the Bayesian approach (Stephens et al. 2001), are well represented in haplotype inference. And these methods have been incorporated into different software programs for the estimation of haplotype frequencies, including haploview (Barrett et al. 2005), WHAP (Purcell et al. 2007a), PLINK (Purcell et al. 2007b), etc. Generally in haplotype association analysis, an omnibus test is performed over all haplotypes prior to testing for individual haplotype-phenotype association, as a test for individual haplotype is valid only if the omnibus test is statistically significant.

1.4.6 **Correction for multiple testing**

When multiple markers are tested in association study, such multiple testing will increase the false-positive (type I error) rate under nominal significance thresholds, e.g., α=0.05. Thus, in order to reduce false-positives, original P values should be corrected. A simple and commonly used method is the Bonferroni correction (Tarone 1990), in which individual marker is tested at a significance level of α/n, where n is the number of markers studied. However, this method is highly conservative because some of the markers may be correlated (e.g., in LD). Recently, a simple correction method was proposed for multiple testing of SNPs in LD, based on the spectral decomposition (SpD) of matrices of pairwise LD between SNPs (Nyholt 2004). Using this method, an effective number of independent SNPs (M_{eff}) is estimated and then the significance threshold is corrected for M_{eff} tests rather than n tests, where $M_{eff} \leq n$. This method is available in the SNP spectral decomposition (SNPSpD) website (http://gump.qimr.edu.au/general/daleN/SNPSpD/). Other approaches for correction of multiple comparisons include the estimation of false discovery rate (FDR) (Sabatti et al. 2003), etc.

1.5 Bioinformatics

Apart from statistical genetics, bioinformatics is also an important tool in genetic studies. Bioinformatics is predominantly a discipline for handling genetic information. It involves research, development or application of computational tools and approaches to acquire, store, visualize and interpret medical or biological data. Bioinformatics serves mainly for: (1) genome sequencing, annotation and comparison, (2) sequence analysis, (3) expression data analysis, (4) protein structure prediction, and (5) modeling of biological networks (Akalin 2006). For each application, different bioinformatics tools are available.

Efficient access to these tools and analyses of bioinformatics data are important for integrating research findings and suggesting new hypotheses.

1.5.1 Analysis of variants

In the absence of *in vivo* functional assays of a gene product, the pathogenicity of an observed variation is usually inferred by assessing both the predicted effect of the variation on the structure of the gene product, and the distribution of the variant among patient and control populations (Alward et al. 2002).

Different web-based programs are available for predicting the impact of a missense variant to the protein, such as PolyPhen and SIFT. PolyPhen (Polymorphism Phenotyping; http://genetics.bwh.harvard.edu/pph/) predicts the functional effect of amino acid changes based on evolutionary conservation, physiochemical differences, and the proximity of the substitution to predicted functional domains and/or structural features (Ramensky et al. 2002; Rudd et al. 2005). PolyPhen uses empirically derived rules to predict that an nonsynonymous SNP is probably damaging, i.e., it is with high confidence supposed to affect protein function or structure; possibly damaging, i.e., it is supposed to affect protein function or structure; benign, most likely lacking any phenotypic effect; or unknown, when in some rare cases, the lack of data do not allow PolyPhen to make a prediction. SIFT (Sorting Intolerant From Tolerant; http://sift.jcvi.org/) predicts whether an amino acid substitution affects protein function based on sequence homology and physical properties of amino acids (Kumar et al. 2009; Ng and Henikoff 2001, 2003). SIFT predicts a substitution to affect protein function if the scaled probability, also termed SIFT score, lies below a certain threshold value. Generally, a highly conserved position is intolerant to most substitutions, whereas a poorly conserved position can tolerate most substitutions (Kumar et al. 2009).

For variants detected in splice sites, i.e., splice donor sites or splice receptor sites, different tool sets could also be applied. For example, the intron analysis tool set in the Alternative Splicing Database (ASD; http://www.ebi.ac.uk/asd-srv/wb.cgi) (Stamm et al. 2006) could be used to predict the potential functional impacts of the variants to alternative splicing, such as Donor Score analysis, which computes Donor Site Scores, and Acceptor Score analysis, which computes Acceptor Site Scores. In these analyses, the individual donor (and acceptor) sites are scored using a weight matrix approach (Clark and Thanaraj 2002). This results in an information content score for each donor spice site. The score is essentially a measure of the binding affinity for U1 snRNA. The donor sites scoring less than 3 bits is considered as weak (Clark and Thanaraj 2002). Another web-based program Automated Splice Site Analyses (ASSA; https://splice.uwo.ca/) (Nalla and Rogan 2005; Rogan et al. 1998) is also useful to evaluate changes in splice site strength based on information theory-based models. In the analysis, the information content (Ri) changes obtained at the donor or acceptor sites, which are caused by the mutation, are categorized into increased, decreased, and no change. Furthermore, the initial Ri, i.e., initial information content measured at the base before the mutation is made, and the final Ri, i.e., final information content measured at the base after the mutation is made, are presented. Finally, the table cells indicating the information contents of predicted binding sites are color-coded by direction and type of change in Ri value. Mutations that inactivate or create leaky, cryptic sites and display preexisting cryptic sites whose Ri values are unchanged are separately coded to facilitate interpretation. A legend indicating the codes and their significance is presented at the end of each analysis (Nalla and Rogan 2005).

Chapter 2

Objectives

Although under intensive investigations, understanding of POAG genetics remains limited. The genes that have been identified only explain a small portion of cases. Causal genes at most of the linkage loci remain to be identified. Many genetic association signals, especially for those reported recently, remain to be verified. Moreover, it is conceivable that new genes will be discovered by using various genetic methodologies. Thus, in the work of my thesis, we aimed to identify new genes for POAG by using a three-tiered strategy.

In the first part, we aimed to identify new genetic profiles of reported loci, genes, or markers for POAG, including:

1) Evaluation of candidate SNPs at or near the *CYP46A1*, *LMX1B*, *PLXDC2*, *TLR4*, *TMTC2* and *ZP4* genes and the 2p16.3 locus in POAG. As introduced above, makers around these genes have recently been associated with POAG. Through a candidate-SNP association approach, we would be able to verify the initial association signals, identify new association patterns, and confirm one or more genes (or markers) as disease genes (or markers) for POAG.

2) Evaluation of multiple SNPs at the *CAV1/CAV2* locus in POAG by using a candidate-SNP association approach. SNP rs4236601 at this locus has recently been associated with POAG across different populations. Through our study of this SNP and five other SNPs, we would be able to further verify the associations of these SNPs with POAG, and dissect the haplotype phase of the risk and protective alleles at different SNPs to assess the role of the genes in the disease.

3) Evaluation of the *NTF4* gene as a disease gene for POAG by using a candidate-gene resequencing approach. Through this work, we would be able to investigate its mutation spectrum, identify new mutations, compare the mutation spectrums across different populations, and assess the role of the gene in POAG.

In the second part, we aimed to search for new genes for POAG by assessing functional candidates, including:

1) Evaluation of the *CNTF* gene as a disease gene for POAG by using a candidate-gene resequencing approach. Through this work, we would, for the first time, investigate the allelic spectrum of *CNTF* in the context of POAG, and assess *CNTF* as a gene for the disease.

2) Evaluation of the *SPARC* gene as a disease gene for JOAG and as the causal gene at the GLC1M locus for JOAG, by using candidate-gene resequencing and copy number analysis. Through this work, we would, for the first time, investigate the allelic spectrum and copy number variation of *SPARC* in the context of JOAG, and assess *SPARC* as a gene for the disease.

In the third part, we aimed to identify the causal gene at the GLC1N locus, by using:

1) A regional candidate-gene resequencing approach to identify gene variant(s) that are cosegregated with the disease in the GLC1N-linked Hong Kong pedigree with JOAG.

2) A candidate-gene resequencing approach to depict the mutation spectrum of the *MEGF11* gene in unrelated POAG patients and controls to verify the gene as a disease gene for POAG.

Chapter 3

Materials and Methods

3.1 Study participants

3.1.1 Research ethics

All studies described in this thesis have been approved by the Ethics Committee for Human Research, the Chinese University of Hong Kong; the Ethics Committee at the Joint Shantou International Eye Center; and the Ethics board of the Beijing Tongren Hospital. All procedures conducted conformed to the tenets of the Declaration of Helsinki. Venous blood was collected from each subject after collection of written Informed Consent, and stored in -80℃ prior to subsequent laboratory analyses.

3.1.2 Recruitment of study subjects

Inclusion criteria were the same at the three patient recruitment centers. Complete ocular examinations were performed for each participant before enrolment, including measurement of visual acuity, refraction, intraocular pressure, gonioscopy, fundus examination, and visual field examination. Patients with POAG were diagnosed according to the following criteria: (1) exclusion of secondary causes, e.g., trauma, uveitis or steroid-induced glaucoma. Also excluded were patients with congenital glaucoma, whose IOP was raised in the first five years of life, with clinical findings consistent with prenatal or infantile onset of glaucoma, such as breaks in Descemet's membrane, enlarged cornea, or buphthalmos; (2) Shaffer grade III or IV open iridocorneal angle on gonioscopy; and (3) glaucoma was defined based on the presence of visual field defects regardless of the optic nerve head and retinal nerve fiber layer appearance. Visual field testing was performed using static automated white-on-white threshold perimetry (SITA Standard 24-2, Humphrey

Field Analyzer II). A visual field was defined as reliable when fixation losses, and false-positive and false-negative errors were <20%. Average visual field sensitivity was expressed in mean deviation (MD), as calculated by the perimetry software. A visual field defect was defined as having ≥3 significant ($P<0.05$) nonedge contiguous points with ≥1 at the $P<0.01$ level on the same side of horizontal meridian in the pattern deviation plot and confirmed with ≥2 consecutive examinations. Family histories of the subjects were self-identified. Except for the members of the two pedigrees in which the GLC1M and GLCIN loci were identified, no patient has known family history of glaucoma.

Intraocular pressure level was not a diagnostic criterion but a parameter for classification of POAG. Patients with untreated IOP consistently greater than 21 mmHg were classified as HTG. Patients were classified as NTG if six median untreated IOP readings were consistently less than 21 mmHg, with no more than 1 reading equal to 23 or 24 mmHg and no single measurement more than 24 mmHg, as per the Collaborative NTG Study (Anderson et al. 2001). At least 2 readings were obtained at a different time of the day from the rest.

For POAG, the age of onset is difficult to determine because of the absence or unawareness of symptoms in the early stages. Therefore the age at diagnosis (AAD) was recorded and used as a surrogate for age of onset in our study. The AAD was defined as the age at which the initial diagnosis of glaucoma was made. All the patients have records of no obvious vision impairment one year before diagnosis.

Control subjects were recruited from the elderly participants to the eye clinics for senile cataract, floaters or mild refractive errors. In order to reduce the chance of developing glaucoma later in life, only subjects with age of ≥60 years were recruited. They had gone through complete ocular examinations and were diagnosed free of glaucoma, having a normal visual field and no history of intraocular pressure >21 mmHg. They had no local or

systemic illness that might cause glaucoma or optic disc changes. They had no other major eye diseases such as macular diseases. They had no myopia or mild myopia with refractive errors of -3.00 D or less. Individuals with known family history of glaucoma were excluded.

In order to reduce potential confounding from other genetic diseases, subjects with known clinical history of systemic diseases, such as tumor, diabetes, cardiovascular diseases, severe hypertension, asthma, or neurological diseases, were excluded.

3.1.3 **Descriptions of overall study subjects**

Three cohorts of Chinese Han subjects were included. Study subjects in the Hong Kong cohort were recruited from the eye clinics in the Hong Kong Eye Hospital and the Prince of Wales Hospital, Hong Kong. Study subjects in the Shantou cohort were recruited from the Joint Shantou International Eye Center of Shantou University and the Chinese University of Hong Kong, Shantou, China. Shantou is located at the southeast coastal area of Guangdong Province. Thus, subjects in these two cohorts may represent southern Han Chinese. In contrast, study subjects from the third cohort were recruited at the Beijing Tongren Eye Center. This cohort is comprised of participants from northern China.

Totally, 1645 unrelated Chinese subjects were recruited. The Hong Kong cohort is composed of 281 sporadic patients with HTG, 311 with NTG and 248 controls. The Shantou cohort included 102 sporadic patients with HTG, 28 with NTG and 298 controls. The Beijing cohort comprised 177 sporadic HTG patients and 200 controls. Demographic and clinical characteristics of the study subjects are shown in Table 3.1.

Also included were the family members from the GLC1M-linked Philippine pedigree with JOAG and the GLC1N-linked Hong Kong pedigree with JOAG. Clinical features of these familial subjects were described in sections 3.4.5 and 3.4.6.

Table 3.1. Demographic and clinical characteristics of study subjects

	Hong Kong cohort			Shantou cohort			Beijing cohort	
	HTG	NTG	Control	HTG	NTG	Control	HTG	Control
Sample size	281	311	248	102	28	298	177	200
Female (%)	38.1	47.9	55.2	20.0	25.0	56.0	21.5	25.0
Mean age ± SD (years)*	52.4 ± 19.1	64.3 ± 13.2	73.6 ± 7.4	45.2 ± 20.8	50.7 ± 20.0	70.4 ± 8.7	38.9 ± 16.3	69.4 ± 6.0
Mean IOP ± SD (mmHg)	31.0 ± 9.4	18.2 ± 2.8	13.5 ± 3.0	36.5 ± 9.6	18.3 ± 3.1	12.7 ± 2.9	36.8 ± 11.0	13.0 ± 3.0
Mean VCDR ± SD	0.7 ± 0.2	0.7 ± 0.1	0.3 ± 0.1	0.8 ± 0.2	0.7 ± 0.2	0.3 ± 0.1	0.9 ± 0.1	0.3 ± 0.1
Mean MD ± SD (dB)	-12.2 ± 9.5	-11.0 ± 9.0	WNR	-20.5 ± 14.6	-16.8 ± 11.1	WNR	-16.9 ± 10.0	WNR
Mean PSD ± SD (dB)	7.3 ± 4.2	6.6 ± 4.0	WNR	7.2 ± 4.1	7.4 ± 4.6	WNR	9.6 ± 4.0	WNR

Note:

* For patients, age at diagnosis was used for calculate the mean and standard deviation, while for controls the age at recruitment was used.

IOP: intraocular pressure; HTG: high-tension glaucoma; MD: mean deviation; NTG: normal-tension glaucoma; PSD: pattern standard deviation; SD: standard deviation; VCDR: vertical cup/disc ratio; WNR: within normal range.

3.2 General study design

The studies of this thesis are cross-sectional, case-control, candidate-gene resequencing or candidate-SNP association studies. The study design for individual investigation will be described in detail in section 3.4.

3.3 Laboratory methods

3.3.1 DNA extraction

Genomic DNA was extracted from 200µl EDTA-blood using Qiagen QIAamp DNA blood mini kit (Qiagen, Hilden, Germany) according to the manufacturer's blood and body fluid spin protocol. The final genomic DNA was eluted with 150µl ddH_2O. Concentration of the extracted DNA was measured by a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

3.3.2 Genotyping

3.3.2.1 Polymerase chain reaction (PCR)

PCR was carried out on a Perkin-Elmer 9700 thermal cycler (Applied Biosystems [ABI], Foster City, CA, USA). PCR primers were designed, using the software Primer3 (v.0.4.0; http://frodo.wi.mit.edu/primer3/) (Rozen and Skaletsky 2000), to amplify the target sequence, including coding sequence, exon-intron boundaries, promoter sequence, and sequence of a part of the 5'-UTR and the 3'-UTR, according to the published gene sequences of the candidate genes from the Ensembl database (Hubbard et al. 2002). PCR was carried out in a total volume of 25µl containing 20 mM Tris-HCL (pH 8.4), 50 mM KCL, amplicon specific concentration of $MgCl_2$, 0.4µM of each amplicon specific primer, 200µM dNTPs, 1 U TaqGold DNA polymerase (ABI) and 200ng template DNA. The thermal cycling program consisted of an initial denature step at 94°C for 12 minutes, a 40-

cycle reaction comprising a denature step at 94℃ for 40 seconds, an annealing step at amplicon specific temperature for 40 seconds and an extension step at 72℃ for 1 minute, and a final extension step at 72℃ for 10 minutes. Annealing temperatures for the primers are listed in the tables for each specific study.

After PCR, 5μl of each PCR product was used for agarose gel electrophoresis to check the size of the product and the quality of the amplification.

3.3.2.2 Direct DNA sequencing

Amplified PCR products were sequenced using the cycle sequencing protocol on an ABI 3130XL automated DNA sequencer (Applied Biosystems [ABI], Foster City, CA, USA). Briefly, 10μl of each PCR product were treated with 2μl ExoSAP-IT (GE Healthcare Life Sciences, USA) at 37℃ for 45 minutes to remove excess primers, and dephosphorylate excess dNTPs before the sequencing reaction. The enzymes were then inactivated by incubation at 80℃ for 15 minutes. Next 2.5μl of this treated PCR product was used for sequencing reactions using the BigDye® Terminator v3.1 Cycle Sequencing Kit (ABI) in a total volume of 5μl using a default condition according to the manufacturer's instructions. After the reaction, the products were precipitated by 3.5μl 7.5M ammonium acetate, 34μl 100% ethanol, and 5.0μl H_2O at -80℃ for 15 minutes and washed for two times with 70% ethanol. The precipitated DNA samples were air-dried and resuspended in 10μl Hi-Di™ formamide, and further diluted with Hi-Di™ to make a 1:10 dilution. The diluted samples, contained in a sequencing plate, were denatured at 94℃ for 2 minutes and then put on ice immediately. Next the plate was loaded onto the 3130XL sequencer. The standard program was used for capillary electrophoresis and data collection. The sequencing data were compared manually with the published reference gene sequence for detection of variants.

3.3.2.3 TaqMan SNP genotyping

For SNPs that were not genotyped by direct DNA sequencing, genotyping was carried out by using TaqMan technology. Briefly, validated genotyping assays containing specific PCR primers and TaqMan MGB probes were purchased from the company (Applied Biosystems [ABI], Foster City, CA, USA). Reactions were performed in 96-well optical microplates using the ABI 9700 thermal cyclers (ABI) with a default condition according to the manufacturer's instructions. Fluorescence was measured using the ABI Prism 7000 real-time PCR system and analyzed using the Sequence Detection System (SDS) software (ver. 1.2.3, ABI). Clustering of the genotypes was conducted manually.

3.3.3 **Copy number analysis**

Copy number analysis was performed using the TaqMan® Copy Number Assays (Applied Biosystems [ABI], Foster City, CA, USA). According to the manufacturer's instructions, the DNA samples were diluted to a concentration of 5.0ng/μl. Each PCR reaction mix contained 5.0μl of 2×TaqMan® Genotyping Master Mix, 0.5μl of TaqMan Copy Number target assay, 0.5μl of TaqMan Copy Number reference assay (RNase P), which is known to exist only in two copies in a diploid genome, 2.0μl of Nuclease-free water and 2.0μl of DNA. The reactions were processed in an ABI 7900HT Fast real time PCR System using a 384-well reaction plate, with each DNA sample analyzed in duplicates, and on 95°C/10min for 1 cycle followed by 92°C/15sec and 60°C/1min for 40 cycles. Data was collected by the SDS software (version 2.3, ABI) using the standard absolute quantification method. After the reaction, raw data was analyzed using a manual cycle threshold of 0.2 with the automatic baseline on, and then imported to the CopyCaller™ Software (version 1.0, ABI) for post-PCR data analysis. In the software, copy numbers

were estimated using a maximum likelihood algorithm. The analytical setting was the same for the three assays.

3.4 Specific materials and methods in individual study

3.4.1 Investigation of multiple gene polymorphisms in POAG

3.4.1.1 Study subjects

This study involved 1247 out of the 1645 total subjects. This sample included a Hong Kong cohort of 185 HTG patients, 206 NTG patients and 230 controls, a Shantou cohort of 102 HTG patients and 147 controls, and a Beijing cohort of 177 HTG patients and 200 controls. Demographic and clinical features of these subjects are shown in Table 3.2.

Among the three study cohorts, the Hong Kong cohort was used as the exploratory cohort, while the Shantou and Beijing cohorts were used for validation. For exploratory purpose, a sample size of 200 cases and 200 controls provides 80% of power to detect association at α level of 0.05, assuming the minor allele frequency of the variant was 30% and the odds ratio 1.8. Thus, the sample size of the Hong Kong cohort could provide a good statistical power for the replication of most reported associations, except for some variants that were present at much lower frequencies in our population. Any significant association detected in the Hong Kong cohort was verified in the Shantou cohort then the Beijing cohort. An association was considered replicable if the trend was at the same direction and the P value became smaller when the samples from different cohorts were pooled together.

3.4.1.2 Strategies for SNP selection and genotyping

In this work, a total of 15 SNPs around 7 genes/loci were investigated. SNPs in *TLR4* were selected using a haplotype-based tag-SNP strategy. The Tagger algorithm in Haploview (Barrett et al. 2005) version 4.1 was used to choose SNPs that optimally capture

Table 3.2. Demographic and clinical characteristics of the study subjects involved in the investigation of multiple gene polymorphisms

Group	Sample Size	Female (%)	Age at Diagnosis (years)*		Highest Recorded IOP (mmHg)		Vertical Cup/disc Ratio at Enrolment	
			Range	Mean (SD)	Range	Mean (SD)	Range	Mean (SD)
Hong Kong Cohort								
HTG	185	64 (34.8)	11-88	59.7 (16.6)	22-69	31.0 (9.4)	0.2-1.0	0.7 (0.2)
NTG	206	99 (48.1)	20-85	61.6 (12.9)	10-21	18.2 (2.8)	0.2-1.0	0.7 (0.1)
Control	230	124 (53.9)	60-94	73.5 (7.5)	6-20	13.5 (3.0)	0.1-0.5	0.3 (0.1)
Shantou cohort								
HTG	102	20 (20.0)	11-85	45.2 (20.8)	22.5-58	36.5 (9.6)	0.2-1.0	0.8 (0.2)
Control	147	94 (63.9)	63-96	74.0 (6.4)	7-21	12.7 (2.9)	0.2-0.5	0.3 (0.1)
Beijing cohort								
HTG	177	38 (21.6)	10-82	38.9 (16.3)	22-70	36.8 (11.0)	0.4-1.0	0.9 (0.1)
Control	200	50 (25.0)	61-85	69.4 (6.0)	10-21	13.0 (3.0)	0.2-0.5	0.3 (0.1)

Note:

* For the control subjects, the age refers to the age at study enrolment.

common haplotypes within the gene, using the algorithm based on an r^2 threshold of 0.8 between SNPs in the Chinese population (International HapMap Project). Four SNPs were tagged: rs12377632, rs1927907, rs11536889 and rs7037117. Except for *TLR4*, SNPs in other genes/loci were selected according to the initial reports. In the *LMX1B* gene, four SNPs (i.e., rs944103, rs16929236, rs10733682 and rs867559) that conferred the highest odds ratios for POAG (Park et al. 2009) were selected. For SNPs showing protective effects, rs7854658 was selected because of its strong association with pooled HTG and NTG (Park et al. 2009), although it occurs at very low frequency in Chinese (HapMap). In *CYP46A1*, rs754203 was the only SNP investigated in the initial report (Fourgeux et al. 2009). At chromosomal region 2p16.3, the two SNPs rs1533428 and rs12994401 were the most strongly associated with POAG in the initial study cohort (Jiao et al. 2009). And, SNPs rs693421, rs7081455 and rs7961953 at chromosomal regions 1q43 (nearest gene: *ZP4*), 10p12.31 (nearest gene: *PLXDC2*) and 12q21.31 (*TMTC2*) respectively were selected as they gave the highest GWA signals in the initial study (Nakano et al. 2009). The other three SNPs rs547984, rs540782 and rs2499601 at 1q43 that also showed similar associations (Nakano et al. 2009) were not selected because they were well tagged by rs693421.

All selected SNPs were genotyped in the Hong Kong cohort using TaqMan® SNP genotyping assays (ABI). SNPs showing significant association in the Hong Kong cohort were genotyped in the Shantou cohort for verification. Finally two SNPs, i.e., *TLR4* rs7037117 and 2p16.3 rs1533428, which showed consistent associations with POAG, were genotyped in the Beijing cohort.

3.4.2 Investigation of *CAV1* and *CAV2* polymorphisms in POAG

3.4.2.1 Study subjects

This study is the latest one included in this thesis, involving 1549 of the overall 1645 subjects. The study subjects in this study included a Hong Kong cohort of 185 HTG patients (the same with that used in the multiple gene study as described in the previous section; Table 3.2), 311 NTG patients and 248 controls (Table 3.1), a Shantou cohort of 102 HTG, 28 NTG and 298 controls (Table 3.1), and a Beijing cohort of 177 HTG patients and 200 controls (Table 3.1). Because some study subjects were newly recruited after the completion of the previous study, the sample size was therefore larger in this study.

3.4.2.2 SNPs selection and genotyping

Across the *CAV1* and *CAV2* gene, six SNPs were selected according to the findings of our GWA study for NTG (unpublished data). Briefly, the Illumina HumanHapCNV370 bead chip was used for genotyping. After data filtering, genotype data from 212 cases with NTG and 226 controls were qualified for association analysis. The genomic inflation factor was 1.00045 suggestive of weak population stratification. In the region of the *CAV1* and *CAV2* genes, 36 SNPs passed the filtering procedure and were included for association analyses. These SNPs span a 260kb region flanked by rs4483091 and rs1049337. The association of each SNP with NTG, presented as a minus logarithm of the P value, was plotted against the SNP location in Figure 1.6. Three SNPs, i.e., rs926201 (location: 7q31, 115,793,341), rs6975771 (115,910,081) and rs959173 (115,969,290), showed significant disease association (P=0.048, 0.038 and 0.027 respectively). SNP rs4236601 (115,949,965), detected in the GWA study of Thorleifsson et al., showed a trend towards an increased risk of the disease (OR=2.69, P=0.22). In our data, this SNP showed strong LD with two flanking SNPs, rs4730742 (115,925,128) and rs6466587 (115,985,237). Thus, these 6 SNPs were selected for further investigation using a larger sample in this study.

All of the six candidate SNPs were genotyped in both the Hong Kong and Shantou subjects using TaqMan® SNP genotyping assays (ABI). Three SNPs rs4236601, rs6975771 and rs959173 that showed the most significant associations in the southern cohorts were genotyped in the Beijing subjects. For validation of the genotyping results, the genotypes of the Hong Kong subjects that overlapped in the GWA study and this present study (n=438) were compared. All genotypes were consistent.

3.4.3 **Mutation screening of the *neurotrophin-4* gene**

3.4.3.1 Study subjects

This study firstly involved subjects described in section 2.4.1, except the controls of the Shantou and Beijing cohorts because no putative mutation was detected in the case subjects of these cohorts. After the 28 Shantou NTG patients were recruited, they were also added into this study. Thus, the *NTF4* gene was screened in 928 subjects, including a Hong Kong cohort of 185 HTG patients, 206 NTG patients and 230 controls, a Shantou cohort of 102 HTG and 28 NTG patients, and a Beijing cohort of 177 HTG patients. Demographic features of these subjects were shown in Tables 3.1 and 3.2.

3.4.3.2 Mutation screening strategies and technologies

PCR primers were designed referring to published gene sequence of *NTF4* (ENSG00000167744) from Ensembl (Table 3.3). The target sequences covering the entire coding region and exon-intron boundaries were amplified using PCR and analyzed by direct DNA sequencing. The method in detail has been described in section 3.3.2.

3.4.4 **Mutation screening of the *ciliary neurotrophic factor* gene**

3.4.4.1 Study subjects

Table 3.3. Primer sequences and PCR conditions for *NTF4* sequencing

Amplifying target	Primer sequence		MgCl2 (mM)	Ta (°C)	Size (bp)
	Forward primer (5'→3')	Reverse primer (5'→3')			
NTF4-1	ACTTGAAGAGGAACTCTGGGAAG	TCAAAACTGCCACTAAGGAGGTA	1.5	58	412
NTF4-2A	CTTCTTTCCCCACTGAAGTTTTT	CACCTTCCTCAGCGTTATCAG	1.5	58	524
NTF4-2B	CCCCGAGTAGTCCTGTCTAGG	CTCTCAGCATCCAGCTCTGTTAT	1.5	58	544

Note:

The primers were designed according to the sequence of *NTF4* (ENSG00000167744) from the Ensembl database.

Ta: annealing temperature; bp: base pairs.

In this work, the hypothesis that variants in the *CNTF* gene are likely to be causative for or associated with POAG was tested by screening the gene in a subset of study subjects, including 102 randomly selected NTG patients (mean age 65.6 ± 13.1 years) and 103 randomly selected controls (mean age 75.1 ± 7.1 years) from the Hong Kong cohort, and the 102 HTG patients (mean age 45.2 ± 20.8 years), 28 NTG patients (mean age 50.7 ± 20.0 years) and 134 randomly selected controls (mean age 74.0 ± 6.4 years) from the Shantou cohort. In a lack of consistent evidence to support our hypothesis, no additional subjects were involved in this investigation.

3.4.4.2 Mutation screening strategies and technologies

PCR primers were designed referring to published gene sequence of *CNTF* (ENSG00000242689) from the Ensembl database (Table 3.4). The target sequences covering the coding region and exon-intron boundaries were amplified using PCR and analyzed by DNA sequencing. The method in detail has been described in section 3.3.2.

3.4.5 Mutation screening of *SPARC* gene for JOAG

3.4.5.1 Study subjects

First, the *SPARC* gene was screened in the GLC1M-linked Philippine pedigree as described in section 1.2.1.6. This is a five-generation family consisted of 95 members, of which 22 were affected. Peripheral venous blood was collected from 27 members who gave consent to the study and underwent complete ophthalmic examinations. Nine of the 27 subjects were affected, with age at diagnosis ranging from 12 to 33 years, the highest IOP between 24 and 44 mmHg, VCDR ranging from 0.7 to 0.9, and visual field damage compatible with glaucoma in two consecutive tests. The unaffected members aged from 3 to 73 years, with IOP <22 mmHg, VCDR between 0.2 and 0.5, and normal visual field.

Table 3.4. Primer sequences and PCR conditions for *CNTF* sequencing

Amplifying target	Primer sequence		MgCl2 (mM)	Ta (°C)	Size (bp)
	Forward primer (5'→3')	Reverse primer (5'→3')			
Exon-1	GAGAGAGATGGAGGCAGACG	GAGCTGATGCGTGATGGTAA	1.5	58	448
Exon-2	ATCCTTGGCCAGAGAGATGA	GAGGCCCCAGGGAACTAC	1.5	58	661

Note:

The primers were designed according to the sequence of *CNTF* (ENSG00000242689) from the Ensembl database.

Ta: annealing temperature; bp: base pairs.

Also enrolled were 46 unrelated Chinese patients with sporadic JOAG and 95 randomly selected controls from the Hong Kong cohort. Of the patients, the age at diagnosis ranged from 6 to 40 years (mean±SD: 25±8.5 years), highest recorded IOP in the more severely affected eye between 23 and 69 mmHg, and VCDR 0.5-0.9. Control subjects aged from 61 to 94 years (mean 75.1±7.1 years).

3.4.5.2 Mutation screening strategies and technologies

The *SPARC* sequences were screened in the 27 participants from the Philippine pedigree, the 46 Chinese JOAG patients and 95 controls. Primers were designed referring to the published gene sequence of *SPARC* (ENSG00000113140) in the Ensembl database (Table 3.5). The amplified sequences covered part of the promoter (-1 to -318bp from the transcription initiation site), the 5'-UTR (+1 to +314bp on the mRNA, noncoding), the entire coding region (c.1 to c.912), exon-intron boundaries, and part of the 3'-UTR (c.912+1 to +94bp). The target sequences were amplified using PCR and analyzed by direct DNA sequencing. The method in detail has been described in section 3.3.2.

Copy number analysis of *SPARC* was performed for all of the 27 family subjects and 18 randomly selected Chinese JOAG patients (10 females) and 18 controls (9 females), using TaqMan® Copy Number Assays (ABI). Three assays were selected for this purpose (Table 3.6), with one being located in proximity to the 5'-end of *SPARC*, one near the 3'-end, and one within the gene. The method has been described in detail in section 3.3.2.

3.4.6 Candidate genes screening at GLC1N and mutation screening of *MEGF11*

3.4.6.1 Study subjects

As described in section 1.2.1.7, a linkage locus GLC1N had been identified by our group in a Hong Kong pedigree with JOAG (Wang et al. 2006b). In this current study, a

Table 3.5. Primer sequences and PCR conditions for *SPARC* sequencing

Amplifying target	Primer sequence		MgCl2 (mM)	Ta (°C)	Size (bp)
	Forward primer (5'→3')	Reverse primer (5'→3')			
SPARC-1 (promoter + exon 1)	CCAGTTCCAAATCATCAAGGA	GGGGTTGGTGCAACTATAGAA	1.5	59	668
SPARC-2 (exon 2)	AAATGGAACCAACCTCCTCA	CAATGGTCCTCATCCCAGTT	1.5	60	388
SPARC-3 (exon 3)	AGCTCCCCTAGCCTGTATCC	CCCTAATTTCTCAGGGCACA	1.5	60	225
SPARC-4 (exon 4)	CTTTCCCTAACACCCCTGGT	TCATGTAGGCTGTCCTCGTG	1.5	60	367
SPARC-5 (exon 5)	TGTGCTAGTCCAGGTGATGC	TGTATTCCGAAGTGCCCAAT	1.5	60	222
SPARC-6 (exon 6)	CAGTGTCCCCATCTCTGAAA	CCCAAGACAGGAGTCTGGAA	1.5	60	250
SPARC-7 (exon 7)	AAGAAACTGTGGCCTGGAGA	CTGGTGCTCAGGGGTAAATG	1.5	60	396
SPARC-8 (exon 8)	CTGGCTAGTCTCTGCCTGCT	TCACTCTAGGGTCTGGGGTCT	2.0	60	279
SPARC-9 (exon 9)	GGGTGTGGAGCTTTTCCAT	CCCCTTGCTTCTTTGTTCAG	1.5	60	229
SPARC-10 (exon 10)	TCCACTGACTCCTTGGGAAG	GGCAGAACAACAAACCATCC	1.5	60	198

Note:

The promoter (-1 to -318bp from the transcription initiation site), 5'-untranslated region (the noncoding exon 1), coding regions, exon-intron boundaries, and a portion of the 3'-untranslated region (+1 to +94bp downstream the stop codon) were covered by the amplimers.

The primers were designed according to the sequence of *SPARC* (ENSG00000113140) from the Ensembl database.

Ta: Annealing temperature; bp: base pairs.

Table 3.6. TaqMan Copy Number Assays used for copy number analysis of *SPARC*

Assay ID	Reporter dye	Context Sequence	Location on NCBI Assembly
Hs02667978_cn	FAM	GTCTCAAAACCCCAGCTCAAAATAC	151021358
Hs06106867_cn	FAM	GTCAGAAGGTTGTTGTCCTCATCCC	151027253
Hs06124887_cn	FAM	CTTCCCAGAGGTGTGGATTAATGGT	151046100

Note:

Assay Hs02667978_cn is located in proximity to the 5'-end of the SPARC gene, Hs06106867_cn is located within the gene, and Hs06124887_cn is in proximity to the 3'-end of the gene.

candidate gene screening was performed in the pedigree, seeking to identify the causal gene at GLC1N. To date, there are 39 members in this pedigree, among which eight were affected (Figure 3.1). The clinical features of these affected members were shown in Table 3.7. For this current investigation, all the eight affected members and another 22 unaffected members were included. Moreover, two additional unaffected subjects were included, namely the mother (II:15M) and sister (II:15S) of subject II:15 (Figure 3.1).

After the identification of a putative causative gene, i.e., *multiple epidermal growth factor-like domains protein 11* (*MEGF11*, OMIM *612454), three groups of subjects were screened for mutations, including 181 HTG patients and 182 controls from the Hong Kong cohort, 177 HTG patients and 200 controls from the Beijing cohort, and 95 HTG patients and 147 controls from the Shantou cohort. Notably, this study is the earliest research work involved in this thesis. The number of subjects involved was smaller than that of the total subjects. Especially for the Hong Kong cohort, 96 of the 181 HTG subjects were not included in the preceding 5 studies mainly because of the exhaustion of DNA samples. Among the 181 HTG patients, 73 (40.3%) were female. The age at diagnosis ranged from 8 to 73 years, with a mean of 42.1 (±14.9) years. Among them, 77 (42.5%) patients had an AAD of ≤40 years. For the other subject groups, the characteristics were similar to that described in section 3.4.1 and Table 3.2. Moreover, no NTG patients were involved in this study because the affected subjects in the pedigree featured typical HTG.

3.4.6.2 Candidate genes selection and mutation screening strategies and technologies

Four candidate genes located within the GLC1N locus (15q22-q24), namely *dipeptidyl-peptidase 8* (*DPP8*; OMIM *606819), *DENN/MADD domain containing 4A* (*DENND4A*), *MEGF11*, and *RB11A*, were selected for mutation screening in the pedigree based on their chromosomal positions, expression profiles and possible functional relevance to glaucoma.

Table 3.7. Clinical features of affected members in the GLC1N-linked Hong Kong family

Subject	Sex	Age at diagnosis (years)	Highest IOP (OD/OS) (mmHg)	Cup/disc Ratio (OD/OS)	Visual filed Score*	Axial Length	Visual acuity	
							OD	OS
I:1	M	32	25/25	0.8/0.8	5	32.6/33.1	20/200	0.5/200
II:4	F	25	28/28	0.9/0.9	4	25.1/25.1	20/30	20/30
II:6	F	25	27/27	0.8/0.5	4	22.9/22.9	20/20	20/20
II:9	F	22	32/34	0.7/0.5	3	26.4/25.7	20/50	20/20
II:13	F	23	26/28	0.7/0.5	4	22.3/22.6	20/10	20/15
II:15	F	22	30/30	0.9/0.9	4	30.3/31.3	20/100	20/100
III:4	M	17	31/31	0.7/0.7	4	24.1/24.2	20/20	20/20
III:6	M	12	32/32	0.75/0.75	4	24.6/22.9	20/30	20/30

Note:

* Visual field score were defined into five categories: 1. No alteration; 2. Early defect; 3. Accurate defect; 4, advanced scotoma; and 5, light perception only or no visual. Fixation losses, false-negative error, visual field pattern, visual field deviation, and visual field pattern standard deviation were taken into account during the evaluation of the visual field scores.

(Table modified from (Wang et al. 2006b))

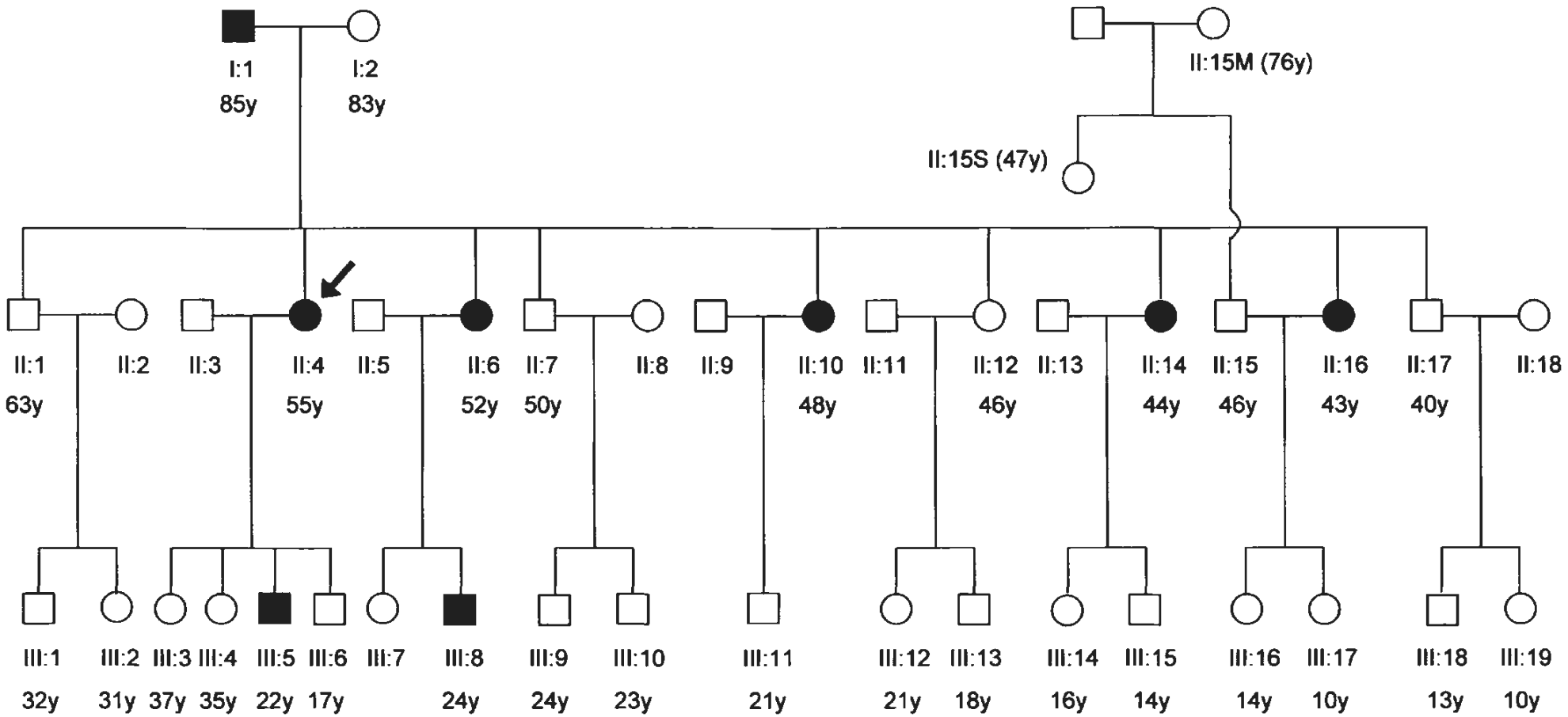


Figure 3.1. Pedigree of the GLC1N-linked Hong Kong family with JOAG

Filled symbols indicate affected members, whereas open symbols indicate unaffected members. The age at study recruitment was shown beneath the symbol of each subject. Age at diagnosis of the affected subjects was shown in Table 3.7. The arrow indicates the proband.

The candidate genes are located consecutively at chromosomal region next to the markers (D15S153 (Z_{max}=3.02) and D15S125 (Z_{max}=3.31)) that gave the highest LOG scores in our previous linkage analysis (Wang et al. 2006b). Around this region, the *SMAD6* gene, which is the nearest gene to marker D15S125, had been excluded (Wang et al. 2006b). In this current study, we evaluated another 4 candidates around this region based on their functional relevance. The *DPP8*, *DENND4A* and *MEGF11* genes are expressed in the retina (BioGPS; http://biogps.gnf.org/). Moreover, DPP8 had been found to influence cell-extracellular matrix interactions, and may regulate tissue remodeling and enhance induced apoptosis (Yu et al. 2006). MEGF11 might be involved in the phagocytosis of apoptotic cells (Suzuki and Nakayama 2007a). The remainders *DENND4A* and *RB11A* were selected mainly because they were in close proximity to the other two functional candidates.

A set of 74 pairs of primers were designed for the 4 candidate genes (Table 3.8). The primers were designed referring to the published sequence of respective gene from the Ensembl database. The target sequences covering the coding region and exon-intron boundaries were amplified by PCR and analyzed by direct sequencing. The method in detail has been described in section 3.3.2.

3.5 Statistical analysis

3.5.1 Hardy-Weinberg Equilibrium test

Hardy-Weinberg Equilibrium for each sequence variant was tested independently in the HTG, NTG and control groups by using the exact tests of Hardy-Weinberg Equilibrium. The tests were run in PLINK (version 1.07; http://pngu.mgh.harvard.edu/purcell/plink/), which is a free, open-source whole genome association analysis toolset (Purcell et al. 2007b). For the test, the command "--hardy" was used.

Table 3.8. Primer sequences for screening of the candidate genes at the GLC1N locus

No.	Amplifying target	Primer sequence		Ta (°C)	Size (bp)
		Forward primer (5'→3')	Reverse primer (5'→3')		
1	DPP8-1	CTATATTGGGCTACACATCTCTG	GGGTCAGTGTACCTTCTCAGC	55	431
2	DPP8-2	ACAGAGAAGGGAGAGCTGTTG	ACTTTTGAGTGTCAATTATACCTAGAC	58	523
3	DPP8-3	TTATGGCATATGGCATAAGCAC	CTTCAAATATAAAATCCCAACCTC	55	362
4	DPP8-4	GAGTCAATAATGTTGGAGATTCTAGG	TTTCTTTGTTGGTGGGTAAACTG	55	386
5	DPP8-5	TTGTTCATCTTATAGGAAGGGTGC	ACAAGTTATCCTCTGTACTTTTCTCC	55	413
6	DPP8-6	ACCCCAGATTTGAACTCAGTCC	CCGCTTTCAAATCTATTATCCAAG	58	360
7	DPP8-7	CATCTCCAGTTGTTTTCCCTCC	GACCAATGTTGTTGGCTACACC	58	438
8	DPP8-8	ACTGCACCCGACCTATACG	ATGATCCACCGATATCTCAGC	60	300
9	DPP8-9	TAGGCTGTGTTAAAAGTGCTTATAAC	AATTTACATGTGAAGGCAGCTCC	58	435
10	DPP8-10	TATAATTGATAGGTGCCAAAGGAG	CTGGAACCATCACTCCAATTG	58	426
11	DPP8-11	AATAAATCTGAAAGCCCCAAGC	AAAGCATGCAAAAGATACATTCC	58	419
12	DPP8-12	AATGCTGTGCAACCATCATCTCT	CATTTTTAGCACTGCATTCACACAG	58	301
13	DPP8-13	CATAAGTCAAGGAGTGGCTCAAG	GCTTTTGCAGGCCAATTTCA	58	349
14	DPP8-14	AAATTGGCCTGCAAAAGCC	GTGAGAGATGGAAAGACTTGTCC	58	423
15	DPP8-15	TGGTCAGTTTGTGTTGGCGG	AAAGCAAATGCCCAACAATGCT	58	372
16	DPP8-16	AAGGTAAAATAATAGTGAAATTAAGC	CTCACTCACTCATTCATAGCC	58	369
17	DPP8-17	GACAGAGTGAGACTCCATCTCAA	AAAATAGACATTTTCATATATCCTCC	55	385
18	DPP8-18	GAGTTGCCTAAGACATGTATGCG	GAGTGAGACCCTGTCTCAATTTAAAA	58	338
19	DPP8-19	TTCATTGCATGTGGCATCTGG	CTTTAGTTCTCTTAAGCAGAGGGTG	58	415
20	DPP8-20	TCTGGAAAGCTTTGGAAGAAGATG	ATGGTATTGCTGGGTCTCTCAGG	58	436
21	DENND4A-3	ACAATGCTACTGTGTTTATCAAGG	CAGTTAATCTCCAAATCTACAAGGC	58	531
22	DENND4A-4	TTACCATGCTACTACATTTAAGGATC	AAAATTATGCTCTTTTCATCAGTGG	58	408
23	DENND4A-5	AACTGAAAATAGCTAGCAGAGGG	CAGAAGATACGGATTGTAGTCCC	58	330
24	DENND4A-6	CATATATATAATGCTTTGGCATTCC	GGATACAGTTATTTTAATCAACTGGA	58	430
25	DENND4A-7	TAATACCTGGCCTCTCACCTGG	CCATATAAAATTTACCCAGTTGCC	60	441
26	DENND4A-8	GTCACTGGGTTTAGGCAGAAG	CTGGTTCTCTCTAAATCTCATTAGG	58	382
27	DENND4A-9	AAGTGTTCTGATACTGGTAAATGG	TAGTAGTTAAATGAGAAGTATTTCCATC	58	311
28	DENND4A-10	TGCCCATTGTTTTAGATAATGAGG	AGCATAATTTATCAGCACACACAA	58	457
29	DENND4A-11	TTGTGTGTGCTGATAAATTATGCT	TGATTACCCCACATAACAAGGAA	58	377
30	DENND4A-12	GTATATTCAACATGCCAATTTACCC	TAATGATCATCTGCCTTTAAAATGG	58	413
31	DENND4A-13	AGAGTTGAGGTAGCTTGGGTCC	ACAGACAGCAAATGAATCATCACA	58	399
32	DENND4A-14	AGGTATAGATGACATGGTTGTTCAG	CTTTCATTTAAGTGGTCAACGATG	58	385
33	DENND4A-15	TTTATTTAACCACTTCCCTTTATATG	AAAGCTACGTCACAGATGATGG	58	281
34	DENND4A-16	ATTTGTTGAGTGTATATCACAGCCTG	GTGATAAAGCATATTACGGGAGG	58	374
35	DENND4A-17	GTGACTTGTACTTGTTTAGCACTCAG	ATGACTGCATCACTGCACTCC	58	377
36	DENND4A-18	AATTACAGGCATGAGCCACTGT	GAGAACTAAAGTCTTCCTTGATTGAC	58	396
37	DENND4A-19	GGCCTACTTTGATATTCTTTAGC	ATTTCTAAAAATGTAAACTGTAGCT	58	308
38	DENND4A-20	AATGCTTTAAGTTGGAAGTTGTCCA	GCTGAGAGGGCACGTAATGG	58	391
39	DENND4A-21	AGGTGTTCAATATTTTCTTGTCACC	TTACTAAGCTATGCCATGAGAGGC	58	401

Table 3.8. continued

No.	Amplifying target	Primer sequence		Ta (°C)	Size (bp)
		Forward primer (5'→3')	Reverse primer (5'→3')		
40	DENND4A-22A	CAGACACTCAGTGAGTGCTTGC	CTGCTGTTCAGTCTCAGCAACC	58	707
41	DENND4A-22B	GATTCAACGTATGAACAGCAGC	GGCTTTTGGTGGTGATGGATAT	58	677
42	DENND4A-23	TGATCATCTGGGTAGTTAGGTG	GAAAGTGTCACAATTCAAATGAAAC	58	362
43	DENND4A-24	ATCTGAGAACTTATGGAAGTAAAA	TTTAACTTCTATTACTCTACCTGGG	58	279
44	DENND4A-25/26	TATCCAATGAATTTATGAATTGAAATGG	ACATTTAAACCTGGAAATGGTCAAC	58	598
45	DENND4A-27	ATCACACCACTGCAGTCCAGC	TGGGATAAGAGACTCTTCTTGGC	60	467
46	DENND4A-28	GCCAAGAAGAGTCTCTTATCCCA	TAAAATAAATTTAGCATAAAAACAGACC	58	491
47	DENND4A-29	TTTTCTGTCTCGCACATATACATCC	AAAATCAAACACTCACTCTCAAACAA	58	391
48	DENND4A-30/31	TAACTTAATATCTGTCAGTTGCTGC	CAGATTTAATATTACTAATAAAGTATAA CTACT	58	584
49	DENND4A-32	CCTCTAATGTTGTGTTTCTGTAGTATTA	AAATGAGAAACAAAGTGGCTTTC	58	436
50	MEG11-2	CACTTGGCTGTGGAGCTTGG	GCCATCAGCAGAGCATCTTG	58	354
51	MEG11-3	TGGATTATGCTCCTTGACCAGAT	CCCTGATGTTCACACAGGCC	58	311
52	MEG11-4	CGCTGCAAAGATGGGAAGG	AGGCACCCTCCAGGTCCC	64-60	409
53	MEG11-5	ATGCTGTGCCTAATACAGATCC	TGGGATTACAGTCACGAGTACC	58	353
54	MEG11-6	TGGAGCCACAGCAGGTGTG	GAAAGGAACTGGAAGGCTGGC	60	330
55	MEG11-7	CCAGGAGGACCACATCCTG	TGGATGTCCATGCTAGAGGTG	60	400
56	MEG11-8	TAGGCAAGCCAGGCTTGG	TTGAGAACAGCTTCAGGGGC	60	425
57	MEG11-9	ATTAGGACATGGACATGTTGGG	AGCTGGCAGCACCACCTG	60	295
58	MEG11-10	AATGTGTGTTCTAACCCAGCCC	AAGTGTCCCATCACCTAGCTTAG	55	417
59	MEG11-11	CAAGATCCAGTAGATCTCACTGG	GATGCATACTCAGGGATCAAG	58	341
60	MEG11-12	ACCTCTTCATACTGCACTGTCC	AGCCCAGCCTCTCTCAGG	60	334
61	MEG11-13	AGGGAAGGATGCAGGTCTCC	CTTCCTTCCCTGCCACTTTC	58	317
62	MEG11-14	AACTTCTAGGGATGAGGCTGTG	ATGCAGAAGGAGATTTCATCCC	58	331
63	MEG11-15	ATCTTGGCATCTTGGCTCTGAC	CTGGGGCTGCCTGCATTC	58	341
64	MEG11-16	TAGTCTTCATCCCATGCTCAGC	TGTAGGCATGACCTTCCTCAGC	62	408
65	MEG11-17	AACAGGCTTATTGCCATAGCTG	GTATCATCAAATCCAGCTGTTCG	58	312
66	MEG11-18	CAGATCTCCTAACATTAGAGGGG	ACTGAGCTTCCACATTCAGCC	58	450
67	MEG11-19	AAGCAAGAAAAGAAAGAAGAACAC	ACACACAGTTTGTCTCAAGTGG	58	241
68	MEG11-20	AGCTCTAAGATCCTGAAGGCAC	AAAAGAAGGATGTGTGGTCAAGG	62	421
69	MEG11-21	TGAGCAAGCCTCAGTCCTAACC	ACGCTCTTCTTTAGTTCCAGGTG	58	455
70	RAB11A-1	AGACTATGGTAGCTAGGAGTTCCAGG	AGCTGGGTAGGAGACGGAACC	58	492
71	RAB11A-2	CCAAACTTCATTCTGTTGAAAGCA	TTCCAATACCAAAAGTCAGCTAATTC	58	353
72	RAB11A-3	CATATTTTGAGTTCTTCCTGGTG	TTCTTGCTAAGCATTCATGTTTACC	58	386
73	RAB11A-4	TACTGTCCCAGAAGTTGAGGAGG	CTCAAATGATAGTCACTCTCGGC	58	323
74	RAB11A-5	CTTTGATGCAAATATATCTCCTACC	CTAAAATCAAAAGACTTAGGACATGG	58	381

Reference sequence	Ensembl Gene ID
DPP8	ENSG00000074603
DENND4A	ENSG00000174485
MEGF11	ENSG00000157890
RAB11A	ENSG00000103769

3.5.2 Individual SNP association analysis

Allelic, genotypic and model-based associations of each SNP with HTG, NTG and pooled POAG were analyzed by χ^2 test in PLINK. For allelic association analyses, the "--assoc" and "--fisher" options were used, while for model-based genotype association analyses, the "--model" option was used. In model-based analyses, the tests included Cochran-Armitage trend test, genotypic (2 degree-of-freedom, df) test, dominant gene action (1df) test, and recessive gene action (1df) test. The genotypic test provides a general test of association in the 2-by-3 table of disease-by-genotype. The dominant and recessive models are tests for the minor allele. That is, if d is the minor allele (and D is the major allele), then a dominant model tests for (dd, Dd) versus DD, and a recessive model tests for dd versus (Dd, DD). Besides PLINK, association was also analyzed by using the SPSS software package (version 16.0, SPSS Inc., Chicago, IL) where applicable.

Odds ratio and its 95% confidence interval were estimated by the Mantel-Haenszel method. Population attributable risk (PAR), which estimates the proportion of cases in the total population attributable to a given risk factor (e.g., the higher-risk allele or genotype), could be computed using the equation: $PAR=f(R-1)/[1+f(R-1)]$ where f is the fraction of controls with the risk allele or genotype(s), R is the measure of odds ratio. In practice, the PAR and the 95% CI were estimated using the PAR program from the Statistical Genetics Utility program package (freely available at: http://linkage.rockefeller.edu/ott/util.htm).

When a SNP was genotyped in more than one cohort, its association with the disease was evaluated in individual cohort and then the pooled subjects from different cohorts. Where applicable, the genotype data were pooled by using a Mantel-Haenszel model with a fixed/random effect in the Review Manager software (version 5.0.24; the Cochrane Collaboration, Copenhagen, Denmark).

3.5.3 Linkage disequilibrium and haplotype-based association analyses

Pairwise LD between SNPs and haplotype frequencies were estimated using Haploview (version 4.1; http://www.broadinstitute.org/haploview/) (Barrett et al. 2005).

Haplotype-based association analysis was performed using the WHAP software (ver. 2.09; http://pngu.mgh.harvard.edu/~purcell/whap/) according to the author's instructions (Purcell et al. 2007a). First, an omnibus H-1 degree-of-freedom (df) test was used to test for global haplotype association. After detection of a significant global effect, a haplotype-specific (HS) test was performed to evaluate whether a haplotype has an independent effect using a likelihood ratio test in WHAP followed by a permutation test (no. of iteration = 10,000). A sole-variant (SV) conditional test was used to control for a haplotype (or SNP) and test whether it can explain the total association. In this test, a haplotype (or SNP) yielding a P value of >0.05 indicates that it can explain the total association. An independent effect (IE) test was used to test whether a SNP still has an effect after controlling for others. For the SNPs in *CAV1/CAV2*, as the minor allele frequencies were low, the frequency threshold was set to 0.1% in order to include all haplotypes with a frequency of >0.1% in all haplotype-based tests.

3.5.4 Genotype-phenotype correlation analysis

Genotype-phenotype correlation analysis was performed by comparing the means of the parameter of interest (e.g., AAD, IOP and VCDR) between groups of patients with the higher-risk or lower-risk genotypes of a SNP of interest, using the analysis of covariance (ANCOVA) in the SPSS software package (version 16.0, SPSS Inc., Chicago, IL). In the analyses, the AAD, peak-IOP and VCDR, respectively, were used as dependent variables, and the genotypes of the SNP were taken as fixed (grouping) factor. Covariates, such as AAD and gender, were introduced into the ANCOVA model where appropriate.

3.5.5 Gene-gene interaction analysis

Gene-gene interactions were estimated with a multistep strategy. Pairwise SNP-SNP interactions were analyzed by logistic regression using the epistasis algorithm in PLINK with a threshold P value of 0.05. In parallel, pair-wise and high-order interactions were analyzed by multifactor dimensionality reduction (MDR) (Ritchie et al. 2001) using the MDR software package (version 1.1.0, www.epistasis.org/software.html).

3.5.6 Correction for multiple testing

In association analysis, a P value of <0.05 was considered statistically significant. Regarding the correction for multiple comparisons, the conventional Bonferroni method was used to correct the P values obtained from the association analyses in the study of multiple gene polymorphisms because the SNPs (even for those in the same gene) were not highly correlated (refer to section 1.4.6 and 4.1.7). For individual SNP association, P values were corrected by the total number of SNPs analyzed (n=14). And for pairwise SNP interactions, the P values were corrected by the number of all valid SNP×SNP tests (n=77).

In the study of *CAV1/CAV2* SNPs, because the SNPs are highly correlated, the effective number of independent SNPs (M_{eff}) was estimated using the SNPSpD program (Nyholt 2004) (http://gump.qimr.edu.au/general/daleN/SNPSpD/), and the significance threshold was corrected for M_{eff} tests (refer to sections 1.4.6 and 4.2.1).

For variants detected in the *NTF4*, *CNTF*, *SPARC* and *MEGF11* genes, correction for multiple testing was not applied because no variant revealed significant association.

3.5.7 Analysis of variant

In these studies, a coding variant was assumed to be causative mutations if it (1) would be expected to alter the amino acid sequence of the gene product, and (2) were more

commonly observed in patients with glaucoma than in controls. To meet the latter criterion, a variant needed to be completely absent from the control population or significantly more common ($P<0.05$ using the Fisher exact test) in the glaucoma population. Moreover, if DNA samples and medical information of the index patients' family members are available, e.g., the Philippine pedigree described in section 3.4.5 and the Hong Kong pedigree described in section 3.4.6, segregation of the variants in pedigree was analyzed. If a variant is cosegregated with glaucoma in the family, it is considered disease-causing.

For a missense variant detected exclusively in patient subject but could not be confirmed as a causative mutation because of a lack of family members for segregation analysis, two web-based analysis programs (i.e., PolyPhen and SIFT) were used to predict the functional impact of the amino acid substitution on the structure and function of the protein (refer to section 1.5.1).

For variants detected in the gene splice sites, i.e., splice donor sites or splice receptor sites, the intron analysis tool sets in the Alternative Splicing Database (Stamm et al. 2006) and another standalone web-based software Automated Splice Site Analyses (Nalla and Rogan 2005; Rogan et al. 1998) were applied to evaluate the impact of the variant on the splice site strength (refer to section 1.5.1). In this thesis, the ASD and ASSA were used only for the splice-site variants detected in the *CNTF* and *MEGF11* genes.

Chapter 4

Results

4.1 Investigation of multiple gene polymorphisms in POAG

4.1.1 Allelic association of individual SNP with POAG in the Hong Kong cohort

All of the 15 SNPs selected from the 7 genes/loci were first genotyped in the Hong Kong cohort as an exploratory process. SNP rs12377632 in *TLR4* deviated from HWE in controls (P<0.001) and was excluded for further analysis. In allelic association analyses, only one SNP, i.e., *LMX1B* rs944103, showed nominally significant association with HTG (P=0.035, Table 4.1). Only heterozygous genotype was detected. The risk genotype AG conferred a 2.38-fold (95% CI: 1.07-5.30, P=0.029) of increased risk to HTG (Table 4.2). However, the association could not withstand correction for multiple testing (P_{corr}=0.41 after the conventional Bonferroni correction). Other SNPs did not show significant allelic association with HTG, NTG, or pooled POAG in the Hong Kong cohort alone.

4.1.2 Genotype association of individual SNP with POAG in the Hong Kong cohort

Regarding model-based genotypic association, rs7037117 in *TLR4* was marginally associated with HTG (P=0.049) in a full model. It showed a trend towards an increased risk of HTG in a recessive model (P_{rec}=0.063; OR_{rec}=2.29, 95% CI: 0.94-5.57, Table 4.2). Another SNP rs1533428 at 2p16.3 was associated with HTG in a dominant model (P_{dom}=0.030; OR_{dom}=1.58, 95% CI: 1.04-2.38, Table 4.3). The higher-risk genotypes TT+CT also showed a trend toward an increased-risk of NTG (OR_{dom}=1.36, 95% CI: 0.92-2.02; P=0.12). However, these associations were not significant after correction for multiple testing by the Bonferroni method. The other 11 SNPs, at any model, were not statistically associated with HTG, NTG or pooled POAG in the Hong Kong cohort.

Table 4.1. Associations of 14 selected SNPs with HTG and NTG in the Hong Kong cohort

Region	Gene	dbSNP ID	Allele*	Minor allele frequency			Association (P)		Genotype counts†			Association (P)	
				HTG	NTG	Control	HTG	NTG	HTG	NTG	Control	HTG	NTG
1q43	*ZP4*‡	rs693421	T/G	0.459	0.456	0.465	0.89	0.84	37/95/52	46/96/64	48/118/64	0.98	0.61
2p16.3	*AC007682.3*	rs1533428	T/C	0.443	0.430	0.384	0.10	0.19	34/95/55	39/99/68	39/98/92	0.088	0.30
2p16.3	*AC007682.3*	rs12994401	T/C	0.369	0.308	0.343	0.46	0.31	22/91/70	24/79/103	28/101/100	0.49	0.40
9q33.1	*TLR4*	rs1927907	T/C	0.209	0.175	0.172	0.18	0.93	10/57/117	8/56/142	8/63/159	0.40	0.97
9q33.1	*TLR4*	rs11536889	G/C	0.294	0.272	0.307	0.70	0.26	20/68/96	15/82/109	19/103/108	0.24	0.46
9q33.1	*TLR4*	rs7037117	G/A	0.201	0.192	0.202	0.99	0.73	14/46/124	8/63/135	8/77/145	0.049	0.80
9q33.3	*LMX1B*	rs944103	G/A	0.049	0.024	0.022	0.035	0.82	0/18/166	0/10/196	0/10/220	0.029	0.80
9q33.3	*LMX1B*	rs7854658	T/C	0.003	0	0	0.44	N/A	0/1/183	0/0/206	0/0/230	0.44	N/A
9q33.3	*LMX1B*	rs16929236	G/A	0.393	0.388	0.409	0.67	0.58	25/94/64	27/106/73	36/116/78	0.85	0.75
9q33.3	*LMX1B*	rs10733682	G/A	0.269	0.240	0.253	0.63	0.69	11/77/96	11/77/118	21/74/134	0.10	0.22
9q33.3	*LMX1B*	rs867559	T/C	0.302	0.333	0.298	0.94	0.27	17/77/90	23/91/92	21/95/114	0.99	0.55
10p12.31	*RP11-337N19*	rs7081455	G/T	0.155	0.141	0.128	0.31	0.62	3/51/130	1/56/149	1/57/172	0.35	0.85
12q21.31	*TMTC2*	rs7961953	A/G	0.408	0.396	0.374	0.35	0.53	30/90/64	32/99/75	29/114/87	0.54	0.68
14q32.2	*CYP46A1*	rs754203	C/T	0.304	0.331	0.339	0.29	0.83	17/78/89	25/85/94	27/100/100	0.56	0.88

Note:

* Minor allele/major allele;

† The genotype counts are presented as Homozygote/Heterozygote/Wildtype;

‡ *ZP4* is the nearest gene to SNP rs693421.

Table 4.2. Model-based association of *TLR4* rs7037117 and *LMX1B* rs944103 with HTG

SNP		Hong Kong cohort		Shantou cohort		Pooled southern subjects*		Beijing cohort	
		HTG (n=185)	Control (n=230)	HTG (n=102)	Control (n=147)	HTG (n=287)	Control (n=377)	HTG (n=177)	Control (n=200)
rs7037117	GG	14 (7.6)	8 (3.5)	15 (14.7)	7 (4.8)	29 (10.1)	15 (4.0)	10 (5.7)	12 (6.0)
	AG	46 (25.0)	77 (33.5)	41 (40.2)	54 (36.7)	87 (30.4)	131 (34.7)	74 (42.0)	79 (39.5)
	AA	124 (67.4)	145 (63.0)	46 (45.1)	86 (58.5)	170 (59.4)	231 (61.3)	92 (52.3)	109 (54.5)
P (recessive)		0.063		0.0066		0.0016		0.90	
OR (95% CI)		2.29 (0.94-5.57)		3.45 (1.35-8.79)		2.72 (1.43-5.18)		0.94 (0.40-2.24)	
rs944103	GG	0 (0)	0 (0)	1 (1.0)	0 (0)	1 (0.35)	0 (0)	NI	NI
	AG	18 (9.8)	10 (4.3)	5 (5.0)	11 (7.5)	23 (8.1)	21 (5.6)	NI	NI
	AA	166 (90.2)	220 (95.7)	95 (94.0)	136 (92.5)	261 (91.6)	356 (94.4)	NI	NI
P (dominnat)		0.029		0.64		0.15			
OR (95% CI)		2.38 (1.07-5.30)		0.78 (0.28-2.18)		1.56 (0.85-2.86)			

Note:

* Only data of the pooled southern samples were shown. When the genotype data from the Beijing cohort was combined to the southern samples, the P value of the association between rs7037117 and HTG in overall Chinese subjects was 0.013, with a OR of 1.88 (95% CI: 1.13-3.12).

NI: not investigated.

Table 4.3. Association of rs1533428 at 2p16.3 with POAG in all and in different age groups

Age group*		Hong Kong cohort			Shantou cohort		Beijing cohort		Pooled Chinese subjects		
		HTG (n=185)	NTG (n=206)	Control (n=230)	HTG (n=102)	Control (n=147)	HTG (n=177)	Control (n=200)	HTG (n=464)	HTG+NTG (n=670)	Control (n=577)
Any age	TT	34 (18.5)	39 (18.9)	39 (17.0)	16 (15.7)	26 (17.7)	19 (10.8)	28 (14.0)	69 (14.9)	108 (16.2)	93 (16.1)
	CT	95 (51.6)	99 (48.1)	98 (42.8)	55 (53.9)	60 (40.8)	91 (51.7)	85 (42.5)	241 (52.2)	340 (50.9)	243 (42.2)
	CC	55 (29.9)	68 (33.0)	92 (40.2)	31 (30.4)	61 (41.5)	66 (37.5)	87 (43.5)	152 (32.9)	220 (32.9)	240 (41.7)
P (dominant)		0.030	0.12		0.074		0.24		0.0038	0.0015	
OR (95% CI)		1.58 (1.04-2.38)	1.36 (0.92-2.02)		1.63 (0.95-2.77)		1.28 (0.85-1.94)		1.46 (1.13-1.88)	1.46 (1.15-1.83)	
	T allele	163 (44.3)	177 (43.0)	176 (38.4)	87 (42.6)	112 (38.1)	129 (36.6)	141 (35.3)	379 (41.0)	556 (41.6)	429 (37.2)
P value		0.089	0.17		0.31		0.69		0.079	0.026	
OR (95% CI)		1.27 (0.96-1.68)	1.21 (0.92-1.58)		1.21 (0.84-1.74)		1.06 (0.79-1.43)		1.17 (0.98-1.40)	1.20 (1.02-1.41)	
< 35 years	TT	0 (0.0)	1 (14.3)		5 (13.9)		5 (6.7)		10 (7.8)	11 (8.1)	
	CT	10 (58.8)	3 (42.9)		19 (52.8)		38 (50.7)		67(52.3)	70 (51.9)	
	CC	7 (41.2)	3 (42.9)		12 (33.3)		32 (42.7)		51 (39.8)	54 (40.0)	
P (dominant)		0.94	0.99		0.37		0.90		0.71	0.72	
OR (95% CI)		0.96 (0.35-2.61)	0.90 (0.20-4.09)		1.42 (0.66-3.05)		1.04 (0.61-1.77)		1.08 (0.73-1.59)	1.07 (0.73-1.57)	
	T allele	10 (29.4)	5 (35.7)		29 (40.3)		48 (32.0)		87 (34.0)	92 (34.1)	
P value		0.30	0.84		0.73		0.48		0.33	0.33	
OR (95% CI)		0.67 (0.31-1.43)	0.89 (0.29-2.70)		1.10 (0.65-1.86)		0.86 (0.58-1.29)		0.87 (0.65-1.15)	0.87 (0.66-1.15)	
35-60 years	TT	12 (19.4)	19 (23.2)		4 (10.8)		11 (13.8)		27 (15.1)	46 (17.6)	
	CT	24 (38.7)	32 (39.0)		22 (59.5)		42 (52.5)		88 (49.2)	120 (46.0)	
	CC	26 (41.9)	31 (37.8)		11 (29.7)		27 (33.8)		64 (35.8)	95 (36.4)	
P (dominant)		0.80	0.71		0.19		0.13		0.16	0.15	
OR (95% CI)		0.93 (0.53-1.64)	1.11 (0.66-1.86)		1.68 (0.77-3.65)		1.51 (0.88-2.60)		1.28 (0.91-1.82)	1.25 (0.92-1.69)	
	T allele	48 (38.7)	70 (42.7)		30 (40.5)		64 (40.0)		142 (39.7)	212 (40.6)	
P value		0.95	0.34		0.70		0.29		0.41	0.19	
OR (95% CI)		1.01 (0.67-1.52)	1.19 (0.83-1.71)		1.11 (0.66-1.86)		1.23 (0.84-1.79)		1.11 (0.87-1.41)	1.15 (0.93-1.42)	
> 60 years	TT	22 (21.0)	19 (16.2)		7 (24.1)		3 (14.3)		32 (20.6)	51 (18.8)	
	CT	61 (58.1)	64 (54.7)		14 (48.3)		11 (52.4)		86 (55.5)	150 (55.1)	
	CC	22 (21.0)	34 (29.1)		8 (27.6)		7 (33.3)		37 (23.9)	71 (26.1)	
P (dominant)		0.00058	0.042		0.16		0.37		5.03×10^{-5}	1.14×10^{-5}	
OR (95% CI)		2.53 (1.48-4.34)	1.64 (1.02-2.65)		1.86 (0.77-4.48)		1.54 (0.60-3.98)		2.28 (1.52-3.41)	2.02 (1.47-2.78)	
	T allele	105 (50.0)	102 (43.6)		28 (48.3)		17 (40.5)		150 (48.4)	252 (46.3)	
P value		0.0049	0.19		0.15		0.50		3.7×10^{-4}	3.7×10^{-4}	
OR (95% CI)		1.60 (1.15-2.23)	1.24 (0.90-1.71)		1.52 (0.86-2.67)		1.25 (0.65-2.39)		1.58 (1.23-2.03)	1.45 (1.18-1.79)	

Note: *Age groups were defined by age at diagnosis of the patients. The controls were not stratified and were used as the reference for all comparisons.

4.1.3 Age-varying association of rs1533428 with POAG in the Hong Kong cohort

In light of the fact that certain genes may contribute predominantly to a specific age group of glaucoma patients, e.g., *MYOC* to JOAG, we stratified the patients by the age at diagnosis. Patients aged <35 years were classified as JOAG, aged between 35 and 60 years as AOAG and, aged >60 years as LOAG. An age-varying association between rs1533428 and POAG was observed, where the TT+CT genotypes conferred a significantly increased risk towards the late-onset form of HTG ($P_{dom}=5.8\times10^{-4}$, $P_{corr}=0.0081$; OR=2.53, 95% CI: 1.48-4.34) and NTG ($P_{dom}=0.042$, $P_{corr}=0.59$; OR=1.64, 95% CI: 1.02-2.65), while showing no significant association with the juvenile- and adult-onset forms of disease (Table 4.3). Such an age-varying patter of association was not found at the other 13 SNPs.

4.1.4 Association of three disease-associated SNPs with HTG in the Shantou cohort and overall Hong Kong/Shantou subjects

Based on the findings in the Hong Kong cohort, SNPs rs944103, rs7037117 and rs1533428 were genotyped in the Shantou cohort of 102 HTG and 147 controls. *LMX1B* rs944103 was not associated with HTG in the Shantou cohort alone ($P_{dom}=0.64$, Table 4.2) or in the pooled Hong Kong and Shantou subjects ($P_{dom}=0.15$). In contrast, *TLR4* rs7037117 was significantly associated with HTG in the Shantou cohort. The higher-risk genotype GG in a recessive model conferred a 3.45-fold (95% CI: 1.35-8.79; $P_{rec}=0.0066$, $P_{corr}=0.092$, Table 4.2) of increased risk to HTG. In the pooled subjects, the GG genotype was still strongly associated with HTG ($P_{rec}=0.0016$, $P_{corr}=0.022$; OR=2.72, 95% CI: 1.43-5.18). The PAR of the risk genotype GG was 6.42% (95% CI: 2.20-10.45%) for HTG.

Of rs1533428, the higher-risk genotype TT+CT showed a trend towards an increased risk of HTG in the Shantou cohort, but the association did not reach statistical significance ($P_{dom}=0.074$; OR=1.36, 95% CI: 0.92-2.02; Table 4.3). Notably, the genotype distributions

of this SNP in the patient and control groups respectively were similar to that in the Hong Kong cohort. The odds ratios conferred by the higher-risk genotypes also increased with ascending AAD of HTG, although no significance was reached (Table 4.3). In the pooled Hong Kong/Shantou subjects, the association of this SNP with HTG was enhanced for patients aged >60 years ($P_{dom}=1.5\times10^{-4}$, $P_{corr}=0.0021$; OR=2.38, 95% CI: 1.51-3.75) but remained insignificant in younger age groups.

4.1.5 Association of rs7037117 and rs1533428 with HTG in the Beijing cohort and overall Chinese subjects

With the findings on rs7037117 and rs1533428 in the southern Chinese, these two SNPs were genotyped in the Beijing cohort of 177 HTG and 200 controls. Rs7037117 showed no disease association ($P_{rec}=0.90$), even to a reverse tendency ($OR_{rec}=0.94$, Table 4.2). Of rs1533428, the genotype distributions in the patient and control group respectively were similar to that in the southern cohorts. And a trend of the age-varying association was also observed (Table 4.3).

In view of this, all Chinese samples were simply pooled altogether to increase statistical power, with a total of 462 HTG patients, 206 NTG patients and 577 controls. A stronger association was observed between rs1533428 and overall HTG ($P_{dom}=0.0038$, $P_{corr}=0.053$, Table 4.3). Furthermore, when the Hong Kong NTG was compared with the pooled controls, the SNP showed a nominally significant association ($P_{dom}=0.029$, $P_{corr}=0.41$; OR=1.45, 95% CI: 1.04-2.03). The age-varying association was even stronger, with the higher risk genotype TT+CT conferring a 2.28-, 1.74- and 2.02-fold of increased-risk to HTG ($P_{dom}=5.03\times10^{-5}$, $P_{corr}=0.00070$), NTG ($P_{dom}=0.011$, $P_{corr}=0.15$) and overall POAG (i.e., HTG+NTG, $P_{dom}=1.14\times10^{-5}$, $P_{corr}=0.00016$) respectively for patients aged >60 years, yet imposing no risk upon JOAG ($P_{allele}=0.33$, OR=0.87) and having a trend towards a mild

but insignificant increase in risk of AOAG (P_{allele}=0.19, OR=1.15). The TT+CT genotypes had a PAR of 42.7% (95% CI: 22.9-57.4%) for HTG and 30.3% (95% CI: 5.9-48.3%) for NTG among subjects aged >60 years.

4.1.6 Genotype-phenotype correlation at rs7037117 and rs1533428

Correlations between the genotypes of rs7037117 and rs1533428 and AAD, peak-IOP and VCDR were analyzed in individual cohort and pooled subjects (Table 4.4). Only the AAD was significantly correlated with rs1533428. The mean AAD of HTG patients possessing the higher-risk genotypes (TT or CT) was approximately 6 years older than patients with the CC genotype (P=0.0082). In individual cohort, mean AAD of patients with the higher-risk genotypes were consistently greater than those with the lower-risk genotype, but significant difference was found only in Hong Kong HTG patients (P=0.019, details not shown). Such genotype-AAD correlation is likely resulted from the age-varying association as described above. Notably in the pooled HTG subjects, such genotype-AAD correlation was not observed when only patients with AAD >60 years were included, where the mean AAD of patients with the TT or CT genotype was 70.47±6.67 years in contrast to 70.49±5.86 years in patients with the CC genotype (P=0.98).

No significant genotype-phenotype correlation was detected for rs7037117 (Table 4.4).

4.1.7 Linkage disequilibrium and haplotype association analysis

Pairwise LD analysis showed weak LD between the SNPs at individual locus in the Hong Kong cohort (Figure 4.1). In particular, the D' values between SNPs rs1533428 and rs12994401 at 2p16.3 varied from 0.096 in HTG to 0.30 in controls, with the r^2 values varying from 0.007 to 0.075. For confirming the LD pattern, rs12994401 was genotyped in

Table 4.4. Genotype-phenotype correlation in pooled HTG subjects

SNP	Genotype	AAD* mean (SD)	P value	IOP† mean (SD)	P value	VCDR† mean (SD)	P value
rs1533428	TT+CT	50.5 (19.5)	0.0082	34.70 (10.6)	0.31	0.75 (0.20)	0.23
	CC	44.5 (20.0)		34.69 (10.2)		0.79 (0.19)	
rs7037117‡	GG	50.1 (20.7)	0.83	33.4 (8.1)	0.62	0.71 (0.21)	0.15
	AA+AG	48.4 (19.8)		34.8 (10.7)		0.77 (0.19)	

Note:

* Mean AAD was compared between the two genotypic groups, adjusted for study cohort in the ANCOVA model;

† Mean peak-IOP and VCDR were compared between the two genotypic groups, adjusted for AAD and study cohort in the ANCOVA model;

‡ When only the Hong Kong and Shantou samples were pooled, still no significant genotype-phenotype correlation was detected.

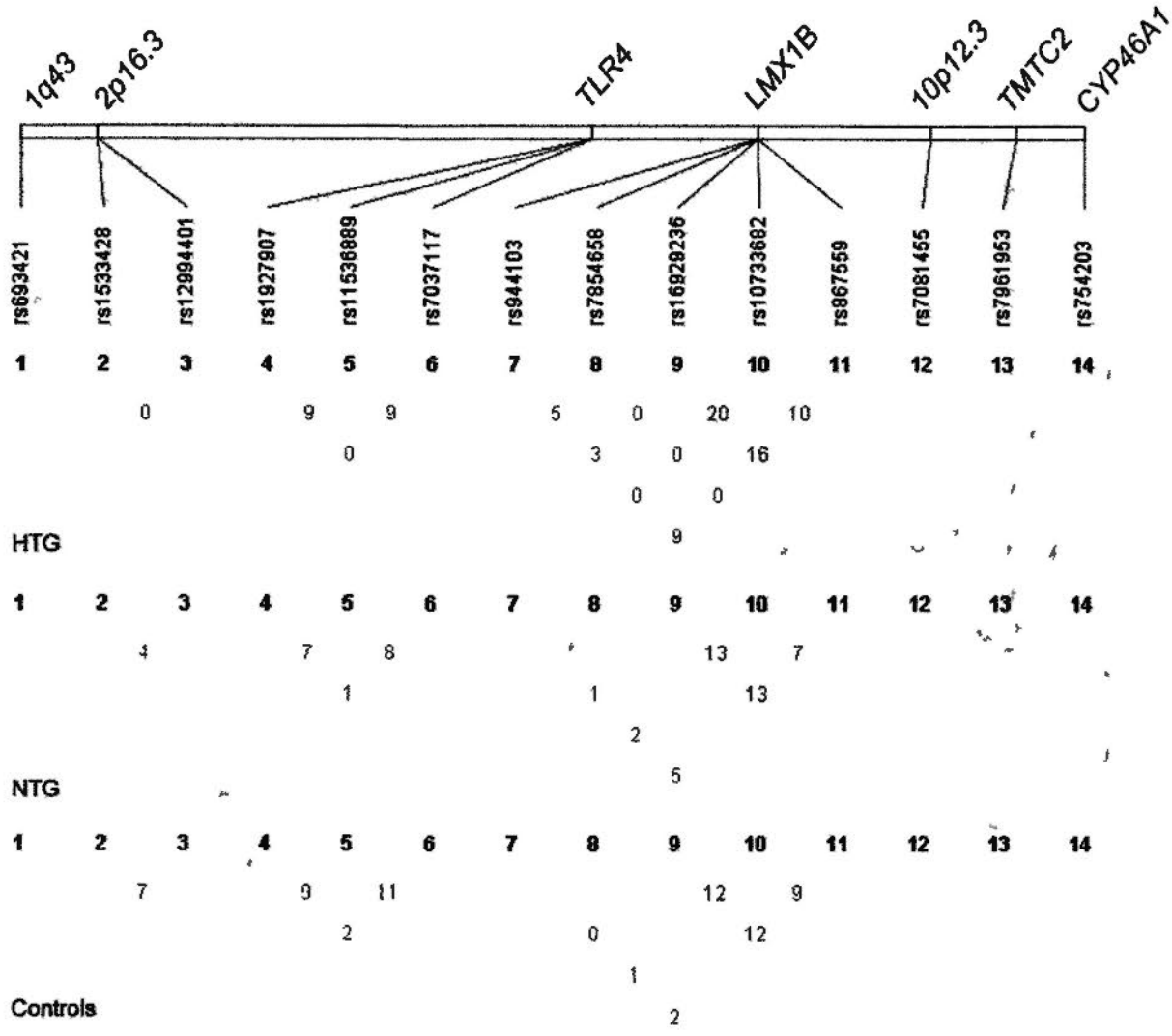


Figure 4.1. Gene-based linkage disequilibrium of the 14 SNPs in the Hong Kong cohort

The LD between the polymorphisms was analyzed in HTG, NTG and controls separately, using Haploview (ver. 4.1) (Barrett et al. 2005). The numbers in the cross cells indicated the estimated statistics of the coefficient of determination (r^2), with darker shades indicating stronger LD. The positions of the SNPs are not to scale.

the Shantou cohort. Still, weak LD was found between rs1533428 and rs12994401 in patients (D'=0.23, r^2=0.037) and controls (D'=0.25, r^2=0.053).

In haplotype-based association analyses, significant omnibus haplotype association was found between the two SNPs at 2p16.3 and HTG in the Hong Kong cohort (P=0.043) and pooled Hong Kong/Shantou subjects (P=0.044). A common haplotype (C-C) defined by the wild-type alleles of rs1533428 and rs12994401 conferred a significantly reduced risk to HTG in the Hong Kong cohort (P=0.030; OR=0.74, 95% CI: 0.56-0.97) and pooled subjects (P=0.019; OR=0.76, 95% CI: 0.61-0.95). The other haplotypes defined by these two SNPs were not significantly associated with the disorder. Haplotype-based sole-variant tests revealed that this C-C haplotype can explain the global haplotype association in the Hong Kong cohort (P_{SVh}=0.24) and pooled subjects (P_{SVh}=0.41). However, the association with the C-C haplotype could not withstand the correction by permutation test (P_{perm}>0.05). In SNP-based sole-variant test, the omnibus association was not statistically significant after controlling for rs1533428 (P_{SVs}=0.075 and 0.065 in the Hong Kong and pooled subjects, respectively), while remaining significant after controlling for rs12994401 (P_{SVs}=0.021 and 0.036, respectively), suggesting that rs1533428 is responsible for the global association.

Haplotypes defined by SNPs in the *TLR4* or *LMX1B* genes did not reveal significant omnibus association with POAG.

4.1.8 Gene-gene interactions and joint effect of the SNPs on POAG

Pairwise SNP×SNP interaction analysis by PLINK revealed that four pairs of SNPs have marginal interactions in HTG (i.e., rs11536889 and rs944103, rs1533428 and rs754203, rs693421 and rs7081455, rs10733682 and rs7081455) and two pairs in NTG (i.e., rs7037117 and rs7961953, rs693421 and rs12994401). However, these interactions were

insignificant after corrections for multiple comparisons. The most significant interaction was between rs7037117 and rs7961953 (P_{int}=0.0087, OR=2.11). We additionally genotyped rs7961953 in the Shantou cohort, but no interaction was found. Next, MDR was used to verify the two-locus interactions and search for high-order interactions. However, the interactions detected by PLINK were not present in the best two-locus models in MDR. Moreover, no significant 2-locus or higher-order interaction was detected by MDR. Hence, there was no promising statistical interaction among the SNPs studied.

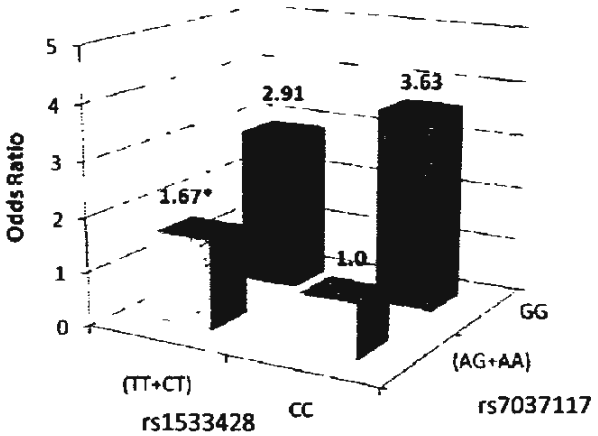
The joint two-locus effects of rs1533428 and rs7037117 on HTG in the Hong Kong and Shantou cohorts were examined. Since the association occurred in dominant (rs1533428) and recessive (rs7037117) model respectively, we tested the joint effect in only one mode. The joint ORs for each combination of the risk genotypes versus the combination of lower-risk genotypes were plotted in Figure 4.2 with details shown in Table 4.5. In each individual cohort and the pooled subjects, a similar trend was observed. Combination of the both higher-risk genotypes conferred a greater risk to HTG than that with only one risk genotype. In particular among the pooled subjects, those possessing both higher-risk genotypes had a strongly increased risk of HTG (OR_{joint}=4.53, 95% CI: 1.90-10.83; P=0.00028), followed by those with either risk genotype (OR_{joint}=2.73 and 1.61 respectively). These results suggest an additive genetic effect between these two SNPs.

4.2 Investigation of *CAV1/CAV2* polymorphisms in POAG

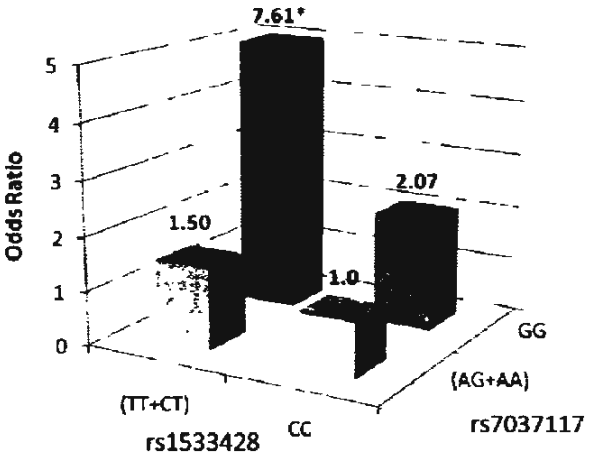
4.2.1 Association of the six *CAV1/CAV2* SNPs with POAG in southern Chinese

The six candidate SNPs, namely rs926201, rs6975771, rs4730742, rs4236601, rs959173 and rs6466587, were genotyped in 1172 study subjects, including 185 HTG, 311 NTG patients and 248 controls form the Hong Kong cohort and 102 HTG, 28 NTG and 298 controls from the Shantou cohort. No deviation from HWE was detected in either the case

A. Hong Kong cohort



B. Shantou cohort



C. Pooled subjects

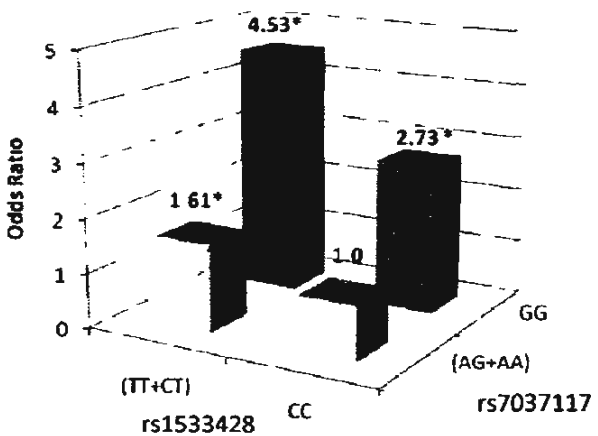


Figure 4.2. Joint ORs between rs1533428 and rs7037117 for HTG in southern Chinese

The numbers on the top of the bars represent the odds ratios for the combination of genotypes with at least one at-risk genotype of the SNP rs1533428 (dominant model) or rs7037117 (recessive model) versus the combination of both non-risk genotypes.

* indicates $P<0.05$.

Table 4.5. Joint effect of SNPs rs1533428 and rs7037117 on the genetic risk of HTG

		rs1533428 (dominant)			
		HTG (%)		Control (%)	
		TT+CT	CC	TT+CT	CC
Hong Kong cohort					
rs7037117	**GG**	8 (4.3)	6 (3.3)	5 (2.2)	3 (1.3)
(recessive)	**AG+AA**	121 (65.8)	49 (26.6)	132 (57.6)	89 (38.9)
		P values		**OR (95% CI)**	
		0.077	0.080	2.91 (0.90-9.37)	3.63 (0.87-15.17)
		0.019	1.0	1.67 (1.09-2.55)	1.0
Shantou cohort					
rs7037117	**GG**	11 (10.8)	4 (3.9)	3 (2.1)	4 (2.7)
(recessive)	**AG+AA**	60 (58.8)	27 (26.5)	83 (56.8)	56 (38.4)
		P values		**OR (95% CI)**	
		0.0010	0.44	7.61 (1.93-29.53)	2.07 (0.48-8.93)
		0.16	1.0	1.50 (0.852-2.64)	1.0
Pooled subjects					
rs7037117	**GG**	19 (6.6)	10 (3.5)	8 (2.1)	7 (1.9)
(recessive)	**AG+AA**	181 (63.3)	76 (26.6)	215 (57.3)	145 (38.7)
		P values		**OR (95% CI)**	
		0.00028	0.043	4.53 (1.90-10.83)	2.73 (1.0-7.45)
		0.0063	1.0	1.61 (1.14-2.26)	1.0

or control groups (P>0.05). Genotype distributions of these SNPs in the Hong Kong, Shantou and pooled Hong Kong/Shantou subjects were shown in Tables 4.6, 4.7 and 4.8. Among all subjects, only one patient was homozygous for the minor T allele at rs926201, while at the other SNPs only heterozygous genotypes were found. Thus, all associations of the *CAV1/CAV2* SNPs in this study were estimated in dominant genetic models only.

In the Hong Kong cohort, statistically significant association was observed between NTG and the 3 significantly protective SNPs detected by our GWA study, i.e., rs926201, rs6975771 and rs959173 (Table 4.6). The strongest association was with *CAV2* rs6975771, where the protective AG genotype was present in 7.4% of NTG compared with 14.1% of controls (P=0.010), conferring an OR of 0.49 (95% CI: 0.28-0.85). The other two SNPs (i.e., rs926201 and rs959173) also showed significant associations (P=0.042 and 0.020, respectively) with NTG. In this investigation, because the *CAV1/CAV2* SNPs are correlated, the SNPSpD program was used to calculate the effective number of independent loci (M_{eff}, refer to section 1.4.6), which in turn was estimated to be 4.4466. Thus, an experiment-wide significance threshold of 0.011 (0.05/4.4466) was required to keep the type I error rate at 5%. As such, only the association with rs6975771 withstood correction for multiple testing (P_{dom}=0.010). For HTG, although these three SNPs showed a similar trend towards a reduced risk, the associations did not reach statistical significance (P>0.053, Table 4.6). When the HTG and NTG patients were pooled, the associations with the three SNPs became more significant, but still only the association with rs6975771 withstood correction for multiple testing (P=0.0053, OR=0.51; Table 4.6). Of the other three SNPs studied, i.e., rs4730742, rs4236601 and rs6466587, no statistically significant association was observed with either HTG, NTG or pooled POAG. Rs4236601, the responsible marker for the GWA signal in the study of Thorleifsson et al, was detected in 3.2% of HTG patients, 1.9% of NTG patients and 0.81% of controls. The minor genotype AG showed a trend towards an

increased risk of HTG (OR=4.12, P=0.078) and NTG (OR=2.42, P=0.31). The other two SNPs (i.e., rs4730742 and rs6466587) showed a similar but milder trend (Table 4.6).

In the Shantou cohort, each SNP showed a similar trend of association to that in the Hong Kong cohort. The strongest trend was observed at rs4236601 and rs4730742, whose genotype distributions were the same. The higher-risk genotype was found in 2.9% of HTG, 3.6% of NTG and 0.67% of controls, conferring a trend towards an increased disease risk (OR=4.49, 5.48 and 4.70 for HTG, NTG and overall POAG, respectively; and P=0.11, 0.24 and 0.072 respectively, Table 4.7). Of rs926201, rs6975771 and rs959173, the minor genotypes showed a trend towards a reduced risk (OR=0.89, 0.81 and 0.69, respectively, for overall POAG, Table 4.7), but no statistical significance was reached.

In view of the similar findings in the Hong Kong and Shantou cohorts, the subjects were pooled (Table 4.8). The risk SNP rs4236601 showed a significant association with HTG (P=0.02, OR=4.26) and overall POAG (P=0.03, OR=3.56), while the two protective SNPs rs6975771 and rs959173 showed significant associations with NTG (P=0.007 and 0.03, OR=0.48 and 0.55) and overall POAG (P=0.01 and 0.01, OR=0.58 and 0.58).

4.2.2 *CAV1/CAV2* SNPs associated with HTG in northern Chinese

The three SNPs showing the strongest associations, namely rs6975771, rs4236601 and rs959173, were genotyped in the Beijing cohort of 177 HTG patients and 200 controls. Rs4236601[A] also showed a trend towards an increased risk (OR_{dom}=2.09, P=0.19, Table 4.9). Notably, the frequency of the risk genotype AG was higher in the Beijing subjects (5.1% in patients versus 2.5% in controls) than in the southern subjects (2.6% in patients versus 0.73% in controls). Of rs6975771 and rs959173, the minor genotypes showed a trend towards an increased risk of HTG (OR_{dom}=1.28 and 1.08; P=0.45 and 0.82, respectively) in contrast to the protective effects that they conferred to HTG in the southern cohorts.

Table 4.6. Genotype associations of the six SNPs in *CAV1/CAV2* with POAG in the Hong Kong cohort

SNP	Gene (location)	Genotype	Hong Kong subjects			P value and OR (95% CI) in dominant model		
			HTG (n=185)	NTG (n=311)	Control (n=248)	HTG	NTG	POAG
rs926201	*CAV2*	TT	1 (0.5)	0 (0)	0 (0)	0.16	0.042	0.035
	(upstream)	CT	15 (8.1)	24 (7.7)	32 (12.9)	0.64 (0.34-1.20)	0.56 (0.32-0.99)	0.59 (0.36-0.97)
		CC	169 (91.4)	287 (92.3)	216 (87.1)			
rs6975771	*CAV2*	AA	0 (0)	0 (0)	0 (0)	0.053	0.010	0.0053
	(upstream)	AG	15 (8.1)	23 (7.4)	35 (14.1)	0.54 (0.28-1.02)	0.49 (0.28-0.85)	0.50 (0.31-0.82)
		GG	170 (91.9)	288 (92.6)	213 (85.9)			
rs4730742	*CAV2*	GG	0 (0)	0 (0)	0 (0)	0.41	0.47	0.35
	(upstream)	GT	4 (2.2)	5 (1.6)	2 (0.81)	2.71 (0.49-15.00)	2.01 (0.39-10.45)	2.27 (0.49-10.60)
		TT	181 (97.8)	306 (98.4)	246 (99.2)			
rs4236601	*CAV1*	AA	0 (0)	0 (0)	0 (0)	0.078	0.31	0.16
	(upstream)	AG	6 (3.2)	6 (1.9)	2 (0.81)	4.12 (0.82-20.66)	2.42 (0.48-12.09)	3.05 (0.68-13.73)
		GG	179 (96.8)	305 (98.1)	246 (99.2)			
rs959173	*CAV1*	CC	0 (0)	0 (0)	0 (0)	0.11	0.020	0.016
	(Intron)	CT	15 (8.1)	22 (7.1)	32 (12.9)	0.60 (0.31-1.14)	0.51 (0.29-0.91)	0.54 (0.33-0.90)
		TT	170 (91.9)	289 (92.9)	216 (87.1)			
rs6466587	*CAV1*	GG	0 (0)	0 (0)	0 (0)	0.18	0.74	0.27
	(Intron)	AG	6 (3.2)	6 (1.9)	3 (1.2)	2.74 (0.68-11.09)	1.61 (0.40-6.49)	2.03 (0.57-7.24)
		AA	179 (96.8)	305 (98.1)	245 (98.8)			

Table 4.7. Genotype associations of the six SNPs in *CAV1/CAV2* with POAG in the Shantou cohort

SNP	Gene (location)	Genotype	Shantou subjects			P value and OR (95% CI) in dominant model		
			HTG (n=102)	NTG (n=28)	Control (n=298)	HTG	NTG	POAG
rs926201	*CAV2*	TT	0 (0)	0 (0)	0 (0)	0.78	0.91	0.77
	(upstream)	CT	7 (6.9)	2 (7.1)	23 (7.7)	0.88 (0.37-2.12)	0.92 (0.21-4.12)	0.89 (0.40-1.98)
		CC	95 (93.1)	26 (92.9)	275 (92.3)			
rs6975771	*CAV2*	AA	0 (0)	0 (0)	0 (0)	0.86	0.71	0.61
	(upstream)	AG	8 (7.8)	1 (3.6)	25 (8.4)	0.93 (0.41-2.13)	0.40 (0.053-3.10)	0.81 (0.37-1.79)
		GG	94 (92.2)	27 (96.4)	273 (91.6)			
rs4730742	*CAV2*	GG	0 (0)	0 (0)	0 (0)	0.11	0.24	0.072
	(upstream)	GT	3 (2.9)	1 (3.6)	2 (0.67)	4.49 (0.74-27.23)	5.48 (0.48-62.42)	4.70 (0.85-25.98)
		TT	99 (97.1)	27 (96.4)	296 (99.3)			
rs4236601	*CAV1*	AA	0 (0)	0 (0)	0 (0)	0.11	0.24	0.072
	(upstream)	AG	3 (2.9)	1 (3.6)	2 (0.67)	4.49 (0.74-27.23)	5.48 (0.48-62.42)	4.70 (0.85-25.98)
		GG	99 (97.1)	27 (96.4)	296 (99.3)			
rs959173	*CAV1*	CC	0 (0)	0 (0)	0 (0)	0.36	0.77	0.37
	(Intron)	CT	6 (5.9)	2 (7.1)	26 (8.7)	0.65 (0.26-1.64)	0.81 (0.18-3.58)	0.69 (0.30-1.56)
		TT	96 (94.1)	26 (92.9)	272 (91.3)			
rs6466587	*CAV1*	GG	0 (0)	0 (0)	0 (0)	0.76	0.24	0.59
	(Intron)	AG	1 (0.98)	1 (3.6)	2 (0.67)	1.47 (0.13-16.33)	5.48 (0.48-62.42)	2.31 (0.32-16.60)
		AA	101 (99.0)	27 (96.4)	296 (99.3)			

Table 4.8. Genotype associations of the six SNPs in *CAV1/CAV2* with POAG in the pooled Hong Kong/Shantou subjects

SNP	Gene (location)	Genotype	Pooled subjects			P value and OR (95% CI) in dominant model*		
			HTG (n=287)	NTG (n=339)	Control (n=546)	HTG	NTG	POAG
rs926201	*CAV2*	TT	1 (0.3)	0 (0)	0 (0)	0.20	0.06	0.06
	(upstream)	CT	22 (7.7)	26 (7.7)	55 (10.1)	0.71 (0.43-1.19)	0.60 (0.35-1.02)	0.67 (0.44-1.02)
		CC	264 (92.0)	313 (92.3)	491 (89.9)			
rs6975771	*CAV2*	AA	0 (0)	0 (0)	0 (0)	0.10	0.007	0.01
	(upstream)	AG	23 (8.0)	24 (7.1)	60 (11.0)	0.65 (0.39-1.09)	0.48 (0.28-0.82)	0.58 (0.38-0.88)
		GG	264 (92.0)	315 (92.9)	486 (89.0)			
rs4730742	*CAV2*	GG	0 (0)	0 (0)	0 (0)	0.06	0.22	0.06
	(upstream)	GT	7 (2.4)	6 (1.8)	4 (0.73)	3.38 (0.97-11.79)	2.47 (0.59-10.35)	3.03 (0.94-9.74)
		TT	280 (97.6)	333 (98.2)	542 (99.3)			
rs4236601	*CAV1*	AA	0 (0)	0 (0)	0 (0)	0.02	0.15	0.03
	(upstream)	AG	9 (3.1)	7 (2.1)	4 (0.73)	4.26 (1.27-14.32)	2.82 (0.69-11.57)	3.56 (1.12-11.33)
		GG	278 (96.9)	332 (97.9)	542 (99.3)			
rs959173	*CAV1*	CC	0 (0)	0 (0)	0 (0)	0.07	0.03	0.01
	(Intron)	CT	21 (7.3)	24 (7.1)	58 (10.6)	0.61 (0.36-1.04)	0.55 (0.32-0.94)	0.58 (0.38-0.90)
		TT	266 (92.7)	315 (92.9)	488 (89.4)			
rs6466587	*CAV1*	GG	0 (0)	0 (0)	0 (0)	0.16	0.29	0.18
	(Intron)	AG	7 (2.4)	7 (2.1)	5 (0.9)	2.37 (0.72-7.79)	1.96 (0.56-6.89)	2.09 (0.71-6.16)
		AA	280 (97.6)	332 (97.9)	541 (99.1)			

Note:

* Results for the different sample sets were combined using a Mantel-Haenszel model.

Table 4.9. Associations of rs6975771, rs4236601 and rs959173 with HTG in the Beijing cohort

SNP	Gene (location)	Genotype	Beijing subjects		P value (dominant)	OR (95% CI)
			HTG (n=177)	Control (n=200)		
rs6975771	*CAV2*	AA	0 (0)	0 (0)	0.45	1.28 (0.67-2.43)
	(upstream)	AG	22 (12.4)	20 (10.0)		
		GG	155 (87.6)	180 (90.0)		
rs4236601	*CAV1*	AA	0 (0)	0 (0)	0.19	2.09 (0.69-6.36)
	(upstream)	AG	9 (5.1)	5 (2.5)		
		GG	168 (94.9)	195 (97.5)		
rs959173	*CAV1*	CC	0 (0)	0 (0)	0.82	1.08 (0.56-2.10)
	(Intron)	CT	19 (10.7)	20 (10.0)		
		TT	158 (89.3)	180 (90.0)		

4.2.3 *CAV1/CAV2* SNPs associated with POAG in pooled Chinese

When all Chinese subjects were pooled, rs4236601[A] was significantly associated with an increased risk of HTG (P_{dom}=0.01; OR=2.90, 95% CI: 1.28-6.55, Table 4.10) and overall POAG (P_{dom}=0.01; OR=2.77, 95% CI: 1.25-6.12). It also showed a trend towards an increased risk of NTG (P_{dom}=0.15; OR=2.82, 95% CI: 0.69-11.57). In overall Chinese subjects, the higher-risk AG genotype accounts for 2.71% (95% CI: 0.75-4.62%) of PAR percentage for HTG and 0.87% (95% CI: 0-2.58%) for NTG.

Of SNPs rs6975771 and rs959173, the minor genotypes were associated with a significantly reduced risk of NTG (P_{dom}=0.007 and 0.03, OR=0.48 and 0.55, Table 4.10) while showing a trend towards a reduced risk of HTG (P_{dom}=0.38 and 0.19, OR=0.84 and 0.76). For overall POAG, rs6975771 showed a trend towards a reduced risk (P_{dom}=0.09, OR=0.74) and, rs959173 was marginally associated with the disorder (P_{dom}=0.05, OR=0.70).

4.2.4 Genotype-phenotype correlation

In view of the genetic associations, genotype-phenotype correlation was evaluated. No trend of correlation was observed between the risk or protective SNPs and the maximum IOP or VCDR (data not shown). We then evaluated whether the variants modified the age of onset, for which the AAD was used as a surrogate as described in section 3.1.3. In the pooled sample, HTG patients possessing the risk allele at rs4236601 was 2.6 years younger in mean AAD than those with the wild-type genotype (P=0.60, Table 4.11). In contrast, NTG patients with the risk allele have an older mean AAD by 0.8 years (P=0.89). Regarding rs6975771, NTG patients with the protective allele were 5.0 years older in mean AAD than those with the wild-type genotype, but the correlation fell just short of significance (P=0.10). Such a trend of correlation was milder for HTG patients, with a mean AAD difference of 0.028 years (or 2.5 years in southern subjects, Table 4.11).

Table 4.10. Genotype associations of rs6975771, rs4236601 and rs959173 with POAG in pooled Chinese subjects

SNP	Gene (location)	Genotype	Pooled Chinese subjects			P value and OR (95% CI) in dominant model†		
			HTG (n=464)	NTG (n=339)*	Control (n=746)	HTG	NTG‡	POAG
rs6975771	*CAV2*	AA	0 (0)	0 (0)	0 (0)	0.38	0.007	0.09
	(upstream)	AG	45 (9.7)	24 (7.1)	80 (10.7)	0.84 (0.57-1.24)	0.48 (0.28-0.82)	0.74 (0.52-1.04)
		GG	419 (90.3)	315 (92.9)	666 (89.3)			
rs4236601	*CAV1*	AA	0 (0)	0 (0)	0 (0)	0.01	0.15	0.01
	(upstream)	AG	18 (3.9)	7 (2.1)	9 (1.2)	2.90 (1.28-6.55)	2.82 (0.69-11.57)	2.77 (1.25-6.12)
		GG	446 (96.1)	332 (97.9)	737 (98.8)			
rs959173	*CAV1*	CC	0 (0)	0 (0)	0 (0)	0.19	0.03	0.05
	(Intron)	CT	40 (8.6)	24 (7.1)	78 (10.5)	0.76 (0.50-1.14)	0.55 (0.32-0.94)	0.70 (0.49-1.0)
		TT	424 (91.4)	315 (92.9)	668 (89.5)			

Note:

* The NTG patients were from the Hong Kong and Shantou cohorts only;

† Results for the different sample sets were combined using a Mantel-Haenszel model;

‡ The association was analyzed using the southern samples only. The results are therefore the same with that shown in Table 4.8.

Table 4.11. Age at diagnosis of patients with different genotypes at rs4236601 and rs6975771

SNP	Genotype	Pooled HTG (n=464)				Pooled NTG (n=339)†			
		Frequency (%)	Mean AAD ± SD (years)	P*	Mean difference (95% CI)	Frequency (%)	Mean AAD ± SD (years)	P*	Mean difference (95% CI)
rs4236601	AG	18 (3.9)	46.9 ± 18.7	0.60	-2.6 (-12.1 – 7.0)	7 (2.2)	63.5 ± 13.0	0.89	0.80 (-11.0 – 12.6)
	GG	446 (96.1)	49.5 ± 20.2			332 (97.8)	62.7 ± 14.5		
rs6975771	AG	45 (9.7)	49.4 ± 22.0	0.99	0.028 (-6.2 – 6.2)‡	24 (7.1)	67.8 ± 11.0	0.10	5.0 (-0.96 – 10.97)
	GG	419 (90.3)	49.3 ± 20.0			315 (92.9)	62.8 ± 14.5		

Note:

All patients involved in this study were pooled for the analysis.

* Analyzed by analysis of covariance.

† The pooled NTG subjects were southern Chinese only, because the Beijing cohort did not contain NTG patients.

‡ When only the southern Chinese HTG subjects were considered, because of the different tendencies of association between southern and northern Chinese at this SNP, the mean difference in AAD was 2.5 years (58.1 vs. 55.6 years; 95% CI: -5.9 – 10.9; P=0.56).

4.2.5 Linkage disequilibrium and haplotype-based association analysis

Pair-wise LD structure was constructed (Figure 4.3). The LD patterns were similar in the three cohorts. The protective SNPs rs6975771 and rs959173 were in strong LD (r^2 >0.88), whilst these two SNPs were in weaker LD with rs926201 (r^2 <0.82). The risk SNPs rs4730742 and rs4236601 were in strong LD (r^2 >0.78) and they exhibited weaker LD with rs6466587 (r^2 <0.68). Between the risk and protective SNPs, weak LD was observed (r^2 <0.1).

Haplotype-based association analysis was performed to further evaluate the role of *CAV1/CAV2* in POAG. First, all of the six SNPs in the Hong Kong or Shantou cohort were included in the haplotype analysis and then a sliding-window analysis of 2 to 4 adjacent SNPs across the region was performed to detect the most significant haplotype block. In Block-1 (B1, Table 4.12), where all six SNPs were included, a significant omnibus association was detected with NTG (P=0.031) and overall POAG (P=0.029) in the Hong Kong cohort. In the sliding-window analyses, the block (B3) containing rs6975771, rs4730742, rs4236601 and rs959173 showed the strongest omnibus association in all groups suggestive of a major contribution.

Haplotype-specific associations including all the 6 SNPs with overall POAG in the Hong Kong cohort were shown in Table 4.13. Three haplotypes (i.e., B1H2, B1H5 and B1H7) showed nominally significant association (P=0.034, 0.032 and 0.036 respectively), conferring reduced risk to POAG. These haplotypes contained at least one protective allele at the three protective SNPs, i.e., rs926201, rs6975771 and rs959173, but no higher-risk allele at the three risk SNPs, i.e., rs4730742, rs4236601 and rs6466587. Haplotype-based sole-variant tests showed that more than one haplotype gave a P_{SVh} value >0.05, suggesting that no single haplotype could explain the omnibus association. SNP-based sole-variant tests also showed that no individual SNP could explain the omnibus association.

Independent effect tests showed that SNPs rs6975771 and rs959173 had independent effects after controlling for other SNPs (P_{IE}=0.013 and 0.025 respectively), while the effects of these two SNPs could not be distinguished from each other ($P_{IE(2)}$=0.0069 and 0.017 respectively, Table 4.13).

Haplotype-based association analysis for the SNPs in Block 3, which was found to have major contribution, was performed (Table 4.14). The most common haplotype (B3H1) was present at higher frequencies in the Hong Kong patient groups (~95% in case groups vs. 92.1% in controls) but present at lower frequency in the Shantou patient group (94.6% in POAG vs. 95.3% in controls). And a protective haplotype (B3H4) detected in the Hong Kong cohort (0.1% in POAG vs. 1.0% in controls) was present at higher frequency in Shantou POAG (0.8%) than in controls (0%, P=0.029). Thus, these two haplotypes did not have consistent contribution to POAG. In contrast, two haplotypes, i.e., B3H2 and B3H3, exhibited similar distributions in different study cohorts. Haplotype B3H2, which contained the protective alleles of rs6975771 and rs959173 and the wild-type alleles of rs4730742 and rs4236601, was present consistently at higher frequencies in the case groups. Moreover, this haplotype was nominally associated with a reduced risk of NTG (P=0.044, OR=0.57) and overall POAG (P=0.041, P=0.60) in the Hong Kong cohort. In contrast, haplotype B3H3, which contained the wild-type alleles of rs6975771 and rs959173 and the higher-risk alleles of rs4730742 and rs4236601, showed a consistent trend towards an increased disease risk. And it was nominally associated with overall POAG in the pooled Hong Kong and Shantou subjects (P=0.048, OR=2.85). Moreover, in the pooled subjects this haplotype (B3H3) was the only haplotype that can explain the omnibus association (P_{SVh} =0.052). However, no single SNP could explain the global association and no SNP showed an independent effect, suggesting that every SNP contributed to the global association.

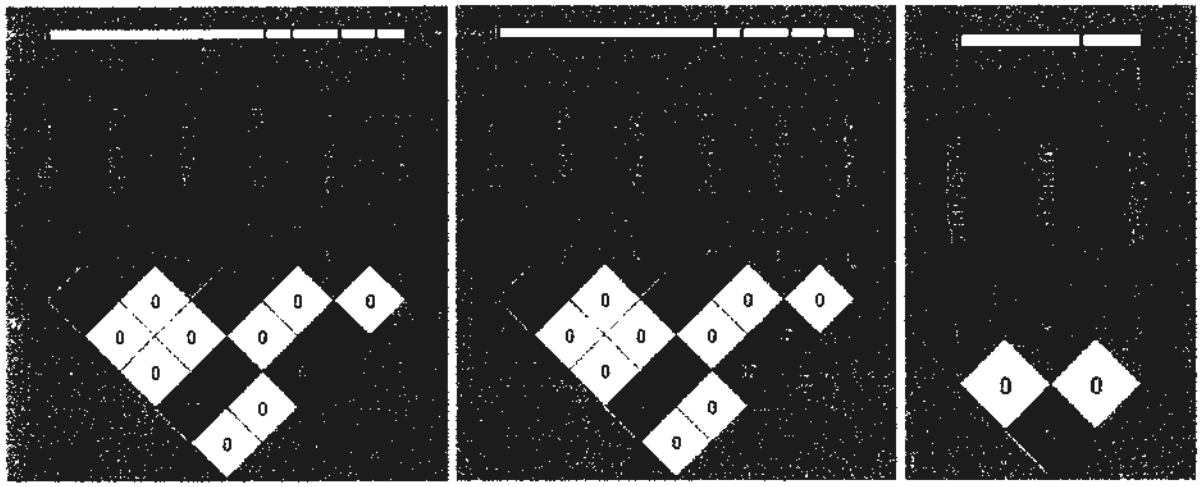


Figure 4.3. Linkage disequilibrium of SNPs at the *CAV1/CAV2* locus

Linkage disequilibrium was measured using data from all subjects from the Hong Kong, Shantou and Beijing cohorts, respectively. Each box provides estimated statistics of the coefficient of determination (r^2), with darker shades representing stronger LD.

Table 4.12. Omnibus haplotype associations in the Hong Kong and Shantou cohorts

Block	Haplotype*						P value (Omnibus test)			
	rs926201	rs6975771	rs4730742	rs4236601	rs959173	rs6466587	Hong Kong HTG	Hong Kong NTG	Hong Kong POAG	Shantou POAG†
B1	x	x	x	x	x	x	0.084	0.031	0.029	0.11
B2	x	x	x	x	-	-	0.031	0.095	0.041	0.39
B3	-	x	x	x	x	-	0.013	0.016	0.0021	0.041
B4	-	-	x	x	x	x	0.14	0.071	0.10	0.27
B5	x	x	x	-	-	-	0.061	0.093	0.055	0.39
B6	-	x	x	x	-	-	0.039	0.028	0.013	0.15
B7	-	-	x	x	x	-	0.067	0.052	0.029	0.11
B8	-	-	-	x	x	x	0.15	0.071	0.072	0.27
B9	x	x	-	-	-	-	0.051	0.061	0.042	0.90
B10	-	x	x	-	-	-	0.078	0.027	0.015	0.15
B11	-	-	x	x	-	-	0.088	0.39	0.15	0.065
B12	-	-	-	x	x	-	0.055	0.040	0.019	0.11
B13	-	-	-	-	x	x	0.17	0.054	0.030	0.48

Note:

* Haplotype with an overall frequency of ≥0.1% was included in the analyses;

† The Shantou patients with HTG or NTG were pooled in haplotype analyses;

The "x" indicates that the SNP was included in the sliding-window haplotype analyses;

The most significant P values from omnibus tests in each group were underlined.

Table 4.13. Haplotype-based association of all six *CAV1/CAV2* SNPs with POAG* in the Hong Kong cohort

	Haplotype†						Frequency (%)		HS (P)‡	P_{perm}	Odds ratio (95% CI)‡	SV_H (P)
	rs926201	rs6975771	rs4730742	rs4236601	rs959173	rs6466587	POAG*	Control				
B1H1	C	G	T	G	T	A	94.4	91.5	0.063	0.14	1.57 (1.03-2.38)	0.059
B1H2	T	A	T	G	C	A	3.3	5.6	0.034	0.14	0.58 (0.35-0.97)	0.082
B1H3	T	G	T	G	T	A	0.7	0.6	0.35	0.99	NS	0.023
B1H4	C	G	G	A	T	G	0.8	0.4	0.46	0.98	NS	0.021
B1H5	C	A	T	G	T	A	0.1	1.0	0.032	0.048	0.10 (0.012-0.86)	0.086
B1H6	C	A	T	G	C	A	0.3	0.2	0.72	0.99	NS	0.018
B1H7	C	G	T	G	C	A	0	0.4	0.036	0.40	NS	0.080
B1H8	C	G	T	A	T	G	0.2	0	0.20	0.90	NS	0.030
B1H9	T	A	T	G	C	G	0.1	0.2	0.63	0.99	NS	0.018
SVs (P)	0.052	0.16	0.023	0.031	0.12	0.024						
IE (P)	0.53	0.013	0.37	1.0	0.025	0.91						
$IE_{(2)}$ (P)	-	0.0069	-	-	0.017	-						

Note:

* POAG represents pooled HTG and NTG; for the six SNPs, results of haplotype analysis in the NTG and HTG group respectively were similar to that in the overall POAG sample and were therefore not shown in the table;

† Haplotype with an overall frequency of ≥0.1% was included in the analysis;

‡ Haplotype-specific test P value and OR estimated by χ^2 test; OR was calculated for each individual haplotype compared to all the other haplotypes;

P_{perm}: P value from permutation test performed in Haploview (no. of iteration=10,000);

SV_H: haplotype-based sole-variant test, testing whether other haplotypes still have an effect after controlling for this haplotype;

SVs: SNP-based sole-variant test P-value, testing whether other SNPs still have an effect after controlling for the tested SNP;

IE: independent effect test P-value, testing whether the tested SNP has any effect after controlling for others;

$IE_{(2)}$: independent effect test P-value for the two SNPs indicated, testing whether rs959173 has an effect after controlling for rs6975771, and vice versa;

Omnibus $\chi^2_{(8)}$=17.11, P=0.029, where 8 is the degree of freedom;

NS: non-significant.

Table 4.14. Haplotype-based association of *CAV1/CAV2* SNPs in Block 3 with POAG in the Hong Kong and Shantou cohorts

Haplotype†					Frequency (%)				
	rs6975771	rs4730742	rs4236601	rs959173	Case	Control	HS (P)	OR (95% CI)	
Hong Kong subjects									
HTG									
B3H1	G	T	G	T	94.3	92.1	0.19	1.42 (0.82-2.45)	
B3H2	A	T	G	C	4.1	6.0	0.17	0.66 (0.35-1.24)	
B3H3	G	G	A	T	1.1	0.4	0.23	2.70 (0.49-14.82)	
B3H4	A	T	G	T	0	1.0	0.018	-	
B3H5	G	T	G	C	0	0.4	0.14	-	
B3H6	G	T	A	T	0.5	0	0.065	-	
NTG									
B3H1	G	T	G	T	95.5	92.1	0.016	1.80 (1.09-2.98)	
B3H2	A	T	G	C	3.5	6.0	0.044	0.57 (0.33-1.004)	
B3H3	G	G	A	T	0.8	0.4	0.39	2.01 (0.39-10.40)	
B3H4	A	T	G	T	0.2	1.0	0.048	0.16 (0.018-1.36)	
B3H5	G	T	G	C	0	0.4	0.071	-	
B3H6	G	T	A	T	0	0	-	-	
POAG									
B3H1	G	T	G	T	95.0	92.1	0.028	1.61 (1.04-2.48)	
B3H2	A	T	G	C	3.7	6.0	0.041	0.60 (0.37-0.99)	
B3H3	G	G	A	T	0.9	0.4	0.26	2.26 (0.49-10.51)	
B3H4	A	T	G	T	0.1	1.0	0.011	0.099 (0.012-0.85)	
B3H5	G	T	G	C	0	0.4	0.036	-	
B3H6	G	T	A	T	0.3	0	0.12	-	
Shantou subjects (POAG)									
POAG									
B3H1	G	T	G	T	94.6	95.3	0.67	0.87 (0.45-1.67)	
B3H2	A	T	G	C	2.7	4.2	0.26	0.63 (0.27-1.48)	
B3H3	G	G	A	T	1.5	0.3	0.065	4.64 (0.85-25.50)	
B3H4	A	T	G	T	0.8	0	0.029	1.01 (1.0-1.019)	
B3H5	G	T	G	C	0.4	0.2	0.56	2.30 (0.14-36.87)	
B3H6	G	T	A	T	0	0	-	-	
Pooled Hong Kong/Shantou subjects (POAG)									**SV_H (P)**
B3H1	G	T	G	T	94.9	93.9	0.27	1.21 (0.85-1.73)	0.017
B3H2	A	T	G	C	3.5	5.0	0.062	0.69 (0.46-1.03)	0.044
B3H3	G	G	A	T	1.0	0.4	0.048	2.85 (0.93-8.78)	0.052
B3H4	A	T	G	T	0.2	0.5	0.37	0.53 (0.13-2.21)	0.014
B3H5	G	T	G	C	0.1	0.3	0.25	0.29 (0.03-2.79)	0.018
B3H6	G	T	A	T	0.2	0	0.052	-	0.049
SVs (P)	0.060	0.052	0.13	0.061					
IE (P)	0.48	0.23	0.055	0.47					
Omnibus test (P): 0.021									

Note:

Underlined were haplotypes that showed a consistent trend towards an increased or reduced risk of POAG.

4.2.6 Interaction between *CAV1/CAV2* SNPs and SNPs in other genes for POAG

Genotype data of the study subjects that overlapped with those included in the study of multiple SNPs described in section 4.1 were extracted for gene-gene interaction analysis using PLINK and MDR. However, no statistically significant interaction was detected between the *CAV1/CAV2* SNPs and the candidate SNPs in or near the *CYP46A1*, *LMX1B*, *PLXDC2*, *TLR4*, *TMTC2* and *ZP4* genes, or in the 2p16.3 locus.

4.3 Mutation screening of the *neurotrophin-4* gene for POAG

4.3.1 Sequence variants detected in *NTF4*

A total of 928 subjects were screened for *NTF4* mutations, including 185 HTG patients, 206 NTG patients and 230 controls from the Hong Kong cohort, 130 POAG patients from the Shantou cohort and 177 HTG patients from the Beijing cohort. Totally, three variants were detected: c.453G>A (Pro151Pro), c.470G>C (Gly157Ala) and c.545C>T (Ala182Val). The Pro151Pro variant was reported in an Indian cohort (Rao et al. 2010), while the other two were new. The synonymous variant Pro151Pro was detected in 1 HTG patient, 1 NTG patient and 1 control subject from the Hong Kong cohort, and in 1 HTG patient from the Beijing cohort. This variant is unlikely to be a disease-causing mutation.

The two missense variants were detected only in the Hong Kong cohort, with Gly157Ala being identified in one NTG patient and Ala182Val in one HTG patient. If they are in fact disease-causing, the *NTF4* mutations likely account for approximately 0.51% (2/391; 95% CI: 0.14-1.84%) of POAG in Hong Kong Chinese or 0.29% (2/698; 95% CI: 0.08-1.04%) in overall Chinese.

No sequence change was detected in the 130 patients from the Shantou cohort.

4.3.2 Analysis of variants and genotype-phenotype correlation

For the missense variants, two programs (i.e., PolyPhen and SIFT) were used to predict their functional impacts to the NTF4 protein. The Gly147Ala was predicted to be "benign" by PolyPhen, while being predicted to "affect protein function" by SIFT, with a median sequence conservation score of 4.32 indicative of low confidence. This variant was detected in a male patient with NTG. The age at diagnosis was 67 years, and the maximum IOP was 17 mmHg in both eyes. The Ala182Val variant was predicted to be "benign" and "tolerated" by PolyPhen and SIFT, respectively. It was present in a female patient with HTG, whose AAD was 28 years and maximum IOP was 36 and 40 mmHg in the right and left eye, respectively. Family members of these two patients were not available for segregation analysis.

Up to the completion of this thesis, the functional characterization of these two variants is still in progress and no consolidated results are yet available to support their properties.

4.4 Mutation screening of the *ciliary neurotrophic factor* gene for POAG

4.4.1 Sequence variants detected in *CNTF*

The *CNTF* gene was screened in a subset of 469 study subjects, including 102 NTG patients and 103 controls from the Hong Kong cohort and, 102 HTG patients, 28 NTG patients and 134 controls from the Shantou cohort. Totally, 7 sequence variations were identified, including 3 missense changes: c.223G>C (Glu75Gln), c.407G>A (Arg136His) and c.545A>G (His182Arg), one 1-bp insertion (c.103_104insA), and 3 changes in the noncoding sequence: c.-135G>T, c.-22C>T and IVS1-6G>A (Table 4.15). Among them, five were new and, the other two, i.e. IVS1-6G>A (rs1800169) and c.545A>G (rs6266), were known SNPs registered in the dbSNP database.

Table 4.15. Genotype distributions of the *CNTF* variants detected in this study

Location	Sequence change	Residue change	SNP ID	Hong Kong cohort		Shantou cohort		
				NTG (n=102)	Control (n=103)	HTG (n=102)	NTG (n=28)	Control (n=134)
5'-UTR	-135G>T	—	Novel	0/1/101	ND	ND	0/1/27	0/1/133
5'-UTR	-22C>T	—	Novel	0/1/101	ND	0/1/101	ND	0/4/130
Exon 1	c.103_104insA	Thr35AsnfsX28	Novel	ND	ND	ND	ND	0/1/133
Intron 1	IVS1-6G>A	N/A	rs1800169	2/21/79 (1.9/20.6/77.5)*	2/29/72 (1.9/28.2/69.9)*	1/28/73 (1.0/27.7/71.3)*	0/14/14 (0/50/50)*	2/31/101 (1.5/23.1/75.4)*
Exon 2	c.223G>C	Glu75Gln	Novel	0/1/101	ND	ND	ND	ND
Exon 2	c.407G>A	Arg136His	Novel	ND	ND	0/1/100	ND	0/1/133
Exon 2	c.545A>G	His182Arg	rs6266	0/3/99	1/3/99	0/6/95	ND	0/12/122

Note:

The genotype counts of the variants in each study group are shown as homozygote/heterozygote/wild-type.

* Genotype frequency.

ND: not detected.

4.4.2 Distribution of *CNTF* variants in the study subjects

All of the 7 variants followed HWE (P>0.05). Among them, the missene change Glu75Gln was detected in one Hong Kong NTG patient but not in the 237 control subjects. In contrast, the 1-bp insertion c.103_104insA was detected exclusively in one control subject from the Shantou cohort. The other five variants were detected in both patients and controls, and there was no statistically significant difference in their distributions between groups, with an exception of SNP rs1800169 in the Shantou cohort, where the minor allele A was present in 50% (14/28) of NTG patients and 24.6% of controls (Table 4.15), conferring a significantly increased risk (P_{dom}=0.007; OR=3.06, 95% CI: 1.32-7.08). The A allele at rs1800169 was not significantly associated HTG in the Shantou cohort (28.7% in HTG vs. 24.6% in controls; P_{dom}=0.48; OR=1.23, 95% CI: 0.69-1.21). Interestingly in the Hong Kong cohort, the A allele at rs1800169 was present in 22.5% of NTG patients and 30.1% of controls (P=0.22; OR=0.68, 95% CI: 0.36-1.27). With these findings, we pooled the genotype data of the two cohorts using a Mantel-Haenszel model with random effects (Figure 4.4). In the pooled subjects, no significant association was found between rs1800169 and NTG (P=0.66; OR=1.40, 95% CI: 0.32-6.15) or overall POAG (P=0.94, OR=1.03, 95% CI: 0.47-2.26). As such, the association found with Shantou NTG might be artifact of a small cohort effect.

4.4.3 Analysis of variants and genotype-phenotype correlation

For the missense mutations Glu75Gln that was detected exclusively in one patient subject from the Hong Kong cohort, PolyPhen and SIFT were used to predict its functional impact. It was predicted to be "benign" by PolyPhen and "tolerated" by SIFT, suggesting that the variant is less likely to be a deleterious mutation. This variant was present in a female patient with NTG diagnosed at the age of 35 years. The maximum IOP was 21

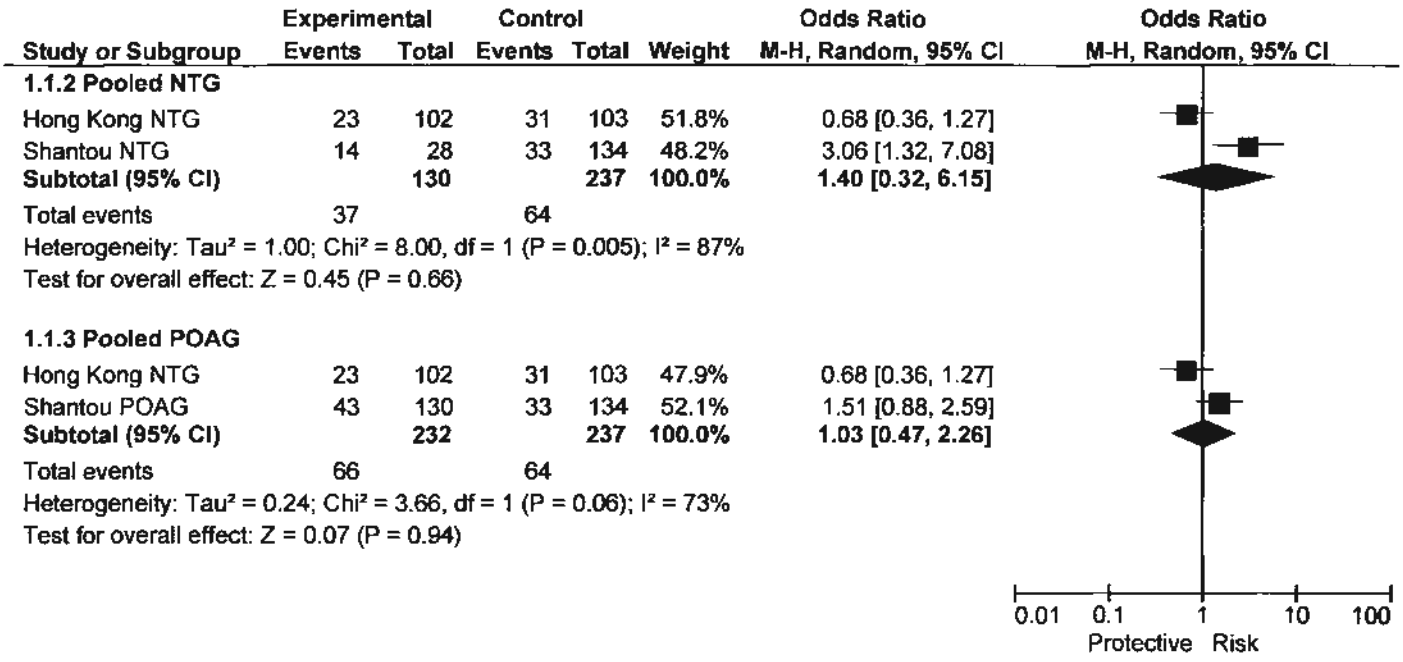


Figure 4.4. Association of *CNTF* rs1800169 with POAG in the Hong Kong, Shantou and pooled subjects

The associations were evaluated in a dominant genetic model. The data from different cohorts were pooled using a Mantel-Haenszel model with random effects.

mmHg in the right eye and 18 mmHg in the left eye, the VCDR was 0.8 and 0.7 in respective eye. The patient has no known family history of glaucoma. But her family members were not available for clinical and genetic investigations.

The 1-bp insertion c.103_104insA is predicted to cause a frameshift and introduce a premature termination codon to the transcript, resulting in a truncation protein designated as Thr35AsnfsX28. The mutant truncates the 200-amino acid CNTF protein by approximately 70% of full length. The mutation was detected in a 94-year old female subject, who visited the eye clinic for senile cataract. The IOP was normal in both eyes (OD: 9 mmHg, OS: 11 mmHg), and no glaucomatous sign was detected. Therefore, the frameshift mutation is unlikely to be causative for POAG.

Since the variant IVS1-6G>A (rs1800169) at the splice receptor site of *CNTF* is a null mutation causing reduced expression of CNTF (Takahashi et al. 1994), and it was found to be a genetic modifier leading to early disease onset of other neurodegenerative disorders, such as familial amyotrophic lateral sclerosis (FALS) with SOD-1 mutations (Giess et al. 2002a), and early onset multiple sclerosis (MS) (Giess et al. 2002b), correlation between this variant and the AAD of glaucoma was evaluated in our patient subjects (Table 4.16). The null variant was significantly correlated with AAD of overall POAG in the Shantou cohort (P=0.013). Mean AAD in patients with at least one copy of the minor allele A was 9.5 years (95% CI: 2.0-17.0) older than patients with the wild-type genotype. In Hong Kong NTG, however, such correlation was not statistically significant (P=0.47). Interestingly, the correlation if any was even at a reverse tendency, with the minor allele being correlated with a younger AAD (mean difference = -2.2 years). In light of these findings, the patients were stratified by AAD as described in section 3.1.3. However, no significant age-varying association was observed in either cohort (data not shown).

Table 4.16. Correlation of the genotypes at *CNTF* rs1800169 with age at diagnosis of POAG

SNP	Genotype	Hong Kong NTG (n=102)				Shantou overall POAG (n=130)†			
		Frequency (%)	Mean AAD (SD, years)	P*	Mean difference (95% CI)	Frequency (%)	Mean AAD (SD, years)	P*	Mean difference (95% CI)
rs1800169	AA+AG	23 (22.5)	64.1 (14.0)	0.47	-2.2 (-8.2 – 3.8)	43 (42.2)	52.6 (18.6)	0.013	9.5 (2.0 – 17.0)
(IVS1-6G>A)	GG	79 (77.5)	66.3 (12.4)			87 (57.8)	43.0 (21.0)		

Note:

* Analyzed by analysis of covariance.

† In the Shantou cohort, the trends of correlation were similar in the HTG and NTG groups, but the difference in mean AAD achieved statistical significance only when all patients were pooled.

4.5 Mutation screening of the *SPARC* gene for JOAG

4.5.1 Sequence variants detected in *SPARC*

The *SPARC* gene was screened in 168 subjects, including 27 members from the GLC1M-linked Philippine pedigree and, 46 unrelated JOAG patients and 95 controls from the Hong Kong cohort. In the pedigree, only one variant, i.e., c.912+29 C>G (rs1053411), was detected in an unaffected subject. No sequence change was found in other members.

In the Chinese study subjects, 11 variants were detected (Table 4.17), among which two were novel: IVS2+8G>T and IVS2+32C>T. The heterozygous variant IVS2+8G>T was detected in two (2.1%) control subjects but not in patients, whilst the IVS2+32C>T was detected in one (2.2%) patient and absent in controls. Owing to the intronic locations, these two variants are less likely to be functional mutations affecting POAG susceptibility. The other 9 variants were known polymorphisms registered in the SNP database. Except for a synonymous SNP rs2304052 (Glu22Glu) detected in exon 2, all other SNPs were located in noncoding regions. All of these SNPs followed HWE in both the control and patient groups. Moreover, the allele or genotype distribution of each SNP was not significantly different between patients and controls indicative of no association with JOAG. Linkage disequilibrium analysis revealed extension of LD throughout the *SPARC* gene. The five most common SNPs, rs2116780 (Intron 3), rs1978707 (Intron 4), rs7719521 (Intron 5), rs729853 (Intron 7), and rs1053411 (3'-UTR), were contained in a LD block spanning approximately 11kb (Figure 4.5A). Haplotype-based association analysis showed that no haplotype was significantly associated with the disorder (Figure 4.5B).

4.5.2 Copy number analysis of *SPARC*

The three assays were unequivocally genotyped in all subjects (n=63) to obtain predicted copy numbers of the target sequences in *SPARC* (Figure 4.6). The confidence of

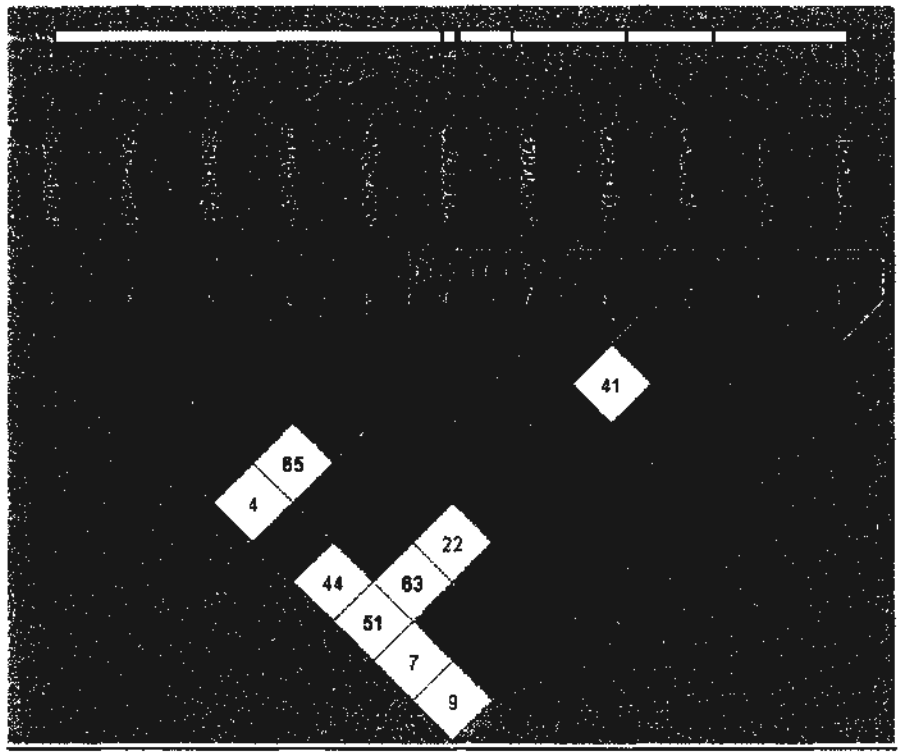
Table 4.17. *SPARC* variants detected in Chinese JOAG and control subjects

Location	Sequence change	Residue change	SNP ID	Minor allele frequency (%)			Genotype counts		
				Case (n=92)	Control (n=190)	P	Case (n=46)	Control (n=95)	P
Exon 1	c.-186G>A	–	rs4958281	5 (5.4)	6 (3.2)	0.35	0/5/41	0/6/89	0.34
Intron 2	IVS2+56G>C	–	rs7714314	3 (3.3)	7 (3.7)	1.0	0/3/43	1/5/89	0.75
Exon 3	c.66A>G	Glu22Glu	rs2304052	2 (2.2)	5 (2.6)	1.0	0/2/44	0/5/90	1.0
Intron 3	IVS3+8G>T	–	novel	0 (0)	2 (1.1)	-	0/0/46	0/2/93	-
Intron 3	IVS3+32C>T	–	novel	1 (1.1)	0 (0)	-	0/1/45	0/0/95	-
Intron 3	IVS3+36T>G	–	rs2116780	36 (39.1)	77 (40.5)	0.82	7/22/17	15/47/33	0.97
Intron 3	IVS3+42T>C	–	rs2304051	4 (4.3)	6 (3.2)	0.73	0/4/42	0/6/89	0.73
Intron 4	IVS4+31C>T	–	rs1978707	45 (48.9)	92 (48.4)	0.94	10/25/11	24/44/27	0.67
Intron 5	IVS5-59T>G	–	rs7719521	44 (47.8)	89 (46.8)	0.88	10/24/12	22/45/28	0.86
Intron 7	IVS7+100G>A	–	rs729853	38 (41.3)	80 (42.1)	0.90	7/24/15	16/48/31	0.97
3'-UTR	c.912+29C>G	–	rs1053411	39 (42.4)	80 (42.1)	0.96	8/23/15	16/48/31	0.99

Note:

3'-UTR: 3'-untranslated region; NS: nonsignificant.

(A)



(B)

Haplotype	Case (%)	Control (%)	P value
TCTGC	45.6	45.8	0.98
GTGAG	38.0	38.9	0.88
TTGGC	9.8	9.5	0.93
TTGAG	1.1	2.7	0.40

Figure 4.5. Linkage disequilibrium and haplotype association analyses for the *SPARC* variants detected in this study

(A) Linkage disequilibrium plot of 11 SNPs of the *SPARC* gene in the combined subjects. D' values corresponding to each SNP pair are expressed as a percentage and shown within the respective square. The five most common SNPs constitute a haplotype block spanning from intron 3 to the 3'-UTR of the gene. (B) Haplotype-based association analysis of the 5 most common SNPs in the LD block with JOAG. The frequencies of each haplotype in the patient and control groups were present in percentage. Only haplotypes with a frequency of >1% were shown.

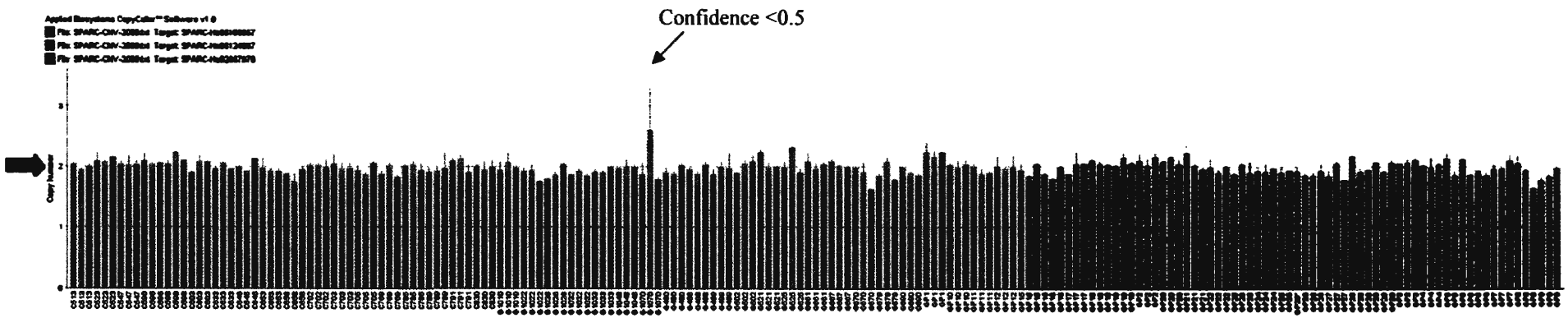


Figure 4.6. Copy number of *SPARC* in the Philippine family members and unrelated Chinese JOAG patients and controls

Each bar represents the copy number prediction of the target sequence in each subject. And each color presents each copy number assay. Thus, each individual is represented by three bars. The red arrow indicates the reference line for two copies. The black arrow indicates the sample (G1070) being predicted to have a copy number of 3 but with a confidence <50%.

prediction was greater than 95% for each assay in each subject, except for the sample G1070, with a confidence <50% at assay Hs06124887_cn. This sample was predicted to have 3 copies of the target sequence by this assay but was predicted to have 2 copies of the target sequences by the other two assays with confidence >99%. Therefore, it could be concluded that this sample only had 2 gene copies. All the other samples were predicted to have 2 copies of the *SPARC* gene by any assay, suggesting no correlation between the copy number and glaucoma.

4.6 Candidate genes screening at GLC1N and mutation screening of *MEGF11*

4.6.1 Mutational screening in the Hong Kong pedigree with JOAG

First, 15 members of the GLC1N-linked Hong Kong JOAG pedigree, including all of the 8 affected and 7 unaffected subjects, were selected for whole gene screening for the *DPP8*, *DENND4A*, *MEGF11* and *RAB11A* genes. A total of 20 sequence changes were identified, with 15 having been reported and 5 being new (Table 4.18). Among these variants, 5 variants were cosegregated with the disease, including *DPP8* IVS6+76insA (novel), *DPP8* IVS10-77A>T (rs389377), *DPP8* c.2616G>T (Ser872Ser, rs11009), *DENND4A* IVS17-68T>A (novel) and *MEGF11* c.1090delT (Cys364ValfsX12, novel). Interestingly, an unaffected subject (III:16) was homozygous for all of the five variant alleles, while the other 6 unaffected members were homozygous for the wild-type alleles. Of these five variants, two are reported common SNPs and are unlikely to be disease causing mutations. Of the three novel variants, two are located in intronic regions and are less likely to be the causal mutations. The remaining novel variant *MEGF11* c.1090delT (Figure 4.7), which is predicted to cause a frameshift, introduce a premature termination codon to the transcript and result in a truncation protein Cys364ValfsX12, is the most likely to be a pathogenic mutation. With these findings, the *MEGF11* c.1090delT was genotyped

in the rest of the family members, all of whom did not have glaucoma at least by the time of recruitment. Four additional members (II:15, III:4, III:6 and III:17) in the pedigree were found to carry the deletion (Figure 4.8). Surprisingly, subject II:15, married-in husband of the affected member II:16, was also found to carry the deletion. Because of this, the kindred members of II:15, i.e., II:15M and II:15S, were recruited and genotyped for the mutation. Subject II:15S was found to be heterozygous for c.1090delT, while II:15M did not carry the mutation (Figure 4.8). According to their family histories and the haplotypes constructed by microsatellite markers used for linkage analysis (Wang et al. 2006b), these two families are not related. Based on these findings, the *MEGF11* c.1090delT variant is likely to be a disease-causing mutation for JOAG in the pedigree, with incomplete penetrance.

4.6.2 *MEGF11* variants detected in unrelated patients and controls

In order to confirm the role of *MEGF11* as a causative gene for POAG, we screened the gene in a set of unrelated subjects, including 181 HTG patients and 182 controls from the Hong Kong cohort, 177 HTG and 200 controls from the Beijing cohort, and 95 HTG and 147 controls from the Shantou cohort. For the Hong Kong and Beijing subjects, all exons were screened. In the Shantou cohort, only the cases subjects were screened for all exons, any putative mutations detected were genotyped in controls.

Totally, 60 sequence changes were identified, of which 8 were known SNPs and 52 were novel (Tables 4.19 and 4.20). Among them, 54 were single base-pair substitutions, 3 were small deletions and 3 were small insertions. Thirty-seven variants were located in the coding region, with 26 being missence changes (Table 4.19). Another 23 variants resided in the introns (Table 4.20). Notably, the c.1090delT mutation, which was detected in the GLC1N-linked Hong Kong pedigree, was identified in one patient from the Beijing cohort.

Table 4.18. Sequence changes detected in the GLC1N-linked Hong Kong pedigree with JOAG

No.	Gene	Sequence change	SNP ID	I:1	II:4	II:6	II:10	II:14	II:16	III:5	III:8	III:16	I:2	II:1	II:5	II:7	II:12	II:17
				2†	2	2	2	2	2	2	2	1	1	1	1	1	1	1
1	DPP8	IVS6+76 INSA	Novel	ins	ins	ins	ins	ins	ins	ins	ins	/	ins *	ins *	ins *	ins *	ins *	ins *
2	DPP8	c.1206 A>G	rs352461	AG	AG	AG	AG	AG	AA	AG	AG	AG	AA	AA	AG	AA	AA	AA
3	DPP8	IVS10-77 A>T	rs389377	AT	AT	AT	AT	AT	AT	AT	AT	TT	AA	AA	AA	AA	AA	AA
4	DPP8	IVS12-59 G>A	Novel	GA	GA	GA	GA	GA	GA	GA	GA	AA	GG	GG	GG	GG	GG	GG
5	DPP8	IVS15+27 A>G	rs3825891	AG	AG	AG	AG	AG	AG	AG	AA	AA	GG	GG	AG	GG	GG	GG
6	DPP8	c.2616 G>T	rs11009	GT	GT	GT	GT	GT	GT	GT	GT	TT	GG	GG	GG	GG	GG	GG
7	DENND4A	IVS6+20 T>C	rs10519304	TC	TC	TC	TC	TC	TC	TC	TC	CC	TT	TT	TT	TT	TT	TT
8	DENND4A	IVS8+80 C>T	rs11071849	TT	TT	TT	TT	TT	TT	TT	CT	TT	TT	TT	CT	TT	TT	TT
9	DENND4A	IVS12-45 G>A	rs2060454	GA	GA	GA	GA	GA	GA	GA	GG	GG	AA	AA	GA	AA	AA	AA
10	DENND4A	IVS13-11 T>A	rs28687655	AA	AA	AA	AA	AA	AA	AA	TA	AA	AA	AA	TA	AA	AA	AA
11	DENND4A	c.2337 G>A	rs17810074	GG	GG	GG	GG	GG	GG	GG	GA	GG	GG	GG	GA	GG	GG	GG
12	DENND4A	IVS17+41 T>C	rs28377658	TC	TC	TC	TC	TC	TC	TC	TT	TT	CC	CC	TC	CC	CC	CC
13	DENND4A	IVS17-68 T>A	Novel	TA	TA	TA	TA	TA	TA	TA	TA	AA	TT	TT	TT	TT	TT	TT
14	DENND4A	IVS18+66 A>G	rs16948954	AG	AG	AG	AG	AG	AG	AG	AA	AA	GG	GG	AG	GG	GG	GG
15	DENND4A	IVS28+58 A>G	rs12591647	AG	AG	AG	AG	AG	AG	AG	AA	AA	GG	GG	AG	GG	GG	GG
16	DENND4A	IVS31+61delTTT	Novel	del	del	del	del	del	del	del	/	/	del *	del *	del	del *	del *	del *
17	MEGF11	IVS4-21C>T	rs7168565	CC	CC	CC	CC	CC	CC	CT	CC	CC	CC	CC	CC	CC	CC	CC
18	MEGF11	c.1090delT	Novel	del	del	del	del	del	del	del	del	del*	/	/	/	/	/	/
19	MEGF11	IVS9+9InsT	rs36088570	ins*	ins*	ins*	ins*	ins*	ins*	ins	ins*	ins*	ins*	ins*	ins	ins*	ins*	ins*
20	MEGF11	IVS10+78C>A	rs333569	AA	AA	AA	AA	AA	AA	CA	AA	AA	AA	AA	CA	AA	AA	AA

Note:

† Affected status: 2 represents affected, 1 represents unaffected.

* Homozygous for the deletion.

Variants that are cosegregated with the disease in the family are highlighted.

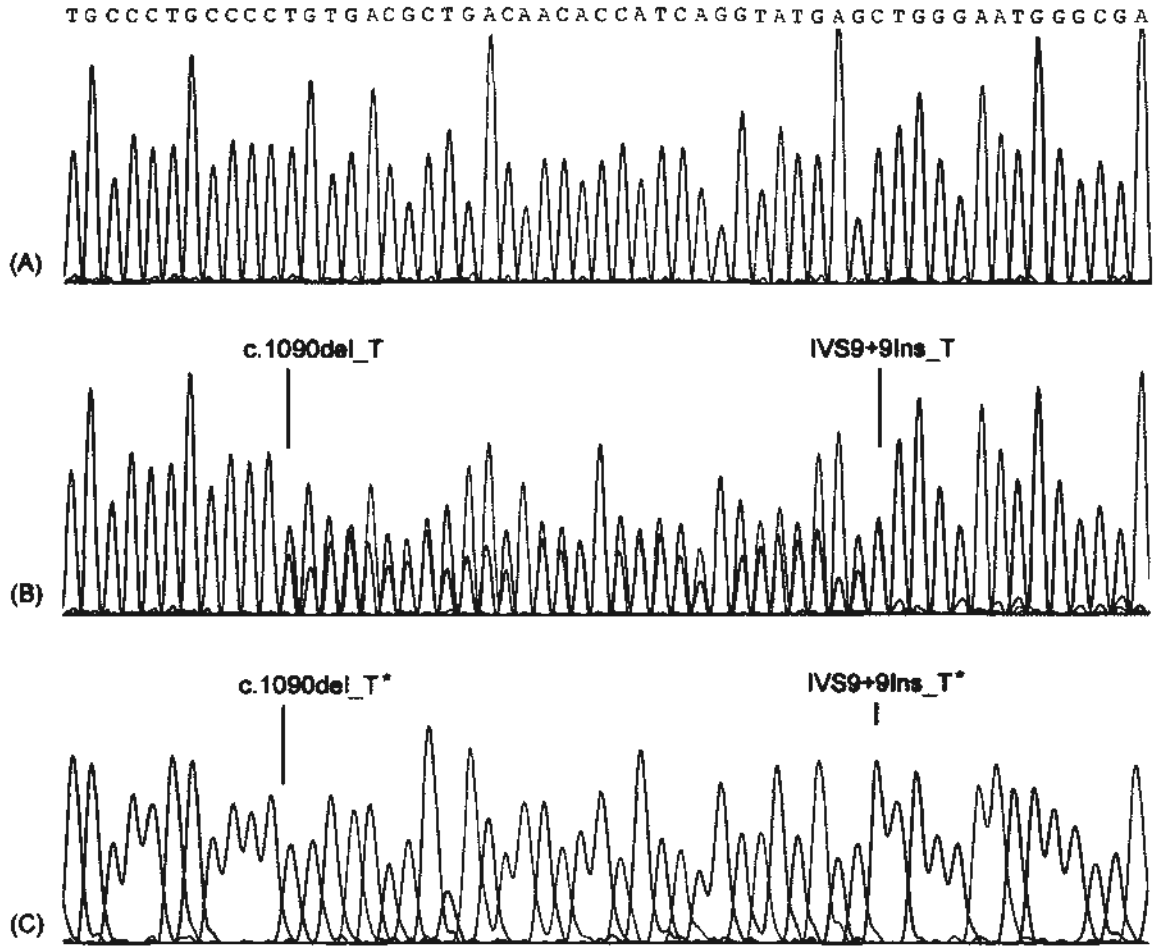


Figure 4.7. Chromatograms of the sequence flanking *MEGF11* c.1090delT mutation

(A) Reference sequence; (B) c.1090delT mutation detected in an affected subject III:5 of the Hong Kong pedigree with JOAG, this subject also carries the IVS9+9insT variant; (C) Homozygous for c.1090delT mutation, detected in subject III:16 in the Hong Kong pedigree with JOAG, who was also homozygous for IVS9+9insT. No detectable glaucomatous change was found in this subject at the last visit when she was 14 years old.

* Homozygous for the substitution.

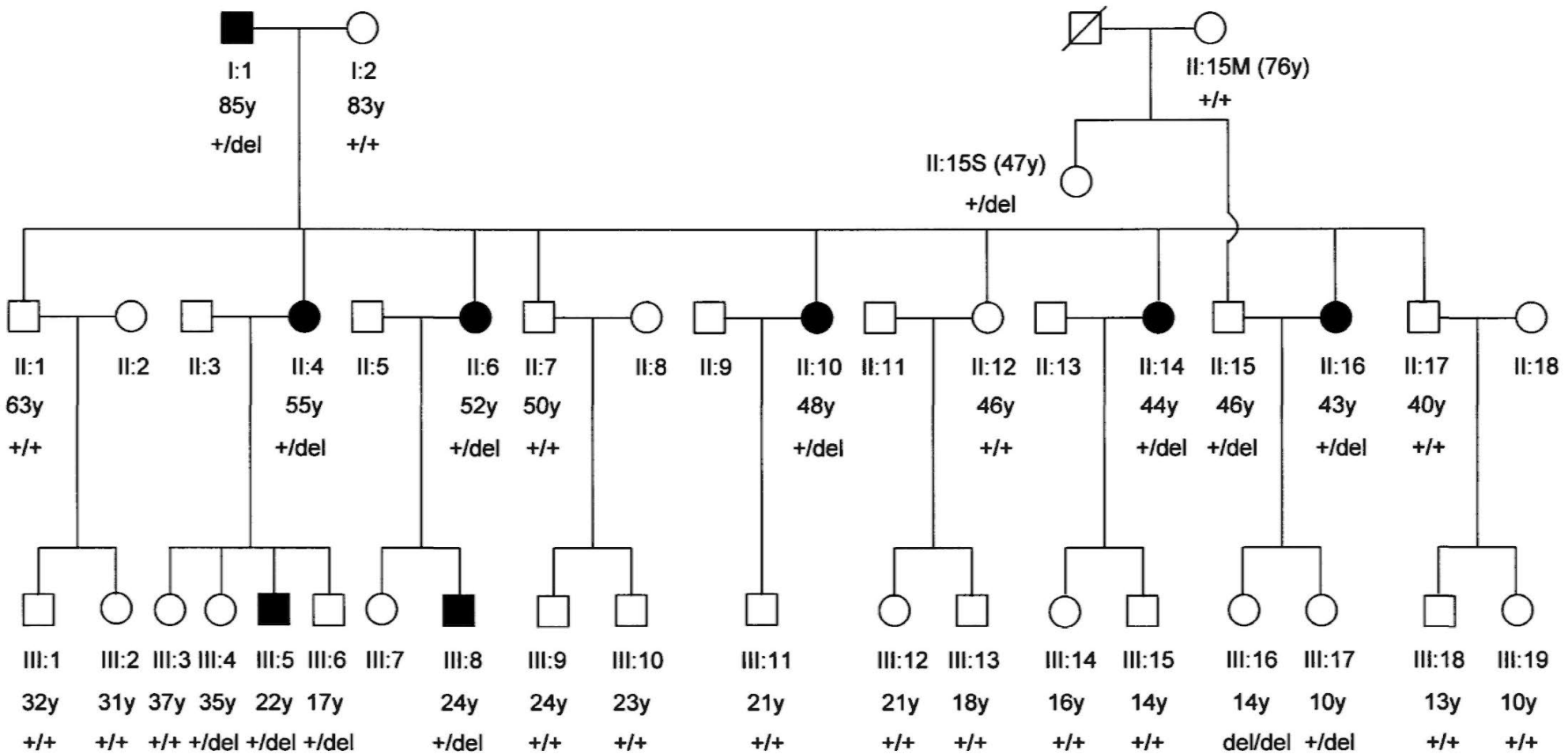


Figure 4.8. Segregation of *MEGF11* c.1090delT in the GLC1N-linked Hong Kong pedigree

This is an enriched version of Figure 3.1. The genotypes beneath the symbol represents the genotype of c.1090delT, where +/+ represents the wild-type, +/del represents heterozygote, and del/del represents homozygote. The absence of a genotype beneath the symbol indicates that the subject was not available for this current study.

Table 4.19. Variants detected in the coding region of *MEGF11* in unrelated study subjects

No.	Location	Domain	Sequence change	Residue change	SNP ID	PolyPhen prediction*	Hong Kong cohort		Beijing cohort		Shantou cohort	
							HTG (n=181)	Control (n=182)	HTG (n=177)	Control (n=200)	HTG (n=95)	Control (n=147)
1	Exon 3	EMI	c.200G>A	Arg67Gln	novel	Possibly	0	1	0	0	0	NI
2	Exon 5	–	c.315G>A	Glu105Glu	novel	–	0	0	1	0	0	NI
3	Exon 5	1st EGF	c.361G>A	Glu121Lys	novel	Benign	1	0	0	0	0	NI
4	Exon 6	–	c.456C>G	Ala152Ala	novel	–	1	0	0	0	0	NI
5	Exon 9	–	c.921C>A	Phe307Leu	novel	Benign	1	0	0	0	0	0
6	Exon 9	–	c.930C>T	Phe310Phe	novel	–	0	0	1	0	0	0
7	Exon 9	–	c.947A>G	Gln316Arg	novel	Benign	0	0	1	0	0	0
8	Exon 9	–	c.1046G>A	Cys349Tyr	novel	Probably	0	0	0	1	0	0
9	Exon 9	–	c.1090delT	Cys364ValfsX12	novel	–	1	0	1	0	0	0
10	Exon 10	8th EGF	c.1289G>A	Gly430Glu	novel	Probably	0	1	0	0	0	NI
11	Exon 11	8th EGF	c.1303G>A	Val435Ile	novel	Benign	0	0	1	0	0	NI
12	Exon 11	–	c.1360A>T	Asn454Tyr	novel	Probably	0	1	0	0	0	NI
13	Exon 11	–	c.1385A>G	Asp462Gly	novel	Possibly	1	0	0	0	0	NI
14	Exon 12	9th EGF	c.1423G>A	Asp475Asn	novel	Benign	1	0	0	0	0	NI
15	Exon 12	10th EGF	c.1542G>T	Trp514Cys	novel	Probably	0	0	0	1	0	NI
16	Exon 13	–	c.1582T>C	Phe528Leu	novel	Probably	0	0	1	0	0	NI
17	Exon 13	–	c.1623T>C	Ala541Ala	novel	–	0	0	0	1	0	NI
18	Exon 13	–	c.1639G>A	Val547Ile	novel	Benign	0	0	1	1	0	NI
19	Exon 13	11st EGF	c.1666G>A	Gly556Arg	novel	Probably	0	0	0	0	1	NI
20	Exon 14	–	c.1709G>A	Arg570His	novel	Benign	3	1	0	0	0	NI
21	Exon 14	–	c.1782C>T	Cys594Cys	novel	–	0	0	0	1	0	NI
22	Exon 14	12nd EGF	c.1783G>A	Glu595Lys	novel	Benign	0	0	1	0	0	NI
23	Exon 16	–	c.2017G>A	Gly673Arg	novel	Probably	0	1	0	0	0	NI

Table 4.19. continued

No.	Location	Domain	Sequence change	Residue change	SNP ID	PolyPhen Prediction*	Hong Kong cohort		Beijing cohort		Shantou cohort	
							HTG (n=181)	Control (n=182)	HTG (n=177)	Control (n=200)	HTG (n=95)	Control (n=147)
24	Exon 17	–	c.2148G>A	Ala716Ala	novel	–	1	0	0	2	2	0
25	Exon 18	–	c.2225C>G	Ala742Gly	novel	Benign	0	1	0	0	0	NI
26	Exon 19	–	c.2472A>C	Gln824His	novel	Benign	1	0	0	0	0	NI
27	Exon 20	–	c.2581C>T	Leu861Phe	rs3803414	Benign	1/12/168	0/19/163	0/12/165	0/9/191	0	NI
28	Exon 20	–	c.2638G>A	Gly880Ser	novel	Possibly	1	0	0	0	0	NI
29	Exon 20	–	c.2642G>A	Arg881Gln	novel	Benign	1	3	1	0	0	NI
30	Exon 22	–	c.2810T>C	Leu937Ser	novel	Benign	0	0	0	0	1	NI
31	Exon 22	–	c.2832C>T	Tyr944Tyr	novel	–	0	1	0	0	0	NI
32	Exon 22	–	c.2901G>A	Ser967Ser	novel	–	1	0	0	0	0	NI
33	Exon 22	–	c.2963T>C	Ile988Thr	rs2303374	Benign	0/4/177	0/8/174	0/16/161	0/10/190	0/4/89	NI
34	Exon 23	–	c.3003T>C	Gly1001Gly	novel	–	0	1	0	0	0	NI
35	Exon 23	–	c.3011A>G	His1004Arg	novel	Possibly	0	1	0	0	0	NI
36	Exon 23	–	c.3046C>T	Pro1016Ser	novel	Probably	0	0	0	2	0	NI
37	Exon 23	–	c.3123C>T	Asp1041Asp	novel	–	0	1	0	2	0	NI

Note:

* PolyPhen was used to predict the functional impacts of the missense changes to MEGF11; Benign indicates the substitution is most likely lacking any phenotypic effect; Possibly represents "possibly damaging", indicating that the change is supposed to affect protein function or structure; Probably represents "probably damaging", indicating that the variant is with high confidence supposed to affect protein function or structure.

NI: not investigated (the Shantou control subjects were screened only for those exons with putative mutations).

Table 4.20. Variants detected in the noncoding region of *MEGF11* in unrelated study subjects

No.	Location	Sequence change	db SNP	Hong Kong		Beijing		Shantou	
				HTG (n=181)	Control (n=182)	HTG (n=177)	Control (n=200)	HTG (n=95)	Control (n=147)
1	Intron 1	IVS2-61G>A	novel	2	1	0	2	0	NI
2	Intron 3	IVS3+34 A>G	novel	2	0	0	1	1	NI
3	Intron 3	IVS3+54C>T	novel	0/12/169	0/16/166	0/23/154	0/23/177	0/8/87	NI
4	Intron 3	IVS4-21C>T	rs7168565	11/88/82	12/89/81	17/74/86	22/80/98	6/39/41	NI
5	Intron 6	IVS6+3G>T	novel	0	0	0	1	0	NI
6	Intron 6	IVS6+29C>T	novel	0	0	1	0	0	NI
7	Intron 9	IVS9+9insT	rs3840018	37/92/52	21/101/60	20/92/65	30/107/63	4/59/32	7/92/51
8	Intron 9	IVS9-7T>C	novel	0/1/80	0/2/180	0/4/173	0/5/195	0	NI
9	Intron 10	IVS10+78C>A	rs333569	35/90/55	32/87/63	27/85/65	32/109/59	19/48/24	NI
10	Intron 10	IVS10+122C>T	rs8041692	0/7/174	0/11/171	0/6/171	0/6/194	0/4/87	NI
11	Intron 11	IVS11+23C>A	novel	0	0	1	0	0	NI
12	Intron 13	IVS13+60A>T	novel	1	0	0	0	0	NI
13	Intron 13	IVS13+66_82del17	novel	1	0	0	0	0	NI
14	Intron 16	IVS16+19G>C	novel	0	1	0	0	0	NI
15	Intron 17	IVS17+2insT	novel	0	0	0	0	1	0
16	Intron 17	IVS17-85insT	novel	1	0	0	0	0	NI
17	Intron 17	IVS17-4C>G	novel	0	0	1	0	0	NI
18	Intron 18	IVS18+64G>C	rs11632730	0/10/171	0/21/159	0/14/163	0/10/190	0/4/91	NI
19	Intron 18	IVS18+119T>C	novel	0	1	2	3	0	NI
20	Intron 20	IVS20+81C>A	novel	0	0	0	2	0	NI
21	Intron 21	IVS21-30T>C	rs2303373	0/30/151	0/31/151	3/33/141	0/38/162	0/6/83	NI
22	Intron 22	IVS22+24T>C	novel	1	0	0	0	0	NI
23	Intron 22	IVS22-93_-91delCCT	novel	0	1	0	0	0	NI

Since coding variants are more likely to be pathological mutations, especially for those located in the functional domains and/or those affect protein function or structure, we mapped the exonic variants to the reported functional domains of the MEGF11 protein and the functional impacts of the missense variants was predicted by using PolyPhen. The 1044-residue MEGF11 contains a signal peptide, an EMI domain (a small cysteine-rich module that may be involved in protein-protein interaction (Doliana et al. 2000)), 17 atypical EGF-like domains (a sequence of about 30 to 40 amino-acid residues found in the sequence of epidermal growth factor), a transmembrane region (a membrane-spanning alpha-helical or beta-sheet domain embedded in a membrane), and a cytoplasmic domain (Suzuki and Nakayama 2007a). Totally, 8 missense variants are located in the functional domains, with one in the EMI domain and 7 in EGF-like domains (Table 4.19). Among the 26 missence variants, 4 were predicted to be possibly damaging and 8 probably damaging by PolyPhen.

4.6.3 Distribution of *MEGF11* variants in patients and controls

Besides the 8 known SNPs, i.e., rs3803414, rs2303374, rs7168565, rs3840018, rs333569, rs8041692, rs11632730 and rs2303373, a new variant IVS3+54C>T was identified in all three study cohorts, with an overall minor allele frequency of about 5% (Table 4.20). It should thus be a common SNP that had not been identified in the HapMap project. The other variants are relatively rare. Of the common SNPs, no individual SNP or haplotype constructed by them was significantly associated with glaucoma in individual cohort or in pooled subjects.

For the relatively rare variants (MAF<1%), we grouped them by using different criteria and compared the frequencies of the variants between patients and controls. No statistically significant difference was detected for any combination of the variants, with the frequencies being similar between groups (Table 4.21). In particular, the damaging changes were

present at higher frequencies in the control groups (2.8% and 2.0%) than in the case groups (1.1% and 0.6%) in both Hong Kong and Beijing cohorts. Within the EGF-like domains, two (0.5%) damaging variants were found in the controls while 1 (0.2%) was found in the patients. These findings suggest that single amino acid substitutions in MEGF11 are less likely to be pathogenic. It is notable that the truncation mutation c.1090delT detected in a sporadic patient from the Beijing cohort was not detected in 529 unrelated controls, supporting its role as a disease-causing mutation, and suggesting that mutations having more drastic impact to the protein may be pathogenic.

Apart from affecting protein function through amino acid substitution, sequence variations can also lead to alternative splicing of gene transcripts, especially for those located at the splice sites. In this study, a variant was considered splice-site variant if it is located within the region comprising of 5 exonic and 10 intronic nucleotides at the exon-intron junction, referring to the Alternative Splicing Database. Accordingly seven variants, including 2 coding and 5 intronic, fulfilled the definition (Table 4.22). Herein, a cohort of Caucasian subjects from Connecticut, USA, is introduced and data shown in Table 4.22. The data were provided by our collaborator Professor Mansoor Sarfarazi. Briefly, this cohort included 93 patients with POAG and 90 controls. Whole gene screening was performed for *MEGF11* and 2 splice-site variants were identified: IVS9+9insT (rs3840018) and IVS17-2A>G (novel). The distributions of these splice-site variants were compared between patients and controls, and their possible impacts to alternative splicing were estimated by using two web-based programs: ASD and ASSA. The most common splice-site variant IVS9+9insT was predicted to mildly reduce the donor site score from 9.216 to 9.087 by ASD, and was predicted to cause no change to the splice site by ASSA, suggesting that this 1-bp insertion is unlikely to result in alternative splicing. This variant was not

significantly associated with HTG in individual Chinese cohort or the pooled subjects (P>0.05). And it was not associated with POAG in the Caucasian cohort (P=0.13).

Among the relatively rare splice-site variants, the IVS9-7T>C was evenly distributed in patients and controls in the Hong Kong and Beijing cohorts while being absent in the Shantou and Connecticut cohorts (Table 4.22). This variant was predicted to mildly alter the donor site score by ASD and information content (Ri) by ASSA. In light of this prediction and its high occurrence in controls, the IVS9-7T>C is unlikely to be a pathogenic mutation.

The other six variants, which were detected in one subject each, were likely to have functional impacts. The predicted products from alternative splicing, their possible impacts, and the clinical manifestations of the carriers were shown in Table 4.23 and depicted in Figure 4.9. Two variants IVS17+2insT and IVS17-2A>G were predicted to be site-abolished (i.e., the mutation may completely abolish normal splicing), with the donor or acceptor site score and Ri values falling well below zero. Thus, it is likely that the mutations cause exon-skipping resulting in a pathogenic protein. IVS17+2insT may lead to skipping of exon 17 (size: 129bp), resulting in in-frame missing of the 15th EGF-like domain. It was detected in a Shantou HTG patient, with an AAD of 43 years and maximum IOP of 26(OD)/21.5(OS) mmHg. Similarly, IVS17-2A>G may cause skipping of exon 18 (129bp), leading to in-frame missing of the 16th EGF-like domain. It occurs in a POAG patient from the Connecticut cohort, whose clinical data in detail are currently not available.

Of the four splice-site variants that were predicted to result in leaky-sites (i.e., the mutation may partially abolish normal splicing), two (i.e., c.200G>A and IVS6+3G>T) were detected in controls and the other two (i.e., IVS17-4C>G and c.2472A>C) in patients (Table 4.22). The IVS6+3G>T is likely to lead to skipping of exon 6 (247bp), cause a frameshift and introduce a premature termination codon to the transcript, resulting in a

Table 4.21. Distribution of rare variants of *MEGF11* in patients and controls

Variation category	Hong Kong cohort		Beijing cohort		Shantou cohort		Pooled Chinese subjects	
	HTG (n=181)	Control (n=182)	HTG (n=177)	Control (n=200)	HTG (n=95)	Control (n=147)	HTG (n=453)	Control (n=382)*
Any coding change (including variants present concurrently in cases and controls)	14 (7.7)	13 (7.1)	9 (5.1)	11 (5.5)	4 (4.2)	N/A	27 (6.0)	24 (6.3)
Missense change	10 (5.5)	10 (5.5)	6 (3.4)	5 (2.5)	2 (2.1)	N/A	18 (4.0)	15 (3.9)
Damaging change	2 (1.1)	5 (2.8)	1 (0.6)	4 (2.0)	1 (1.1)	N/A	4 (0.9)	9 (2.4)
Benign change	8 (4.4)	5 (2.8)	5 (2.8)	1 (0.5)	1 (1.1)	N/A	14 (3.1)	6 (1.6)
In EGF domain	2 (1.1)	1 (0.6)	2 (1.1)	1 (0.5)	1 (1.1)	N/A	5 (1.1)	2 (0.5)
In EGF and damaging	0	1 (0.6)	0	1 (0.5)	1 (1.1)	N/A	1 (0.2)	2 (0.5)
Truncation	1 (0.6)	0	1 (0.6)	0	0	0	2 (0.4)	0/529
Coding changes exclusively present in cases or controls	9 (5.0)	9 (5.0)	7 (4.0)	6 (3.0)	2 (2.1)	N/A	18 (4.0)	15 (3.9)
Missence change	7 (3.87)	6 (3.3)	4 (2.3)	2 (1.0)	2 (2.1)	N/A	13 (2.9)	8 (2.1)
Damaging change	3 (1.7)	5 (2.8)	1 (0.6)	2 (1.0)	1 (1.1)	N/A	5 (1.1)	7 (1.8)
Intronic variants	8 (4.4)	7 (3.9)	10 (5.6)	14 (7.0)	2 (2.1)	N/A	20 (4.4)	21 (5.5)

Note:

The two common coding SNPs, namely rs3803414 (p.Leu861Phe) and rs2303374 (p.Ile988Thr) were not included in this analysis.

For each comparison of the proportion, no significant difference was detected.

* The pooled controls included only the control subjects from the Hong Kong and Beijing cohorts. But for the truncation mutation, controls from the three cohorts were pooled, thus made the sample size up to 529.

EGF domain: epidermal growth factor-like domain.

Table 4.22. Splice-site variants of *MEGF11* in patients and controls

Sequence change	Donor/acceptor site score*		Information content (Ri)†			Hong Kong cohort		Beijing cohort		Shantou cohort		Connecticut cohort (Caucasian)	
	Wild-type	Mutant	Type of change	Initial	Final	HTG (n=181)	Control (n=182)	HTG (n=177)	Control (n=200)	HTG (n=95)	Control (n=147)	POAG (n=93)	Control (n=90)
c.200G>A	10.565	7.413	Leaky Site	10.3	7.3	0	1	0	0	0	0	0	0
IVS6+3G>T	6.099	2.141	Leaky Site	6.9	2.8	0	0	0	1	0	0	0	0
IVS9+9insT	9.216	9.087	No change	8.4	8.4	37/92/52	21/101/60	20/92/65	30/107/63	4/59/32	7/92/51	12/43/38	4/45/41
IVS9-7T>C	4.598	4.474	Leaky Site	10.8	10.6	1	2	4	5	0	0	0	0
IVS17+2insT	4.112	-2.951	Site Abolished	4.6	-14.2	0	0	0	0	1	0	0	0
IVS17-4C>G	7.467	6.964	Leaky Site	5.9	5.7	0	0	1	0	0	0	0	0
IVS17-2A>G	7.467	AG not seen	Site Abolished	5.9	-7.2	0	0	0	0	0	0	1	0
c.2472A>C	7.002	4.578	Leaky Site	6.9	4.8	1	0	0	0	0	0	0	0

Note:

In this analysis, a splice site is defined as the region containing 5 exonic nucleotides and 10 intronic nucleotides at the exon-intron boundary.

* The splice donor site scores and acceptor site scores were predicted using the tools for splice signal analysis in the Alternative Splicing Database, using the U2-type-GT-AG Weight Matrix.

† The impact of the splice site variant to the splice donor site or receptor site was analyzed by the online program Automated Splice Site Analyses, using self-defined sequence according to the reference sequence ENSG00000157890, with a window range of 54.

Table 4.23. Clinical features of the carriers with putative *MEGF11* mutations

Mutation	Predicted mutant product	Predicted functional impact	Subject	Gender	Status	Age at diagnosis (years)	Highest IOP (OD/OS) (mmHg)	Cup/disc ratio (OD/OS)
Truncation mutation								
c.1090delT	Cys364ValfsX12	Haploinsufficiency (NMD)	BJ-G079	M	JOAG	14	25 / 30	0.5 / 0.7
Splice-site mutations predicted to be "site abolished"								
IVS17+2insT	Ala696_Gln738del (Exon 17 skipped)	Loss of function (15th EGF-like domain skipped)	ST-G098	M	JOAG	43	26 / 21.5	0.5 / 0.4
VS17-2A>G	Arg739_Gln781del (Exon 18 skipped)	Loss of function (16th EGF-like domain skipped)	US-POAG	N/A	POAG	N/A	N/A	N/A
Splice-site mutations predicted to result in a "leaky site"								
c.200G>A (Arg67Gln)	Tyr34_Arg67del (Exon 3 skipped)	Loss of function (EMI domain skipped, partially)	HK-C292	M	Unaffected	68	Normal	Normal
IVS6+3G>T	Gly132AlafsX72 (Exon 6 skipped)	Haploinsufficiency (NMD)	BJ-C054	F	Unaffected	71	Normal	Normal
IVS17-4C>G	Arg739_Gln781del (Exon 18 skipped)	Loss of function (16th EGF-like domain skipped)	BJ-G095	M	JOAG	38	32 / 32	0.9 / 0.9
c.2472A>C (Gln824His)	Arg782_Gln824del (Exon 19 skipped)	Loss of function (17th EGF-like domain skipped)	HK-G001	M	JOAG	34	No record	No record

Note:

The Hong Kong subject with the c.1090delT mutation belongs to the JOAG pedigree (Table 3.7) and is not shown in this table.

NMD: nonsence-mediated mRNA decay; N/A: not available (clinical data of the subject from the Connecticut cohort are not yet available).

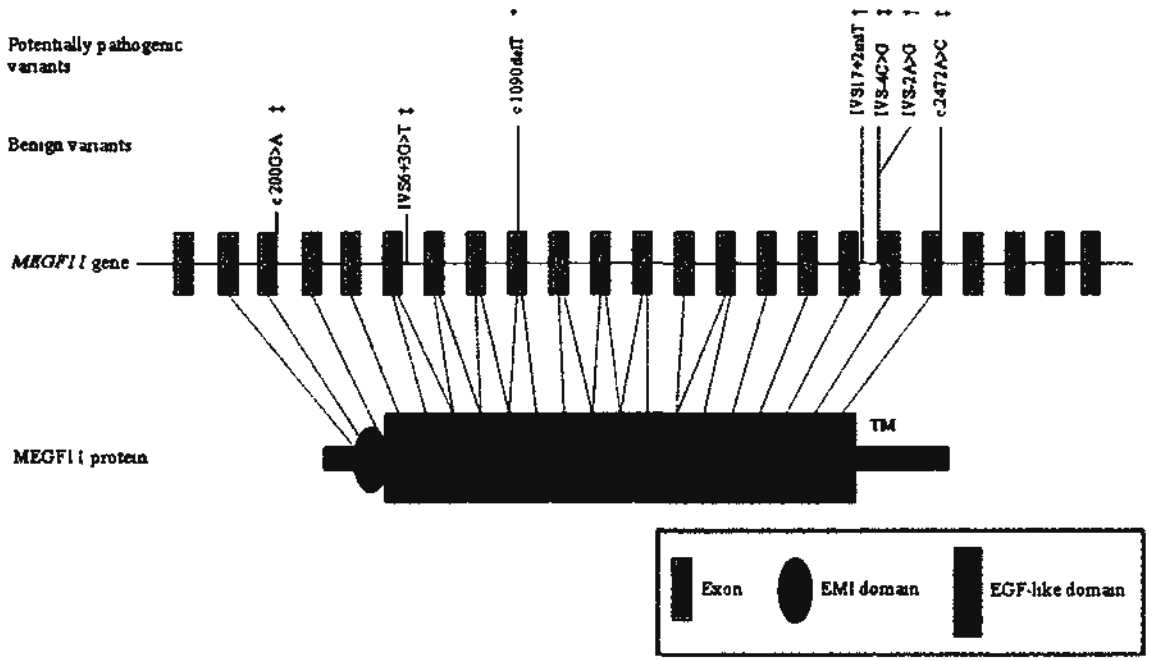


Figure 4.9. Schematic diagram of the *MEGF11* gene and MEGF11 protein

The 1044-residue MEGF11 contains a signal peptide, an EMI domain, 17 atypical EGF-like domains, a transmembrane (TM) region, and a cytoplasmic domain.

The truncation mutation c.1090delT and 6 splice-site variants were mapped to the gene.

* indicates truncation mutation; † indicates splice-site variant leading to abolished site; ‡ indicates splice-site variant leading to leaky site.

truncation protein designated as Gly132AlafsX72. This variant was detected in a 71-year old control subject from the Beijing cohort, suggesting that it is not causative for glaucoma. Also detected in a control subject was the coding variant c.200G>A (Arg67Gln), which may result in skipping of exon 3 (102bp), which encodes part of the EMI domain. It is also not likely to be causative for glaucoma. Another coding variant c.2472A>C (Gln824His), which was found in a 34-year old HTG patient from the Hong Kong cohort, is likely to result in skipping of exon 19 (129bp), which encodes for the 17th EGF-like domain. This variant is likely to be a causative mutation for glaucoma. Besides these three variants, another variant IVS17-4C>G was predicted to just mildly decrease the acceptor site score by 0.5 (Table 4.22) and the Ri value by 0.2. If any, this variant is likely to result in skipping of exon 18 (129bp) and subsequently in-frame missing of the 16th EGF-like domain. This variant was detected in a HTG patient from the Beijing cohort, with an AAD of 38 years and a maximum IOP of 32 mmHg in both eyes (Table 4.23). Thus, this variant is also likely to be disease causative.

If these five variants, i.e., c.1090delT, IVS17+2insT, IVS17-2A>G, IVS17-4C>G and c.2472A>C, are in fact pathogenic, the *MEGF11* mutations in overall may account for approximately 1.1% (6/546, 95% CI: 0.5-2.4%, the proband of the GLC1N-linked pedigree was counted once) of POAG patients, with the mutation frequencies being similar between Chinese (5/453, 1.10%) and Caucasians (1/93, 1.08%).

Chapter 5

Discussion

5.1 Complexity in POAG genetics

Genetic heterogeneity of POAG has rendered the identification of disease genes very challenging. So far, although more than 20 genetic loci have been linked to POAG, only three genes (i.e., *MYOC*, *OPTN* and *WDR36*) have been identified, and mutations in these genes are responsible for only a small fraction of POAG with mutation patterns varying across populations. Moreover, although more than 30 genes have been associated with POAG, the actual contributions of these genes remain to be verified. All these findings highlight the need for further identification of new genes for POAG.

In the research work of this thesis, we aimed to identify new POAG genes and genetic patterns by using a three-tiered strategy. In the investigation of multiple gene polymorphisms, differential association patterns were identified for all genes involved. In particular, an age-varying association pattern was identified for rs1533428 at 2p16.3. By mutation screening, *NTF4* mutations were found to likely account for a small fraction of POAG, while *CNTF* and *SPARC* were excluded as disease genes for POAG. Finally, the *MEGF11* gene was identified as a causal gene at the GLC1N locus for JOAG. These findings provide new insights into the genetics of POAG.

5.2 New genetic profiles identified for POAG

5.2.1 Multiple gene polymorphisms in POAG

In this thesis, association patterns of 14 candidate SNPs with POAG were evaluated. These SNPs were located in either newly reported POAG-associated genes, e.g., *CYP46A1*, *LMX1B*, *TLR4*, or were identified by hypothesis-free approaches, e.g., linkage analysis and

genome-wide association study, and possibly linked to novel POAG genes. Therefore, investigation of these variants in independent cohorts may serve to verify the involvement of the genes in the disease and provide additional evidence for the mapping of the gene. Through the investigation, differential genetic association patterns were identified.

5.2.1.1 Age-varying association of rs1533428 at 2p16.3 with POAG

Among the 14 SNPs studied, replicable association was found only for rs1533428 at 2p16.3 across the three study cohorts from Hong Kong, Shantou and Beijing. Moreover, we have identified an age-varying association between POAG and rs1533428, in which the genetic susceptibility conferred by the TT and CT genotypes was strongly increased in more elderly ages but not in younger ages. Given the consistent findings across all three samples collected at different geographical locations, it is unlikely that the age-varying association is artifact of a cohort effect. Moreover, as subjects with known systemic diseases were excluded at study recruitment, the age effect observed in carriers of the 2p16.3 variant was less likely influenced by interaction with age-related comorbid conditions that increase in frequency with aging, including hypertension, diabetes, and heart failure. Thus, the age-specific effect of rs1533428 could be one of the genetic determinants for late-onset POAG, which is the most common form of glaucoma.

As far as we know, this is the first study to provide consistent evidence supporting a common SNP as a genetic risk factor of a specific age group of POAG. The higher-risk genotypes account for approximately 30-40% of population attributable risk for late-onset POAG with AAD >60 years. Similar phenomena had been observed for certain mutations in the *MYOC*, *OPTN* and *WDR36* genes for glaucoma (Monemi et al. 2005; Rezaie et al. 2002; Stone et al. 1997). Moreover, age-specific genetic associations have been identified in other diseases, such as the association between variants at *UMOD* and chronic kidney

disease (Gudbjartsson et al. 2010), and between the *DMBT1* variants and breast cancer (Tchatchou et al. 2010).

One limitation in the use of age at diagnosis as the surrogate of the age of onset is that POAG is usually diagnosed several or many years after the onset of disease, with the time-gap varying in different patients. However, since the age of onset in POAG patients is difficult to be determined because of the absence or unawareness of symptoms in the early stages, the AAD should be the most appropriate proxy. For the patients who have AAD <35 years, there is no doubt that they developed the disease at an early age. As such, rs1533428 is not a susceptibility marker for JOAG. In contrast, for those patients with an older AAD, there are so far no clear-cut criteria to define the adult- and late-onset forms of disease. Therefore in our study, it could be argued that the age limit of late-onset POAG could have been skewed upward or downward by our stratification method. To address this issue, we expanded or constricted the group of late-onset POAG by moving the lower boundary of AAD downward or upward by 5 years. Interestingly, when this group was altered to include patients aged >55 years or patients >65 years old, the odds ratio conferred by the TT+CT genotypes for overall POAG was 1.88 (95% CI: 1.41-2.51, $P_{dom}=1.68\times10^{-5}$) and 2.15 (95% CI: 1.51-3.06, $P_{dom}=2.08\times10^{-5}$) respectively. However, the smallest P value was obtained from the group of patients with AAD >60 years ($P_{dom}=1.14\times10^{-5}$, Table 4.3). Thus, an AAD of 60 years could be used as the lower boundary for late-onset POAG.

The biological relevance of the age-varying association with POAG is yet unknown. This genetic pattern may represent at least partially the differences in the genetic bases between late- and early-onset POAG. Late-onset POAG is more likely to conform to the common disease-common variant hypothesis. In contrast, juvenile-onset POAG, many of which have a highly penetrant autosomal dominant mode of inheritance, is more likely caused by gene variants with high penetrance (Stone et al. 1997). The adult-onset POAG

may represent an intermediate between or a mixture of the two. Our data that the odds ratio conferred by the higher-risk genotypes of rs1533428 in this age group (35-60 years) was at intermediate level (Table 4.3) also support this assumption. Hence, it is possible that rs1533428 may contribute specifically to the late-onset form of POAG. Another possible explanation is that as the penetrance of common susceptibility variants is usually low, other risk factors are needed to determine the development of the disease. Older age is a major risk factor for glaucoma (Kwon et al. 2009; Rudnicka et al. 2006). It may diminish the protection from or impose additional risk upon the underlying genetic risk factors to cause glaucoma, suggesting an age-dependent penetrance function, being reminiscent of the association of *CFH* and *HTRA1* with age-related macular degeneration (Dewan et al. 2006; Klein et al. 2005). In view of these findings, rs1533428 could be a useful genetic marker for the prediction of POAG risk, especially for individuals with higher baseline IOP, enabling early detection and treatment of the disease.

5.2.1.2 Discrepant association patterns between the SNPs at 2p16.3 with POAG

In this thesis, the associations between the SNPs (i.e., rs1533428 and rs12994401) at 2p16.3 and POAG were investigated in a population other than that of African origin. In the Afro-Caribbean population of Barbados, these two SNPs were strongly associated with POAG, with rs12994401 (OR_{hom}>33.0) conferring a stronger risk than rs1533428 (OR_{hom}>5.5) (Jiao et al. 2009). These two SNPs were in strong LD in the Barbados population (D'=0.72), with the most common haplotype C-C having a strong protection while the T-T haplotype conferring a strong risk (Jiao et al. 2009). However, conflicting findings from African-American and Ghanaian (West Africa) cohorts have been reported. SNP rs12994401, but not rs1533428, was associated with POAG in the African-American cohort, and these two SNPs were in weak LD (Liu et al. 2010b). In contrast to their findings,

we found that rs12994401 was not associated with POAG in our study cohorts, while rs1533428 conferred a significant but milder increased-risk of POAG. There could be several explanations for such discrepancies. First, the Barbados population is of African origin, which was known to have high rates of glaucoma (Leske et al. 2001). Second, the population of Barbados is relatively isolated and homogenous, with no indigenous population and limited European admixture (Benn-Torres et al. 2008; Jiao et al. 2009). Third, the strong LD between the two SNPs suggests that the 2p16.3 region has been conserved in the Barbados population. These phenomena have given advantages in detecting genetic ancestral variants for POAG. In our study, despite all subjects were Han Chinese in China, there is inevitable population admixture in such a large area (Abdulla et al. 2009), so that the effect of the risk founder allele might have been diluted. Moreover, revealed by a weak LD between rs1533428 and rs12994401, which reside approximately 113kb apart, recombination might have occurred between the two SNPs. Thus, the effect of the higher-risk T-T haplotype might have been compromised. However, the ancestral C-C haplotype, as shown in our data, has maintained a mild protective effect. Finally, there could be other yet-to-be-identified factors that have modified the effect of this locus. All these findings suggest that rs1533428, rather than rs12994401, is responsible for the association between the 2p16.3 locus and POAG in Chinese. Since rs12994401 is the major associated-SNP in populations of African origin (Jiao et al. 2009; Liu et al. 2010b), it is likely that another SNP located around rs1533428 and rs12994401 tags these two SNPs, being the causal marker for the association signal detected in this region. A detailed study of the sequence at 2p16.3 is needed to identify this variant.

Apart from this study and the work of Jiao et al., a linkage study in six white families and one Afro Caribbean family had linked adult-onset POAG to the 2p15-16 region (GLC1H) (Suriyapperuma et al. 2007). This region had also been linked with autosomal

dominant JOAG in two Chinese families (Lin et al. 2008). Thus, the 2p16 region should harbor a gene for POAG.

In our study, the higher-risk genotypes of rs1533428 were also found to be significantly associated with an increased risk of late-onset NTG (P_{dom}=0.011, OR=1.74), but the effect size was smaller than that for late-onset HTG ($P_{dom}=5.03\times10^{-5}$, OR=2.28). Hence, rs1533428 may be a common marker for both POAG subtypes. The stronger risk it confers to HTG may be due to the elevation of IOP, which might impose additional risks upon the patients. Moreover, genotype-phenotype correlation analysis revealed that this SNP was not correlated with the peak-IOP level (Table 4.4). These findings suggest that the gene tagged by rs1533428 may be involved in a disease pathway independent of IOP regulation.

5.2.1.3 The gene tagged by rs1533428 at 2p16.3

So far, however, no functional gene at the 2p16.3 region next to rs1533428 has been annotated. The nearest gene is a hypothetical gene *LOC730100* (part of the gene *AC007682.3* in the Ensembl database: ENSG00000231918), encoding a predicted protein with no known conserved domains but with some homology to the TRF1-interacting ankyrin-related ADP-ribose polymerase, which plays a regulatory role in telomere length (Donigian and de Lange 2007) associating with cellular senescence and apoptosis (Rodriguez-Brenes and Peskin 2010). To examine whether rs1533428 is in LD with SNPs in other genes, we searched for its flanking SNPs with $r^2>0.3$ in the JPT+CHB samples from HapMap, using GLIDERS (Genome-wide Linkage Disequilibrium Repository and Search engine, at http://mather.well.ox.ac.uk/GLIDERS/) (Lawrence et al. 2009). A total of 28 SNPs spannig a 150kb-region (84kb upstream and 65kb downstream of rs1533428) within *AC007682.3* were detected, suggesting that *AC007682.3* is the only gene tagged by rs1533428. Further studies are warranted to characterize this gene at 2p16.3.

5.2.1.4 *TLR4* associated with HTG in southern Chinese

Besides rs1533428 at 2p16.3, we also found that *TLR4* rs7037117, in a recessive model, is associated with HTG in southern but not northern Chinese, suggesting a population-specific effect. The other two SNPs rs1927907 and rs11536889 were not disease-associated. As mentioned above, aberrant immunity may be involved in the pathogenesis of glaucoma (Grus et al. 2008; Schwartz 2003; Tezel and Wax 2004; Wax 2000). The toll-like receptor 4 protein functions to mediate immune responses to exogenous and endogenous ligands and interacts with heat shock proteins (Andreakos et al. 2004; Kang et al. 2006), which are reportedly involved in glaucoma (Tezel et al. 2004). Recently, *TLR4* was reported to be associated with NTG in a Japanese cohort, in which rs7037117 was the principal associated-variant conferring a 1.65-fold of increased risk in a dominant model (Shibuya et al. 2008). In our results, although rs7037117 is also the major associated variant in the gene, there were differences in the association pattern. First, rs7037117 is associated with HTG but not NTG in southern Chinese. Second, the association was detected in a recessive model. Thus, it is likely that rs7037117 is involved in the pathogenesis of both HTG and NTG, whilst being modified by other factors. In the Japanese population, NTG is a predominant form of POAG accounting for nearly 90% of cases (Iwase et al. 2004). Therefore, certain factors, e.g., the TLR4 higher-risk allele, might have contributed to the development of glaucoma in people with low IOP within the normal range. In Chinese, however, the rs7037117 risk allele may only exert its effect under a prerequisite of elevated IOP in people homozygous for the allele, suggesting an IOP- and allele dose-dependent manner in the TLR4-related genetic etiology of the disease.

5.2.1.5 Lack of association between other candidate SNPs and POAG

In this study, we have also investigated SNPs at or near the *CYP46A1*, *LMX1B*, *PLXDC2*, *TMTC2*, and *ZP4* genes. Among them, only *LMX1B* rs944103 showed a marginal association (P=0.029) with HTG in the Hong Kong cohort, but it failed to be replicated in the Shantou cohort. *LMX1B* variants have been found to be associated with both HTG and NTG in a Caucasian cohort (Park et al. 2009). However, such associations could not be replicated in our Chinese cohorts, even when the HTG and NTG samples were pooled. The discrepancies may partially be due to the distinct allele frequencies between Caucasian and Chinese (Table 5.1), leading to insufficient power for detecting the association in our study. Especially for rs7854658, it was the most significantly associated both HTG and NTG in the study of Park et al. However, this variant was detected in only 1 subject in our study and thus unlikely to be a major genetic contributor to POAG. However, for the other three common (MAF >25%) SNPs, i.e., rs16929236, rs10733682 and rs867559, our pooled sample size provided >80% of power for detecting significance at 0.05 level, assuming the odds ratio to be 1.7 according to the study of Park et al. Therefore, the lack of association of these three SNPs with POAG was less likely due to inadequate statistical power. As such, the *LMX1B* gene is less likely to be a susceptibility gene for POAG in Chinese.

The three SNPs, i.e., rs693421, rs7081455 and rs7961953, which were found in a genome-wide association study to be associated with POAG in Japanese (Nakano et al. 2009), did not show significant association with POAG in the Hong Kong cohort (Table 4.1). Two common SNPs (i.e., rs693421 and rs7961953) were genotyped in the Shantou cohort but still revealed a lack of association, even when the subjects were pooled (Table 5.1). Moreover, the trends of association with rs693421 and rs7081455 were in reverse directions between Chinese and Japanese. Therefore, these two SNPs were unlikely to be common genetic markers for POAG. Recently, lack of association between these SNPs and

Table 5.1 Allelic distributions of the selected SNPs in the Chinese and initial cohorts

Gene	dbSNP ID	Allele*	Hong Kong cohort			Shantou cohort		Initial cohort				
			HTG	NTG	Control	HTG	Control	Population	Phenotype	Case	Control	Reference
ZP4	rs693421	T/G	0.459	0.456	0.465	0.474	0.526	Japanese	POAG†	0.55‡	0.45‡	(Nakano et al. 2009)
AC007682.3	rs1533428	T/C	0.443	0.430	0.384	0.426	0.381	Barbados	HTG	0.591‡	0.305‡	(Jiao et al. 2009)
AC007682.3	rs12994401	T/C	0.369	0.308	0.343	0.353	0.338	Barbados	HTG	0.413‡	0.125‡	(Jiao et al. 2009)
TLR4	rs1927907	T/C	0.209	0.175	0.172	NI	NI	NI	NI	NI	NI	-
TLR4	rs11536889	G/C	0.294	0.272	0.307	NI	NI	Japanese	NTG	0.232	0.256	(Shibuya et al. 2008)
TLR4	rs7037117	G/A	0.201	0.192	0.202	0.348	0.231	Japanese	NTG	0.252	0.182	(Shibuya et al. 2008)
LMX1B	rs944103	G/A	0.049	0.024	0.022	0.037	0.033	UK	NTG	0.554	0.433	(Park et al. 2009)
LMX1B	rs7854658	T/C	0.003	0	0	NI	NI	UK	NTG/HTG	0.097 /0.180	0.249	(Park et al. 2009)
LMX1B	rs16929236	G/A	0.393	0.388	0.409	0.421	0.394	UK	NTG	0.230	0.116	(Park et al. 2009)
LMX1B	rs10733682	G/A	0.269	0.240	0.253	NI	NI	UK	NTG	0.405	0.527	(Park et al. 2009)
LMX1B	rs867559	T/C	0.302	0.333	0.298	NI	NI	UK	NTG	0.703	81.1	(Park et al. 2009)
PLXDC2	rs7081455	G/T	0.155	0.141	0.128	NI	NI	Japanese	POAG	0.17‡	0.26‡	(Nakano et al. 2009)
TMTC2	rs7961953	A/G	0.408	0.396	0.374	0.442	0.390	Japanese	POAG	0.34‡	0.26‡	(Nakano et al. 2009)
CYP46A1	rs754203	C/T	0.304	0.331	0.339	NI	NI	French	HTG	0.19	0.27	(Fourgeux et al. 2009)

Note:

In the Shantou cohort, not all SNPs were genotyped. Only those showing a signficant or trend of association were selected for verification.

* Minor allele frequency of each SNP was shown. The minor allele was defined referring to the Hong Kong cohort and it could be the major allele in other populations.

† Herein, POAG represents mixed POAG phenotypes, which has not been specified in the initial reports.

‡ Frequency referred to the frequency in the discovery group in the initial report.

NI: not investigated; UK: United Kingdom.

POAG has also been reported in the Indian population (Rao et al. 2009). In contrast, SNP rs7961953 at *TMTC2* showed a similar trend of association in both the Hong Kong and Shantou cohorts to that in Japanese. However, the association was not significant in individual cohort or in the pooled subjects. The sample size in pooled Hong Kong/Shantou subjects (493 cases and 377 controls) provided a 89.3% of statistical power to detect an allelic association at 0.05 level of significance given a minor allele frequency of 0.38 and an odds ratio of 1.37 (refered to the pooled OR in the initial report (Nakano et al. 2009)). In contrast to Chinese and Japanese, the trend of association in Indian was in reverse direction (Rao et al. 2009). As such, *TMTC2* rs7961953 is also less likely to be a common genetic marker for POAG across different populations. Further studies of these SNPs in other Japanese cohorts will help to verify whether the associations were population specific.

Finally, SNP rs754203 in the *CYP46A1* gene has been implicated in HTG in a French Caucasian cohort, and the higher-risk TT genotype was assumed to confer a stronger risk to NTG, although the sample size was small (n=18) (Fourgeux et al. 2009). In the Hong Kong Chinese population, rs754203 was not associated with HTG, NTG, or overall POAG. Since the sample size of the Hong Kong cohort (391 cases and 230 controls) provides a 96.7% of power to detect an allelic association at 0.05 level of significance given a minor allele frequency of 0.34 and an odds ratio of 1.58 (Fourgeux et al. 2009)), this SNP at *CYP46A1* is unlikely to be a risk factor of POAG in Chinese.

In light of the discrepant association patterns, several explanations are likely, including heterogeneous genetic makeup for the phenotype, population stratification, population-specific LD, heterogeneous genetic and epigenetic backgrounds, and heterogeneous environmental influences (Kingsmore et al. 2008). Therefore, further studies of these genes and/or variants in other populations are warranted to verify their involvement in the genetics of POAG.

5.2.1.6 Multiple gene effect in POAG

As a multifactorial disorder, POAG may be resulted from additive and/or interactive effects of multiple genetic and environmental risk factors. In our study, however, we detected no promising gene-gene interaction. Instead, multi-locus analyses revealed an additive effect between rs1533428 and rs7037117 on the risk of HTG. Combination of the both higher-risk genotypes conferred a greater risk to HTG than that with only one risk genotype. In the pooled subjects, carriers with both the risk genotypes had a strongly increased risk of HTG (OR_{add}=4.53) followed by those with one risk genotype (OR_{add}=2.73 and 1.61 respectively). This finding enriches our current understanding of the multifactorial etiology of POAG.

5.2.2 SNPs at the *CAV1/CAV2* locus associated with POAG

5.2.2.1 Ethnic diversities in the association of rs4236601 with POAG

In this thesis, we present the first follow-up study to confirm the association between POAG and the SNP rs4236601 at the *CAV1/CAV2* locus which was detected in a large-scale GWA study in Caucasian. Among the subjects of this present study, all of the 248 Hong Kong and 298 Shantou control subjects, and 123 of the 130 Shantou POAG subjects were involved in that GWA study (Thorleifsson et al. 2010). In this study, an additional group of Hong Kong POAG patients (including 185 HTG and 311 NTG) and a Beijing cohort of 177 HTG and 200 controls were included.

Although a similar trend of association between rs4236601 and POAG was detected in all three cohorts, significance was reached only when the samples were pooled. This implies that the lack of significance in individual cohort is due to inadequate statistical power. The risk allele rs4236601[A] is present at much lower frequencies in Chinese populations but confers a greater risk. Notably, the frequency of rs4236601[A] in the

Beijing subjects (5.1% in HTG and 2.5% in controls, Table 4.9) was higher than in the Hong Kong/Shantou subjects (3.1% in HTG, 2.1% in NTG and 0.73% in controls, Table 4.8), suggestive of a discrepancy in the genetic distributions of rs4236601 across southern and northern Chinese. In view of this, it is desirable to further study this SNP in other populations to better understand its involvement in the genetic diversity of POAG. In particular, rs4236601[A] is present at a high frequency (MAF=45%) in the HapMap population of Yoruba. Thus, the evaluation of rs4236601 associated with POAG in populations of African origin, which is known to have high rates of glaucoma (Quigley and Broman 2006; Racette et al. 2003), should be informative.

5.2.2.2 Implication of rs4236601 in both HTG and NTG

As shown in the GWA study (Thorleifsson et al. 2010), rs4236601 was associated predominantly with, but not limited to, NTG in the Caucasian sample sets with a mixture of HTG and NTG cases. In our study, rs4236601[A] conferred a stronger risk to pooled HTG (P_{dom}=0.01, OR=2.90, Table 4.10) than to NTG (P_{dom}=0.15, OR=2.82), with a minor exception among the Shantou subjects, where the OR_{dom} was 4.49 for HTG and 5.48 for NTG (Table 4.7). In light of these findings, it is possible that rs4236601 or the functional variant it tags may be involved in a disease pathway independent of IOP regulation, reminiscent of the role of rs1533428 at 2p16.3 described above. However, an elevated IOP may impose additional risk to carriers of the risk allele, increasing its effect size.

5.2.2.3 Other *CAV1/CAV2* SNPs in POAG

In the GWA study, rs4236601 remained significantly associated with POAG after controlling for other SNPs in the 7q31 region, while others did not show significant association after adjusting for rs4236601. Moreover, by screening the *CAV1* and *CAV2*

genes, no mutation was found to be tagged by rs4236601. Therefore, rs4236601 is responsible for the GWA signal (Thorleifsson et al. 2010). In our study two SNPs rs926201 and rs6466587, which are in strong LD with rs4236601, showed a similar trend of association. However, their effects were weaker than rs4236601 and may thus be genetic makers tagged by rs4236601. Moreover, the LD was weaker flanking rs6975771 and rs959173. Thus, it is not likely that rs4236601 is tagged by a variant located outside the region defined by the six SNPs studied.

Besides the risk variants, two SNPs rs6975771 and rs959173 were found to confer a significantly reduced risk of glaucoma. The minor genotype, i.e., rs6975771[AG] and rs959173[CT] conferred an approximately two-fold of reduced risk to NTG (P_{dom}=0.007 and 0.03, OR=0.48 and 0.55, Table 4.10) in the pooled subjects. For HTG, these genotypes showed a trend towards a reduced risk in the Hong Kong (OR=0.54 and 0.60, Table 4.6) and Shantou (OR=0.93 and 0.66, Table 4.7) cohorts, but showed an increased HTG risk in the Beijing cohort (OR=1.28 and 1.08, Table 4.9). Therefore, it is likely that the effects of the protective alleles might have been compromised by the elevated IOP in HTG patients and, the protective effects are more obvious for patients with normal IOP. Furthermore, the mean AAD of NTG patients with the protective genotype of rs6975771 was 5 years older than patients with the wild-type genotype (P=0.10, Table 4.11), while such difference was just 0.028 (or 2.5) years in HTG patients (P>0.5). As such, the protective allele might have postponed the manifestation of disease in subjects whose IOP were normal. In Caucasians, the rs6975771[A] and rs959173[C] alleles are also at lower frequencies in POAG cases (12.5% and 13.5%) than in controls (13.8% and 14.8%, P=0.08 and 0.097) (Thorleifsson et al. 2010), suggesting a trend of protection. However, in the GWA study the HTG and NTG cases were not analyzed separately to allow the evaluation of the effects of these SNPs on individual phenotype.

5.2.2.4 Risk and protective haplotypes at the *CAV1/CAV2* locus for POAG

In haplotype analyses, a block (B3) containing the four central SNPs gave the most significant omnibus association (Table 4.12), suggesting that the sub-region defined by these variants has major contribution to the global association. In the Hong Kong cohort, the two protective SNPs rs6975771 and rs959173 showed independent effects after controlling for the other SNPs (Table 4.13). Moreover, the risk and protective alleles, respectively, were located in different haplotypes (Table 4.14). Therefore, it is likely that the risk alleles of rs4730742 and rs4236601 and the protective alleles of s6975771 and rs959173 may function if any independently. Moreover, the higher-risk alleles may mainly contribute to HTG, while the protective ones to NTG. Detection of such phenomenon is largely attributed to the low frequencies of these alleles in Chinese, which allows us to dissect the phase of the haplotypes. These findings, in return, provide further insight into the role of *CAV1/CAV2* variants in the genetics of POAG.

5.2.2.5 Possible biological relevance of CAV1 and CAV2 to POAG

Among the six SNPs studied, three (i.e., rs926201, rs6975771 and rs4730742) are located upstream to the coding sequence of *CAV2*, rs4236601 is located upstream to *CAV1*, and rs959173 and rs6466587 are within intronic region of *CAV1*. The risk and protective haplotypes span the entire coding region *CAV2* and the 5' region of *CAV1*. In this study, rs6975771 and rs4236601 showed the most significant association with NTG and HTG respectively and suggested that CAV2 (downstream of rs6975771) and CAV1 (downstream of rs4236601) may have independent roles. However, as the pairwise SNPs, i.e., rs6975771 and rs959173, rs4730742 and rs4236601, are in strong LD, which gene is responsible for the associations observed cannot be distinguished. In fact, the CAV1 and CAV2 are normally co-expressed and form a stable hetero-oligomeric complex *in vivo* (Parolini et al.

1999; Scherer et al. 1997). These two proteins may thus play a role together. Furthermore, as rs4236601 and rs6975771 are located in the upstream regions of respective gene, they may play a role through the regulation of CAV1/CAV2 expression. However, it has been found that rs4236601 was not correlated with the expression of *CAV1* or *CAV2* in adipose tissue (Thorleifsson et al. 2010), the correlation of these SNPs with CAV1 and CAV2 expression in ocular tissues remains to be investigated, because there could be a tissue specific expression pattern of the genes.

The caveolin proteins (caveolin-1, -2 and -3) are structural components of caveolae. They function as scaffolding proteins, capable of recruiting numerous signaling molecules to caveolae, as well as regulating their activity (Cohen et al. 2004). Caveolin-1 and -2 are often co-expressed, they are mainly abundant in endothelial cells, epithelial cells, adipocytes, fibroblasts and smooth muscle cells, while caveolin-3 is predominantly expressed in skeletal, smooth and cardiac muscles (Cohen et al. 2004; Scherer et al. 1997; Tang et al. 1996). Moreover, all three caveolins are expressed in the mammalian brain (Galbiati et al. 1998; Ikezu et al. 1998). In the eye, expression of caveolin-1 and -2 has been detected in lens epithelium (Lo et al. 2004), RPE (Berta et al. 2007a; Mora et al. 2006), and different layers of the neuroretina (Berta et al. 2007a; Berta et al. 2007b; Kim et al. 2006a). Caveolin-1 was also found in the epithelium of ciliary body (Berta et al. 2007a). Due to the widespread tissue distribution and diverse attributes, the caveolins have been implicated in the pathogenesis of numerous human diseases, such as cancers, muscular dystrophy, type II diabetes and cardiovascular diseases (Cohen et al. 2004). However, knowledge about their involvement in eye diseases is limited. It has been proposed that CAV1 may have a role in retinal degenerative diseases, e.g., retinitis pigmentosa (RP) (Cohen et al. 2004), due partially to that caveolin-1 is a specific component of rod cell outer segments disk membranes (Elliott et al. 2003). However, no direct link between RP and *CAV1* has been

found. As such, the identification of *CAV1/CAV2* polymorphisms associated with POAG shall shed new light on the involvement of caveolins in eye disorders.

So far, although the mechanism underlying *CAV1/CAV2* variants predisposing to POAG remains unknown, multiple pathways are likely. It is likely that, in the context of glaucoma the caveolin-1 and -2, possibly via an alteration in expression levels, may be involved in pathways that affect the viability of retinal ganglion cells. In adult mouse brain, genetic ablation of caveolin-1, -2 or -3 increased neural stem cell (NSC) proliferation in the subventricular zone, suggesting a role of caveolins in the regulation of the proliferation of adult NSC (Jasmin et al. 2009), which can form the three major cell lineages of the central nervous system, namely astrocytes, oligodendrocytes and neurons (Eriksson et al. 1998; Jasmin et al. 2009; Sanai et al. 2004). In the mouse, cerebral ischemia may induce a marked increase in endothelial caveolin-1 and -2 protein levels. Interestingly, cerebral ischemia in the *Cav-1* knockout (KO) mice was found to result in an increased cerebral volume of infarction while in Cav-2 KO mice with reduced infarction volume, as both compared to wild-type mice (Jasmin et al. 2007). These findings supported the functional role of caveolin proteins in the pathogenesis of ischemic injury. Of note, a marked pathological change in glaucoma is the progressive loss of RGCs together with supporting glia and vasculature (Kwon et al. 2009). Ischemia plays a central role in RGC apoptosis. Patients with either HTG or NTG often suffer from inadequate ocular blood flow (Caprioli and Coleman 2010; Harris et al. 1999). Therefore, whether the caveolins are involved in the ischemic injury in the retina of glaucoma patients deserves further investigation. Furthermore, presence of silent cerebral infarct was found to be an independent risk factor for visual field regression in patients with NTG (Leung et al. 2009). As such, an alteration in the expression level of caveolins in human brain and/or retina may be involved in the pathogenesis of POAG.

In contrast to genetic ablation, overexpression of caveolin-1 in PC12 cells (a model for studying neurotrophin signaling, in which nerve growth factor can induce a well-characterized morphologic change to a sympathetic neuron-like phenotype) was found to prevent neurite outgrowth (Bilderback et al. 1999). It was demonstrated that caveolin-1 can interact with both $p75^{NTR}$ (a low-affinity neurotrophin receptor) and TrkA (a member of the Trk tyrosine kinase receptor family). This interaction may regulate neurotrophin responses (Bilderback et al. 1999). Neurotrophins are a family of growth factors that mediate the survival, development, and death of specific populations of neurons and glial cells (Glass and Yancopoulos 1993). It has been proposed that the obstruction of retrograde transport at the optic nerve head results in the deprivation of neurotrophic support to RGC leading to apoptotic cell death in glaucoma (Johnson et al. 2009). As mentioned above, mutations in the *neurotrophin-4* gene have recently been reported to be implicated in POAG, and expression of recombinant NT-4 protein carrying the most frequent mutation (Arg206Trp) was shown to result in decreased activation of its receptor TrkB (Pasutto et al. 2009). In our study of *NTF4*, we also detected two novel putative mutations. Therefore, investigation of the link between caveolins and NTF4 in the context of glaucoma may shed new light on the mechanisms of this disorder.

Also as mentioned, aberrant immunity is implicated in the pathogenesis of glaucoma, probably through autoimmune-mediated injury to the optic nerve (Grus et al. 2008; Schwartz 2003; Tezel and Wax 2004; Wax 2000). Caveolin-1 plays an important role in innate immunity and inflammatory immune response (Medina et al. 2006). It has been shown that the caveolin-1 deficiency in *Cav-1* KO mice dampens the TLR4 signaling, which is a critical event in the immune response (Janeway and Medzhitov 2002; Medzhitov 2001), through the activation of endothelial nitric oxide synthase (eNOS), consequently mitigating inflammatory lung injury (Mirza et al. 2010). In the lung-injury model, the TLR4,

being activated by lipopolysaccharide (LPS), acts through the interleukin-1R-associated kinase 4 (IRAK4) to activate the nuclear factor-κB (NF-κB), a regulator of immunity and inflammatory, which in return results in production of an array of pro-inflammatory cytokines, chemokines and adhesive molecules, such as tumor necrosis factor-α (TNF-α), leading to inflammatory injury (Mirza et al. 2010). As a regulatory process, activity of the TLR signaling pathway is modulated by the nitration of IRAK4 by eNOS-derived nitric oxide. Caveolin-1 is a critical negative regulator of eNOS (Bucci et al. 2000). In the context of Cav-1 deficiency, the activity of eNOS is increased, leading to IRAK4 nitration and resultant impairment of kinase activity, and resulting in less lung injury (Mirza et al. 2010). Besides, it has also been shown that caveolin-1 bound to TLR4 and inhibited LPS-induced proinflammatory cytokine (e.g., TNF-α, interleukin-6) production in murine macrophages (Wang et al. 2009). Interestingly, polymorphisms in *TLR4* (Shibuya et al. 2008), *eNOS* (Ayub et al. 2010; Tunny et al. 1998) and *TNF-α* (Lin et al. 2003a; Razeghinejad et al. 2009) have been implicated in POAG. Recently, significant association between *TNF-α* -308G>A and HTG was detected by our group (Fan et al. 2010). In the multiple gene study described above, SNP rs7037117 in *TLR4* was found to be significantly associated with HTG. Therefore, with the identification of the association between *CAV1/CAV2* variants and POAG, it is likely that the CAV1→eNOS→IRAK4→TLR4-signaling pathway represents one of the disease pathways of this disorder. In our studies, no statistical interaction between SNPs in the *CAV1/CAV2* and *TLR4* genes was detected. Further studies of these molecules in glaucoma are warranted.

5.2.3 **Discrepant mutation profiles of *NTF4* in POAG**

Besides SNPs in susceptibility genes, we have also studied a candidate causative gene *NTF4* to verify its involvement in POAG genetics.

In a total of 928 study subjects, three variants, i.e., Pro151Pro, Gly157Ala and Ala182Val, were detected. The former has been reported (Rao et al. 2010) while the others are new. Thus, the current total number of *NTF4* variants detected among different populations is 18 (Table 5.2), including 17 missense changes and 1 synonymous change. Regarding the distributions of the variants, only three were detected across different cohorts, with Ala88Val occurring in the European, American and Indian cohorts (Liu et al. 2010a; Pasutto et al. 2009; Rao et al. 2010), Arg206Trp in the European and American cohorts (Liu et al. 2010a; Pasutto et al. 2009), and Pro151Pro in the Indian (Rao et al. 2010) and Chinese (this study) cohorts. As such, the variation spectrums of *NTF4* vary across different populations. In particular, the most common variant Ala88Val was not detected in a total of 928 Chinese subjects from 3 geographic regions (i.e., Hong Kong, Shantou and Beijing), indicating specific founder effects for the variant in Caucasians and Indians.

Regarding distributions of the variants in patients and controls, the four most common variants (i.e., Ala88Val, Ser89Asn, Pro151Pro and Arg206Trp) were found in both groups, whereas the others were found exclusively in either patients or controls, with each variant being detected in only 1 individual (Table 5.2). In the initial study of Pasutto et al., overall frequency of the variants was significantly higher in the case group (15/892, 1.7%) than in the control group (1/895, 0.1%, $P<0.001$) (Pasutto et al. 2009). However, when the two common variants (i.e., Ala88Val and Arg206Trp) were removed, the frequencies of the other variants between groups were no longer significantly different (5/882 vs. 1/895, $P=0.12$, Fisher's exact test). In the studies of Liu et al. and Rao et al., the frequencies of overall variants were even higher in controls than in patients (Liu et al. 2010a; Rao et al. 2010). In Chinese, although the 2 missense variants were detected only in POAG, the difference was not statistically significant (2/698 vs. 0/230, $P>0.5$). Thus, the *NTF4* missene variants are at least not a decisive causative factor for POAG.

Table 5.2. *NTF4* variants detected in different study cohorts

Variant	European (Pasutto et al. 2009)		American* (Liu et al. 2010a)		Indian (Rao et al. 2010)		Chinese (This study)	
	POAG	Control	POAG	Control	POAG	Control	POAG	Control
Cys7Tyr	1	0	-	-	-	-	-	-
Ser29Stop	-	-	0	1	-	-	-	-
Glu84Lys	1	0	-	-	-	-	-	-
Ala88Val	5	0	1	5	3	14	-	-
Ser89Asn	-	-	1	1	-	-	-	-
Arg90His	1	0	-	-	-	-	-	-
Arg90Cys	-	-	0	1	-	-	-	-
Arg114Gly	-	-	0	1	-	-	-	-
Arg133His	-	-	1	0	-	-	-	-
Arg140Cys	-	-	0	1	-	-	-	-
Pro151Pro†	-	-	-	-	1	0	3	1
Gly157Ala	-	-	-	-	-	-	1	0
Ala182Val	-	-	-	-	-	-	1	0
Arg206Trp	5	0	1	2	-	-	-	-
Arg206Gln	1	0	-	-	-	-	-	-
Thr207Ile	-	-	1	0	-	-	-	-
Thr207Ser	0	1	-	-	-	-	-	-
Arg209Gly	1	0	-	-	-	-	-	-
Total	15/892	1/895	5/443	12/533	3/140	14/285	2/698	0/230

Note:

* The study subjects involved in the study of Liu et al. were individuals of European ancestry from the southeastern United States, which were referred to as Americans in this table.

† The synonymous variant Pro151Pro was not counted in the total frequency.

In view of the mutation screening results from this study and others, the role of *NTF4* as a disease gene for POAG remains inconclusive. POAG is a multifactorial disease resulted from interactions of multiple genetic and environmental risk factors. The penetrance of certain pathological mutations could be modified by some if not many other factors. Incomplete penetrance, where not all mutation carriers present with the expected phenotype, has been a common phenomenon in mutations causing glaucoma. For example, incomplete penetrance of a common *MYOC* mutation Thr377Met has been reported in POAG, and there was a widely varying range of disease onset in patients with this mutation (Allingham et al. 1998; Craig et al. 2001; Puska et al. 2005). Also, the presence of glaucoma was found to increase with age in *MYOC* Gln368stop carriers in a family with POAG, while some members remained unaffected at advanced age, suggesting that additional risk factors would be needed for the development of the disease (Angius et al. 2000). Therefore, it is not unexpected that a putatively pathological *NTF4* mutation is found in normal subjects. Like *OPTN*, *WDR36* and *MYOC*, in which mutations do not induce glaucoma unless the genetic context is permissive, it is likely that *NTF4* mutations may predispose to the disease only when the genetic and/or environmental backgrounds are permissive.

In our study, two putative *NTF4* mutations were detected in the Hong Kong cohort. Gly157Ala was identified in a patient with late-onset NTG (AAD: 67 years). And Ala182Val was present in a patient with early-onset HTG (AAD: 28 years). If these two variants are in fact pathogenic, *NTF4* mutations should account for approximately 0.51% (2/391; 95% CI: 0.14-1.84%) of overall POAG in Hong Kong Chinese or 0.29% (2/698; 95% CI: 0.08-1.04%) in overall Chinese. So far, family members of these two patients are not available for segregation analyses. According to the self-identified family history, there are no known glaucoma patients in respective family. Thus, whether these variants are *de novo* mutations for POAG or mutations with incomplete penetrance remain unknown.

In the current lack of functional assays, we used two *in silico* programs, i.e., PolyPhen and SIFT, to predict the impacts of the variants to the NTF4 protein. The Gly147Ala variant was predicted to be "benign" by PolyPhen, while being predicted to be "affect protein function" by SIFT. However, in the SIFT report a low-confidence warning was issued because of a median sequence conservation score of 4.32. This implies that this substitution may have been predicted to affect protein function just because the set of sequences used for prediction are too conserved (median conservation value >3.25) (Kumar et al. 2009). The Ala182Val variant was predicted to be "benign" and "tolerated" by PolyPhen and SIFT respectively. Accordingly, these two variants are likely to be benign changes. However, functional assays for these variants would be helpful to verify their impacts to the protein.

The NTF4, a member of the neurotrophin protein family related to the survival of neurons including RGCs, acts by the phosphorylation of TrkB receptors. Based on the findings on the association of *NTF4* mutations with POAG, Pasutto's group has revealed an impaired TrkB activation as a possible pathway in the pathogenesis of glaucoma (Pasutto et al. 2009). As mentioned in the previous section, it has been demonstrated that caveolin-1 can interact with both $p75^{NTR}$ and TrkA to regulate neurotrophin responses (Bilderback et al. 1999). Therefore, the NTF4 and CAV1/CAV2 may act through similar pathways in the pathophysiology of POAG.

5.3 Identification of novel genes for POAG

In the past two decades or so, genetic linkage combined with positional cloning has offered a rational and direct route to identify genetic loci and gene mutations that are implicated in POAG, such as the more than 24 linkage loci and the 3 causal genes. Moreover, standalone candidate gene approach has also led to the identification of a majority of the currently known associated genes for the disorder.

A candidate gene is a gene for which there is evidence supporting its possible role in the trait or disease that is under study. For POAG, candidate genes may include those (1) located in the known linkage loci, (2) involved in the pathways of IOP regulation, (3) involved in the pathways of ganglion cell apoptosis or viability, (4) associated with other eye diseases that have some relevance to glaucoma, (5) associated with other neurodegenerative diseases, and/or (6) associated with other risk factors of glaucoma, etc. With these criteria, a candidate gene that is expressed in eye tissues should be prioritized.

In this thesis, a candidate-gene resequencing approach was applied for the identification of disease genes for POAG.

5.3.1 *CNTF* is not a causative or susceptibility gene for POAG

One major characterization of glaucoma is the loss of RGCs. While IOP-lowering therapies being the mainstay of treatment for glaucoma, development of neuroprotective strategies that seek to directly promote the survival and health of RGCs represents a logical approach. Ciliary neurotrophic factor is potentially one of the candidate agents. CNTF is a 200-amino acid secreted protein expressed in cells of all retinal layers, RPE (Beltran et al. 2003), and the optic nerve head (Liu et al. 2007). Endogenous expression of CNTF in the retina increases with retinal and optic nerve injury (Cao et al. 2001; Valter et al. 2005), as well as experimental IOP elevation (Wu et al. 2007). CNTF is released in the retina by light (Wen et al. 1995). Furthermore, CNTF has neuroprotective properties in various experimental injuries to retinal and central neurons (Cui et al. 1999; Fischer et al. 2004; Honjo et al. 2000; Maier et al. 2004; Watanabe and Fukuda 2002; Zhang et al. 2005), including experimental glaucoma (Ji et al. 2004; Pease et al. 2009). Virally mediated overexpression of CNTF is protective of RGCs after optic nerve injury (Leaver et al. 2006; van Adel et al. 2003) and experimental glaucoma (Pease et al. 2009). And it has been

shown that astrocyte-derived endogenous CNTF is a glial-derived mediator contributing to the axon growth-promoting effects of exogenously applied CNTF on axotomized RGCs *in vivo* (Muller et al. 2009). These findings suggest that CNTF, exogenous and endogenous, probably works in both neuroprotection and promotion of axonal regeneration. Thus, while CNTF being an excellent candidate agent for glaucoma treatment, the *CNTF* gene, once mutated, can be a genetic risk factor for glaucoma. Mutations in this gene may lead to a loss-of-function effect resulting in a reduced protection for the RGCs, or a gain-of-function or dominant negative effect deleterious to RGCs. So far, however, *CNTF* mutations have not been implicated in glaucoma elsewhere.

In this study, the *CNTF* gene has been evaluated as a genetic risk factor for POAG. In a set of 469 study subjects from the Hong Kong and Shantou cohorts, 7 sequence variations were identified (Table 4.15). No consistent association was found between the variants and POAG. In particular, although the most common SNP IVS1-6G>A (rs1800169) showed a significant association with NTG in the Shantou cohort (50% in NTG vs. 24.6% in controls, P=0.007), it was likely due to a small cohort effect (n=28). Moreover, such association was not replicable in the Hong Kong cohort (22.5% in NTG vs. 30.1% in controls, P=0.22). Thus, *CNTF* is less likely to be a susceptibility gene for POAG.

Of note, this variant is located at a splice acceptor site (-6bp upstream of exon 2) of *CNTF*. We therefore used the ASSA program to predict its impact to splicing. The G>A substitution was predicted to create a cryptic acceptor site at positions -5 and -6 of the 3' intron, with the Ri value being increased from -1.8 to 6.4 (Figure 5.1). This will lead to an inclusion of 4 additional intronic nucleotides of the pre-mRNA into the mRNA during splicing, cause a frameshift and introduce a premature termination codon "TGA" to the transcript, resulting in a truncation protein of 62 amino acids, designated as Val39ProfsX25. In fact, it has been demonstrated that this mutated mRNA is efficiently transcribed, but the

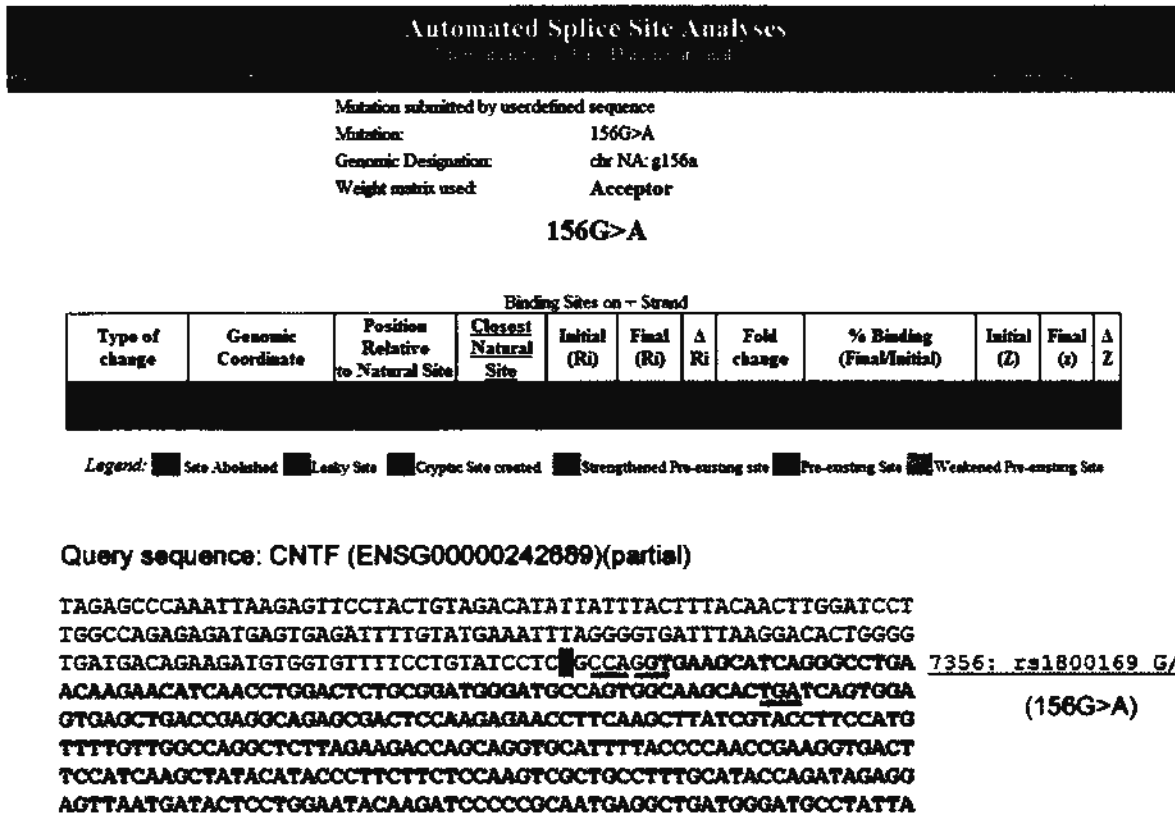


Figure 5.1. Prediction of functional impact of *CNTF* rs1800169 to alternative splicing

The prediction was performed using the Automated Splice Site Analyses program. The G>A substitution at 6bp upstream of exon 2 was predicted to create a cryptic splice acceptor site, which will lead to an inclusion of 4 additional nucleotides of the intronic sequence into the mRNA during splicing, cause a frameshift and introduce a premature termination codon "TGA" at position 63 on the transcript, resulting in truncation protein of 62 amino acids: Val39ProfsX25.

(Figure composed using the output of the ASSA website: https://splice.uwo.ca; and the Ensembl website: http://www.ensembl.org/)

protein is poorly expressed *in vivo*, suggesting that the mutated protein was very unstable and rapidly degraded after translation and subjects homozygous for the null mutation were lacking CNTF (Takahashi et al. 1994). In our study, 4 (1.7%) control subjects (2 from the Hong Kong cohort and 2 from the Shantou cohort) were found to be homozygous for the null mutation. Thus, the lack of association between this mutation and either HTG or NTG suggests that endogenous CNTF insufficiency is not causatively related to glaucoma. Likewise, lack of association with other neurological diseases for the null mutation was found, such as amyotrophic lateral sclerosis (ALS) (Orrell et al. 1995; Takahashi et al. 1994), Alzheimer disease (Gelernter et al. 1997; Takahashi et al. 1994), Parkinson disease (Takahashi et al. 1994), and multiple sclerosis (MS) (Giess et al. 2002b). Thus, our finding of the lack of association between the null mutation and POAG adds into the growing understanding of *CNTF* mutations in human disorders.

It has been proposed that the CNTF protein may be released under pathological conditions, such as nerve injury, in order to promote neuronal regeneration, and that a CNTF deficiency may affect the manifestation, progression and prognosis of neurological diseases (Takahashi et al. 1994). Indeed, MS patients homozygous for the CNTF null mutation was found to have a significantly earlier onset of disease (17 vs. 27 years, P=0.007) with predominant motor symptoms, suggesting that CNTF contributes to time and site of early clinical manifestation (Giess et al. 2002b). The null mutation was found to lead to early onset of familial ALS with SOD-1 mutations (Giess et al. 2002a). Controversially, in other studies no significant correlation was found between CNTF genotypes and the age at onset, course or severity of MS (Hoffmann et al. 2002), sporadic or familial ALS (Al-Chalabi et al. 2003), or Huntington's disease (Rubinsztein et al. 1997). These findings suggest that *CNTF* could be a modifier gene for certain neurological diseases under certain genetic and/or environmental backgrounds. In view of this, we hypothesized that in the

context of glaucoma, patients with the null mutation may have earlier age of onset and/or more severe manifestation. Correlations between the genotypes and AAD, maximum IOP and VCDR in the patient subjects were analyzed. No correlation was found with IOP and VCDR in either the Hong Kong or Shantou cohort. Regarding AAD, in the Hong Kong cohort NTG patients with the null mutation have a mean AAD approximately 2 years younger than those with the wild-type genotype (64.1 vs. 66.3, P=0.47, Table 4.16). Unexpectedly, however, in the Shantou cohort POAG patients possessing the null allele were found to have significant increase in mean AAD by about 10 years (52.6 vs. 43.0, P=0.013). In this sense, the null allele might have postponed the development of disease suggestive of a protective effect. So far, the biological relevance to this phenomenon is unknown. Since the correlation was not found in Hong Kong NTG subjects, its occurrence in Shantou POAG subjects might have been due to chance. However, a cohort specific effect due to certain environmental exposures cannot be excluded. A replication cohort from Shantou is needed for validating this finding.

To date, human clinical trials for retinitis pigmentosa and age-related macular degeneration are under way with an intravitreal implant (NT-501) containing immortalized pigment epithelial cells expressing CNTF (Tao 2006). The fact that CNTF also protects RGCs indicates that NT-501 could be used for glaucoma as well. Since exogenously applied CNTF stimulates RGCs *in vivo* partially and indirectly via a mechanism that depends on astrocyte-derived endogenous CNTF (Muller et al. 2009), whether patients with or without the null mutation will have different pharmacogenetical responses to the regimen deserves investigation in future clinical trials.

In this study, 5 new *CNTF* variants were detected. A 1-bp insertion c.103_104insA was found in a 94-year old normal subject. This mutation is predicted to result in a truncation protein of 61 amino acids, i.e., Thr35AsnfsX28. Similar to the truncation protein resulted

from the null mutation as mentioned above, such mutant protein may also be unstable and degraded rapidly, resulting in a haploinsufficiency rather than a dominant negative effect. Thus, it is not unexpected that the mutation is present in a healthy individual.

For the missense variant Glu75Gln that was detected exclusively in one patient with early-onset NTG, it was predicted to be "benign" by PolyPhen and "tolerated" by SIFT. It is thus less likely to be a pathogenic mutation. The patient has no known family history of glaucoma, but her family members are not available for clinical and genetic investigations. Therefore, the pathogenicity of Glu75Gln remains unverified. Since haploinsufficiency of CNTF is not causative for POAG, other missense mutations in the gene causing dominant negative effect deleterious to RGCs may predispose the carriers to glaucoma.

5.3.2 *SPARC* is not the causal gene at GLC1M nor a susceptibility gene for JOAG

JOAG is a subset of POAG characterized by an early age of onset and severe elevations of IOP. It is often inherited in an autosomal dominant pattern, but sporadic cases exist. Thick, compact tissue and extracellular deposits have been found in trabecular meshwork specimens obtained from JOAG patients during trabeculectomy (Furuyoshi et al. 1997), suggesting that abnormal trabecular meshwork and aqueous outflow could lead to an elevated IOP in JOAG. The identification of *MYOC* mutations accounting mainly for JOAG (Stone et al. 1997) and its mutant products leading to elevated IOP (Kwon et al. 2009) also supports that genes that affect the structure of trabecular meshwork and outflow resistance may function to regulate IOP and, once mutated, predispose to glaucoma. So far, five known loci have been linked to JOAG, including GLC1A (1q21-31, *MYOC*) (Sheffield et al. 1993; Stone et al. 1997), GLC1J (9q22) (Wiggs et al. 2004), GLC1K (20p12) (Wiggs et al. 2004), GLC1M (5q22.1-32) (Pang et al. 2006) and GLC1N (15q22-24) (Wang et al. 2006b) (Table 1.1). However, except for GLC1A, causal genes at other loci remain unidentified.

In the GLC1M-linked Philippine pedigree with JOAG, the affected members had elevated IOP (mean±SD: 32±6.3 mmHg) (Pang et al. 2006). It is likely that this locus harbors a gene involved in IOP regulation. In this study, we selected *SPARC* as a candidate gene mainly on the basis that the SPARC protein functions to promote extracellular matrix deposition (Rhee et al. 2009), and it is rich in eye tissues, especially in the trabecular meshwork and the juxtacanalicular region (Rhee et al. 2003). The SPARC-null mice have lower IOP (Haddadin et al. 2009). Furthermore, *SPARC* is located at 5q31.3-q32 within the GLC1M locus (5q22.1-32). However, no mutation or copy number variant was detected. Thus, although *SPARC* by itself is a good candidate gene for glaucoma, it is not responsible for JOAG in the pedigree. The causal gene at the GLC1M locus remains to be identified.

Also, no missense change in *SPARC* was detected in a group of unrelated JOAG patients. Of the 11 variants detected, none was associated with glaucoma either individually or contained in a haplotype. Our sample of 46 patients might be small to provide adequate statistical power to detect the significance. However, as the distributions of the genotypes were drastically similar between the patients and controls (Table 4.17), it is unlikely that the lack of association was due to insufficient power. Therefore, *SPARC* variants do not have a major contribution to JOAG genetics. However, some rare variants in the gene contributing to a small portion of patients cannot be excluded and can be confirmed by further screening of the gene in a very large cohort. It is also likely that variants located outside the coding region, e.g., 3'-UTR, may have contribution to the disease. It has been reported that a SNP at the 3'-UTR of *SPARC*, i.e., +998C>G (equivalent to rs1053411), was associated with systemic sclerosis in different populations. The CC genotype was correlated with a longer mRNA half-life in normal fibroblasts and may contribute at least in part to increased *SPARC* gene expression (Zhou et al. 2002). In our study, no association was detected for rs1053411, which is in strong LD with the other four common SNPs. According to HapMap,

the SNPs located at the 3'-UTR of *SPARC* are also in strong LD. Therefore, it is not likely that other common SNPs at the 3'-UTR are associated with JOAG (Figure 4.5). As such, if the correlation between CC genotype of rs1053411 and an increased-SPARC expression is real, it is likely that such an extent of increased-SPARC expression is not causative for glaucoma. However, whether a more abnormal expression of the protein, possibly triggered by other pathological factors, can predispose to the disease remains to be investigated.

So far, the involvement of the SPARC protein level in the pathogenesis of glaucoma is unknown. SPARC occurs widely in extracellular matrices and is predominantly expressed during embryogenesis and in adult tissues undergoing remodeling or repair. It is believed to play a modulatory role in cell-cell and cell–matrix interactions, differentiation, ECM production and organization, wound healing, and angiogenesis (Bradshaw and Sage 2001; Lane and Sage 1994; Sage and Bornstein 1991), suggesting a fundamental role of SPARC (Bradshaw and Sage 2001). SPARC has been implicated in multiple systemic as well as ocular conditions. For example, it is expressed at high levels in bone tissue and acts as a major noncollagenous protein of bone matrix (Maillard et al. 1992). SPARC-null mice have low-turnover osteopenia (Mansergh et al. 2007), and it was suggested that SPARC may strengthen bone (Kos and Wilding 2010). Moreover, *SPARC* 3'-UTR polymorphisms had been associated with bone density in Caucasian men with idiopathic osteoporosis (Delany et al. 2008). Likewise, SPARC-null mice have lower IOP (Haddadin et al. 2009), it could thus be hypothesized that increased-SPARC level may elevate IOP and predispose to glaucoma. In the eye, increased SPARC level has been correlated with cataract (Kantorow et al. 2000), corneal wound repair (Berryhill et al. 2003), and proliferative diabetic retinopathy (Watanabe et al. 2009). In glaucoma, elevated SPARC expression has been detected in the iris of POAG and primary angle closure glaucoma patients (Chua et al. 2008). All these findings, suggest that certain SPARC expression level could play a role in

eye diseases. However, the correlation between the expression of SPARC and the occurrence of these eye diseases remained to be clarified. Recently, it has been found that SPARC deficiency in mice resulted in improved surgical survival in a mouse model of glaucoma filtration surgery (Seet et al. 2010). Whether the SPARC level is correlated with the success rate of filtration surgery in glaucoma patients is unknown. If such correlation exists, a pre-operative detection of the SPARC level may help with a better treatment plan.

This study is one of several attempts to evaluate the involvement of copy number variation in POAG. Abu-Amero et al. had screened 27 POAG patients and 12 controls for chromosomal copy number alterations and found no chromosomal deletions or duplications in POAG patients compared to controls (Abu-Amero et al. 2009). Davis et al. performed a whole-genome copy number screening in a cohort of 400 patients with POAG and 100 controls and found that rare copy number variations in the *DMXL1*, *TULP3* and *PAK7* genes may affect development of POAG (Davis et al. 2009). Interestingly, the *DMXL1* gene at 5q23.1 is located within the GLC1M locus. This suggests that copy number of the genes at this locus, including *DMXL1*, may contribute to the genetics of POAG. However, our findings in that all of the 27 subjects from the Philippine family and the 36 Chinese subjects have two copies of the *SPARC* gene indicate that copy number variation of *SPARC* is at least not a common phenomenon in these two populations and is not a major genetic contributor to JOAG. Whether copy number variations in other genes at the GLC1M locus are involved in the genetics of glaucoma remain to be investigated.

5.3.3 **Identification of a novel causative gene for POAG at the GLC1N locus**

5.3.3.1 Identification of *MEGF11* c.1090delT as a causal mutation at GLC1N

As mentioned, genetic linkage combined with positional cloning has greatly facilitated the finding of genetics loci and genes underlying POAG. However, within the 24 known

linkage loci, only 3 causal genes were identified. In this section, the identification of a causal gene for POAG will be described.

In our previous work, a linkage locus (i.e., GLC1N) was identified in a Hong Kong pedigree with JOAG through a genome-wide linkage analysis (Wang et al. 2006b). This locus was mapped to chromosomal region 15q22-q24 within a genetic distance of 16.6Mb. In this region, at least 118 annotated genes have been deposited in the NCBI Map Viewer database (Build 36). Three candidate genes, i.e., *NR2E3*, *CLN6* or *SMAD6*, had been excluded as the causal gene (Wang et al. 2006b). In this present work, four new candidate genes (i.e., *DPP8*, *DENND4A*, *MEGF11* and *RB11A*) located consecutively in the critical region were selected, based on their proximal locations to the makers giving the highest LOD scores, their retinal expression, and possible involvement in cell-extracellular matrix interactions and/or apoptosis-related pathways. A mutation c.1090delT in *MEGF11* is the most likely to be the causal mutation, because it is one of the five variants that were segregated in all patients (Table 4.18), and it is the only variant that is located in the coding region of a gene. However, 4 unaffected mutation carriers (i.e., III:4, III:6, III:16 and III:17) were also found (Figure 4.8). Intriguingly, subject III:16 was found to be homozygous for the mutation and, her father, a married-in normal spouse (II:15), was found to be heterozygous for the mutation. The sister (II:15S) of subject II:15 was also found to be an unaffected carrier. According to their family histories, and the haplotype constructed by the microsatellite markers that were used in the linkage mapping (Wang et al. 2006b), these two families are not related, suggesting different founder effects of the mutation. Of note, the *MEGF11* c.1090delT was detected in an unrelated JOAG patient from the Beijing cohort, and it was absent in a total of 529 unrelated controls. Based on these findings, it is likely that *MEGF11* c.1090delT is the causal mutation for JOAG in the pedigree, expressing an autosomal dominant inheritance with incomplete penetrance.

Arguably, some subjects might still be too young to manifest the disease at the time of study recruitment, e.g., III:6 (17 years), III:16 (14 years) and III:17 (10 years), or even at the last follow-up visit in 2006. However, subjects II:15 (46 years), II:15S (47 years) and III:4 (35 years) were in their adulthood. According to the fact that the patients in the family had the disease diagnosed at an early age (12~32 years of age, Table 3.7), these three subjects should have, if any, manifested the disease, although an age-related penetrance could not be excluded. Thus, although the *MEGF11* c.1090delT mutation is probably responsible for the glaucoma in the family, it may not be a decisive causal factor. Like the phenomenon observed for *MYOC*, *OPTN*, *WDR36* and *NTF4* that a permissive genetic and/or environmental context is needed for the mutations to cause the disease, the *MEGF11* gene may also be subjected to this rule.

The c.1090delT mutation is located in the ninth exon of the *MEGF11* gene, which has 23 exons encoding for a 1044-amino acid protein (ENSP00000414475). The 1-bp deletion is predicted to cause a frameshift to the transcript and introduce a premature termination codon, resulting in a truncation protein with 374 residues, designated as Cys364ValfsX12. However, such an aberrant protein is expected to be sparsely expressed because of the nonsense-mediated mRNA decay (NMD) mechanism. NMD is an mRNA-surveillance mechanism found in all eukaryotic organisms that leads to a degradation of the transcripts with introns in the 3' untranslated region and subsequently prevents the synthesis of truncated proteins that might have toxic effects (Brogna and Wen 2009; Chang et al. 2007; Conti and Izaurralde 2005; Garneau et al. 2007; Isken and Maquat 2007). If a small amount of the mutant protein could be translated, it is expected to be unstable and degraded rapidly, similar to the truncation protein resulted from the *CNTF* null mutation mentioned above. Therefore, the deletion may lead to a haploinsufficiency or loss-of-function effect rather than a gain-of-function or dominant negative effect. Thus, the glaucoma in the GLC1N-

linked family might have been caused by MEGF11 insufficiency. For those normal mutation carriers, the disease might have been arrested or postponed by a complementary mechanism for the reduction of MEGF11. The mechanism is yet unknown, but it may have been introduced by the married-in spouses in generation II (i.e., II:3 and II:15) to the offsprings in generation III, because the penetrance of the mutation is 100% in generation II suggestive of a permissive genetic context in this generation. This assumption may also explain why subjects II:15 and II:15S, who possess the mutation, did not develop glaucoma even at mid 40's. Interestingly, the subject III:16, who is homozygous for the deletion, is expected to have no normal MEGF11. The subject was recruited at the age of 14 years. No identifiable glaucomatous change or elevation of IOP was detected. Unfortunately, whether this subject has developed glaucoma is not currently known because she did not give consent to be followed up since the recruitment visit in 2004. An effort is being made to invite the family subjects for a detailed ophthalmic examination.

5.3.3.2 Evaluation of the contribution of *MEGF11* in POAG

To further verify the role of *MEGF11* as a disease gene for JOAG or POAG and evaluate its contribution to the disease, we screened the gene in a large sample of unrelated patients, including 181 HTG patients from the Hong Kong cohort, 177 HTG patients from the Beijing cohort and 95 HTG patients from the Shantou cohort. Also screened was a group of 182, 200 and 147 control subjects from the Hong Kong, Beijing and Shantou cohort, respectively. The sequence data from a cohort of Caucasian subjects, including 93 POAG and 90 controls, were also introduced for the purpose of comparison.

The *MEGF11* gene is highly polymorphic. A total of 60 sequence changes were identified, with 52 are novel. No individual SNP or haplotype was significantly associated

with POAG in individual cohort or pooled subjects, suggesting that *MEGF11* is not a common susceptibility gene for glaucoma.

We then tested whether rare coding variants in *MEGF11* were associated with POAG. In the absence of *in vivo* functional assays for the variants, the distributions of the coding variants in patients and controls were compared either individually or in combination, under the hypothesis that, if any kind of coding variant is associated with the disease, the accumulated chance that it occurs in patients should be significantly higher than the controls. However, no significant difference in the distributions was detected for any individual variant or any category, including damaging variants and variants in functional domains of MEGF11 (Table 4.21). There were13 missense variants (among which 5 were predicted damaging) that were present exclusively in patients. If they are in fact pathogenic, especially for the damaging ones, *MEGF11* missense mutations may account for 1.1~2.9% of overall POAG in Chinese. However, another 8 (2.1%) missense variants, including 7 (1.8%) damaging ones, were found only in controls. The frequencies of these two groups of variants were not statistically different (2.9% vs. 2.1%, P=0.37). Therefore, the occurrence of the rare variants in patients might have been due to chance rather than driven by a tendency to disease-association. In light of this, single amino acid substitution in MEGF11 is less likely to be causative for POAG.

As mentioned, the c.1090delT mutation was identified in a JOAG patient from the Beijing cohort and absent in controls, supporting its role as a disease-causing mutation. Moreover, since this variant is a null mutation, we hypothesized that, variants in the intronic region (especially for those at the exon-intron junctions) that affect alternative splicing may be related to the disease, because errors in RNA splicing, including aberrant alternative splicing, is a common source of premature termination codons (Chang et al. 2007), and NMD regulates alternative splicing by eliminating unproductive splice variants

that contain premature termination codons (Brogna and Wen 2009; McGlincy and Smith 2008). Moreover, it has been suggested that disrupted splicing by itself has a determinative role in disease, as a direct cause, a modifier of disease severity and a determinant of disease susceptibility (Wang and Cooper 2007). Therefore, we analyzed the variants in the splicing sites by predicting their impact to alternative splicing and comparing their distributions between patients and controls. In this analysis, only variants located at the regions containing 5 exonic and 10 intronic nucleotides at the exon-intron junctions were involved. As such, eight variants, including 2 coding variants and 6 intronic variants, were considered splice-site variants (Table 4.22).

The most common splice-site variant IVS9+9insT was predicted to cause no change to the splice site by ASSA, suggesting that this 1-bp insertion is not likely to cause alternative splicing. Moreover, its distribution in patients and controls were not significantly different in either the Chinese cohorts or the Caucasian cohort, suggesting a lack of disease association. The IVS9-7T>C was also evenly distributed in patients and controls in the Hong Kong and Beijing cohorts while being absent in the Shantou and Caucasian cohorts. Moreover, this variant was predicted to have mild effect on the splice site. Therefore, it is also not likely to be disease-causing.

The other six variants, with each being detected in one subject, are likely to have functional impacts. In particular, 2 variants (i.e., IVS17+2insT and IVS17-2A>G) were predicted to abolish the splice sites, with the information contents values falling well below zero. Therefore, they are the most likely to be pathogenic. In contrast to the aforementioned *CNTF* null mutation IVS1-6G>A, which creates a cryptic acceptor site and leads to an elongated exon, these two mutations are expected to cause exon skipping. IVS17+2insT may lead to an in-frame skipping of exon 17, resulting in a mutant protein without the 15th EGF-like domain. It was detected in a patient with an AAD of 43 years and maximum IOP

of 26 and 21.5mmHg in respective eye. The IVS17-2A>G is likely to cause an in-frame skipping of exon 18 and subsequently the 16th EGF-like domain. It was identified in a POAG patient from the Caucasian cohort, whose clinical data in detail are not yet available.

The other four splice-site variants were predicted to result in leaky splice sites, with the Ri values reduced mildly to moderately. It has been suggested that sites with Ri values ≥2.4 bits but less than the corresponding natural site usually decrease, but do not abolish splicing, and that substitutions producing small changes in Ri values probably do not impair splicing and are often polymorphisms (Rogan et al. 1998). Therefore, it could be expected that three variants, i.e., c.200G>A, IVS6+3G>T and c.2472A>C, which were predicted to moderately reduce the Ri values (Table 4.22), are more likely to cause alternative splicing, resulting in a production of alternative mRNAs and subsequently admixed protein isoforms. The IVS6+3G>T variant is likely to cause a skipping of exon 6. Because this exon contains 247bp, skipping of it may lead to a frameshift and introduce a premature termination codon to the transcript, resulting in a truncation protein. Similar to the c.1090delT mutation, the aberrant transcript should also be subjected to NMD and result in a reduced MEGF11 production. However, as this variant does not abolish splicing, the resultant reduction in MEGF11 is expected to be less than that resulted from c.1090delT, and not necessarily lead to MEGF11 insufficiency. Interestingly, this variant was detected in a 71-year old control individual. Thus, it could be hypothesized that a certain severity of MEGF11 insufficiency is needed to cause glaucoma. However, the threshold is currently unknown. Also detected in a control subject was the coding variant c.200G>A, which may lead to an in-frame skipping of exon 3 encoding for part of the EMI domain. This suggests that partial absence of the EMI domain is not causative for glaucoma. Another coding variant c.2472A>C, which was found in a 34-year old HTG patient, may result in an in-frame skipping of exon 19 and an absence of the 17th EGF-like domain in the protein. This variant is likely to be a

causative mutation for glaucoma. The last splice-site variant IVS17-4C>G, which was predicted to just mildly reduce the Ri value, is likely to result in, if any, a skipping of exon 18 or the 16th EGF-like domain. This variant was present in a HTG patient with an AAD of 38 years and maximum IOP of 32 mmHg in both eyes. In view of these findings, the four splice-site variants, which were predicted to result in a leaky splice site and an absence of a certain EGF-like domain of MEGF11, are likely to be causative mutations. No such mutation was detected in a total of 472 normal subjects through whole gene screening. Moreover, the four putative mutations were not detected in the 147 controls subjects from the Shantou cohort. Therefore, the chance that these mutations are disease-related is statistically significant (4/546 vs. 0/619, P=0.048, Fisher's exact test).

If the five variants, i.e., c.1090delT, IVS17+2insT, IVS17-2A>G, IVS17-4C>G and c.2472A>C, are in fact pathogenic, *MEGF11* mutations may contribute to approximately 1.1% (6/546, 95% CI: 0.5-2.4%) of POAG, with the mutation frequencies being similar between Chinese (5/453, 1.10%) and Caucasians (1/93, 1.08%). Notably, all of the mutation carriers in Chinese could be considered as JOAG patients, with an AAD between 14 and 43 years. The affected subjects in the GLC1N-linked pedigree were JOAG. As such, *MEGF11* is likely to be a disease gene specific for JOAG. In the Chinese cohort, 231 patients were diagnosed of POAG before 40 years of age. Thus, *MEGF11* mutations may account for approximately 2.2% (5/231, 95% CI: 0.93-5.0%) of JOAG in Chinese.

5.3.3.3 Potential biological relevance of *MEGF11* to glaucoma

So far, biological properties of MEGF11 are poorly understood. The gene has not been implicated in diseases. Therefore, the identification of *MEGF11* mutations causative for glaucoma may lead to the discovery of new mechanisms underlying glaucoma.

MEGF11 shares significant similarity with human MEGF10, which is the human ortholog of the *cell death abnormal 1* (*CED-1*) gene in *Caenorhabditis elegans* (Suzuki and Nakayama 2007b). In *C. elegans*, CED-1 is a key component in phagocytosis functioning in clearance of apoptotic cells (Kinchen et al. 2005; Yu et al. 2008). Moreover, CED-1 was found to have axon guidance functions, and mutations in it can cause abnormal axon patterns and commissure branching (Schmitz et al. 2007). Thus, the MEGF10 and MEGF11 may also be involved in apoptotic engulfment pathways. So far, studies on MEGF11 are sparse. MEGF11 is very similar to MEGF10 in its domain organization, and both proteins have similar cellular localization and express a featured irregular, mosaic-like adhesion pattern, which may confer unique functions to the MEGF10 family proteins *in vivo* (Suzuki and Nakayama 2007a). As such, MEGF11 may function similar to MEGF10. However, while localizing throughout the cytoplasmic membrane, MEGF10 does not localize to lamellipodia as does MEGF11, suggesting that MEGF11 is transported into the motility-active zones of a cell, while MEGF10 is not. Moreover, these two proteins localize independently and do not interact (Suzuki and Nakayama 2007a). Therefore, the roles of these two proteins should be partially different.

The MEGF10 and MEGF11 are transmembrane proteins containing a signal peptide, an EMI domain, 17 atypical EGF-like domains, a transmembrane region, and a cytoplasmic domain (Suzuki and Nakayama 2007a). It has been demonstrated that both the EMI and EGF-like domains are required to form MEGF10 polymers responsible for the irregular, mosaic-like adhesion pattern exhibited by full-length MEGF10. Protein lacking the EGF-like domains may lead to an excess production of mutant MEGF10 protein, which forms many oligomers and could not be transported correctly to the cytoplasmic membrane (Suzuki and Nakayama 2007a). If such a phenomenon also occurs to MEGF11, it may provide some insight into the role of the *MEGF11* splice-site mutations that are causative

for glaucoma. As mentioned above, while the c.1090delT mutation may cause a MEGF11 insufficiency due to NMD, the other four splice-site mutations, i.e., IVS17+2insT, IVS17-2A>G, IVS17-4C>G and c.2472A>C, are likely to result in a mixture of normal MEGF11 proteins and proteins lacking a certain EGF-like domain, e.g., the 15th, 16th or 17th EGF-like domain. Thus, it is likely that the mutant proteins may lead to a formation of MEGF11 oligomers containing the mutant proteins or both the mutant and normal proteins and prevent a correct transportation of the proteins to the cytoplasmic membrane. If this is true, the proteins may be sequestered intracellularly, resulting in a cytotoxic effect or an insufficiency of functional proteins on the cytoplasmic membrane. Notably, in the study of Suzuki et al., the mutant MEGF10 protein lacked the entire EGF-like domains (Suzuki and Nakayama 2007a). Thus, whether the lack of single EGF-like domain in MEGF11 mutants can cause a similar effect remains to be further investigated.

MEGF11 is ubiquitously expressed in human tissues, including the retina and brain (BioGPS; http://biogps.gnf.org/). However, whether it is expressed in the trabecular meshwork is unclear. In view of our findings that the *MEGF11* mutations are predominantly causative for juvenile-onset HTG, it is possible that the mutant MEGF11 may impair the apoptotic engulfment pathway at the anterior chamber angle and compromise the aqueous humor outflow, leading to IOP elevation and subsequently glaucoma. However, it is also possible that the mutant proteins may impair the apoptotic engulfment pathway in the retina, inducing the apoptosis of RGCs in subjects with high baseline IOP and predisposing the carriers to glaucoma at an earlier age. If the latter is true, there should be another genetic factor causing the elevation of IOP in the subjects.

The identification of *MEGF11* as a POAG gene suggests that factors involved in apoptotic engulfment pathways may play a role in the pathogenesis of this disease. Unraveling the role of MEGF11 may provide new insights into the mechanism of POAG.

5.4 Strengths and limitations of this study

This present study deserves many advantages. First, a fair amount of new information about POAG genetics is reported. The multiple-gene study represents the first replication study for all candidate genes in Chinese. Discrepant genetic patterns were detected, providing new evidence supporting the ethnic diversity and complexity in the genetics of POAG, and exemplifying the importance of replication study in genetic investigation. Moreover, we provided association data about new SNPs other than rs4236601 at the *CAV1/CAV2* locus. The identification of a protective haplotype suggests a new role of the genes. Furthermore, we have evaluated *CNTF*, *SPARC* as disease genes for POAG, and identified a causal gene *MEGF11* at the GLC1N locus. Second, the coverage of this study is broad. Totally, sequence variants in 16 genes have been studied, either by a candidate SNP approach or by whole gene screening. Third, the sample size was large, involving more than 1600 study subjects. This is by far the largest glaucoma genetic study conducted in Chinese. Moreover, in each individual study, at least two study cohorts were involved for cross-validation of the findings. Furthermore, both HTG and NTG were studied. More importantly, all patients were recruited by experienced glaucoma specialists, with the diagnosis made according to international standard and the phenotypes being relatively homogeneous. This provides us a good chance to identify genotype-phenotype associations. Fourth, detailed data analyses were conducted. In particular, stratification analysis has identified an age-varying association with rs1533428. Finally, the findings of these studies provide new insight into the genetics of glaucoma. The identification of the age-specific association of rs1533428 with late-onset POAG, the different association patterns between HTG and NTG, and the finding that *MEGF11* contributes mainly to JOAG, indicate that glaucoma is a continuum of disease, whereas different genetic factors may contribute specifically to different subphenotypes. These in return exemplify the importance of refined

phenotyping in the genetic studies of glaucoma. The identification of new disease gene and genetic patterns enriches our growing understanding of POAG genetics, which may eventually improve our ability of genetic screening in glaucoma detection and prognosis assessment.

This study also has some limitations. First, on the study design of the multiple gene study, except for the *TLR4* SNPs which were selected using a haplotype-tagged strategy, the other SNPs were simply selected according to the initial reports. Thus, an exhaustive investigation of the SNPs in these genes will enable the evaluation of the involvement of those genes in POAG, especially for *LMX1B* and *CYP46A1*. Second, the sample size of NTG patients in the Shantou cohort was small (n=28), and no NTG patient was available from the Beijing cohort. Therefore, the findings on NTG, especially that *CAV1/CAV2* protective SNPs were predominantly associated with NTG, should be replicated in a larger sample of NTG. Third, the proportions of gender were not perfectly matched in the Hong Kong and Shantou cohorts. However, as the occurrence of POAG is not different between male and female, the disproportion of gender might not be a confounder for the genetic investigations in the study. Fourth, because the studies were conducted over a long period, especially for the earliest work on *MEGF11*, the samples were not overlapped across all studies, rendering difficulty in assessing the candidate genes in all subjects. However, as each study is hypothesis-driven, and the sample size in each study provides a good statistical power, the conclusion of individual study should have been well supported. Finally, for some patients who carry the putative disease-causing mutations in genes such as *NTF4* and *CNTF*, their family members were not available for segregation analysis, leaving the role of the variants unverified. Therefore, further functional assays are needed to confirm the implication of the genes and variants in POAG.

Chapter 6

Conclusions and Future Perspectives

6.1 Complexity in POAG genetics

POAG is complex multifactorial disease with multiple genetic and environmental risk factors. This thesis provides a fair amount of data to support the genetic complexity of POAG. The identification of common associated SNPs and rare mutations reveals that, the genetic basis of POAG conforms to both the "common disease/common variant" and "common disease/rare variant" hypotheses. Thus, different genetic strategies are needed to map the disease genes. Moreover, as the genetic determinants are identified, genetic stratification becomes possible, potentially reducing the genetic complexity of traits and enabling the identification of additional association signals. Finally, assessing the contributions of factors such as epistasis, genocopies and phenocopies enables the evaluation of genetic architecture of POAG with greater precision.

Figure 6.1 summarizes the major findings of this thesis on POAG genetics.

6.1.1 Genetic factors underlying JOAG and AOAG/LOAG

In this study, an age-varying association pattern was identified for rs1533428 at the 2p16.3 locus. This SNP confers a strongly increased risk to late-onset POAG, while the risk was not significant for adult-onset POAG and JOAG. In contrast, the putative mutations in *MEGF11* were identified only in Chinese JOAG. These findings suggest that certain genetic factors may contribute predominantly to a specific age group of POAG. Unraveling the functional roles of these genes will provide important insight into the mechanisms underlying different forms of POAG.

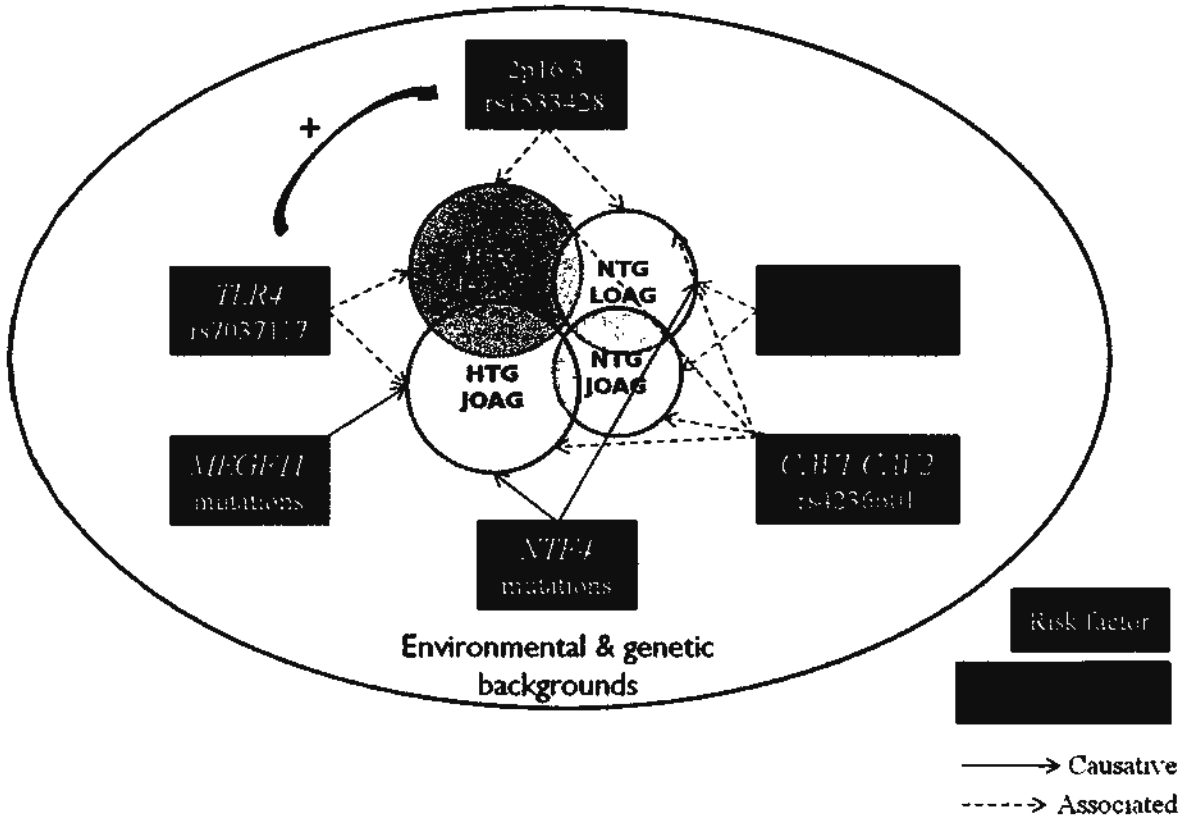


Figure 6.1. Genetic contributors to POAG identified in this thesis.

The entire study reveals the complexity in POAG genetics. Multiple genes and/or makers contributing to POAG have been identified either as risk or protective factors. Some factors are specific for certain groups of the disease, such as late-onset POAG (rs1533428 at 2p16.3) and juvenile-onset HTG (*MEGF11*).

"+" indicates the additive genetic effect between 2p16.3 rs1533428 and *TLR4* rs7037117.

HTG: high-tension glaucoma; NTG: normal-tension glaucoma; JOAG: juvenile-onset POAG; LOAG: late-onset POAG.

6.1.2 Genetic and other risk factors underlying HTG and NTG

In this study, different association patterns were found between HTG and NTG. First, SNP *TLR4* rs7037117 is associated with HTG but not NTG. Second, rs1533428 at 2p16.3 is associated strongly with HTG but moderately with NTG. Third, at the *CAV1/CAV2* locus, the risk SNP rs4236601 is more strongly associated with HTG, while the protective SNPs rs6975771 and rs959173 are more strongly associated with NTG. These findings suggest that different genetic factors may contribute predominately to a certain subtype of POAG, and that the effect size of a gene may be modified by other risk factors, such as IOP. Conceivably, earlier intervention for the risk-allele carriers who have ocular hypertension but not yet developed glaucoma should be beneficial for preventing or postponing the development of the disease.

6.1.3 Ethnic diversities in POAG genetics

In this study, discrepant genetic profiles were found between Chinese and populations in initial reports. In the evaluation of multiple gene SNPs, we found that the association patterns of all SNPs were different. No significant association was found with SNPs around *CYP46A1*, *LMX1B*, *PLXDC2*, *TMTC2* and *ZP4*. *TLR4* rs7037117 showed an association in a different (recessive) genetic model. The two SNPs at 2p16.3, i.e., rs1533428 and rs12994401, showed different LD and association patterns compared with the Barbados population. Of the SNPs at the *CAV1/CAV2* locus, the allele frequencies and effect sizes in Chinese are different from that in Caucasians. In *NTF4*, new putative mutations were found, whereas variants that are common in Caucasians are absent in Chinese, suggesting ethnic diversities in its mutation spectrums. Thus, exhaustive investigation of the genetics of POAG in different populations will facilitate the unraveling of ethnic diversities in its genetic architectures and the identification of population-specific genes for POAG.

6.2 Future researches on glaucoma molecular genetics

The works in this thesis not only emphasize genetic heterogeneity and complexity behind the pathogenesis of POAG, but also open new avenues for glaucoma research.

6.2.1 More genes to identify

In this study, two candidate genes, *CNTF* and *SPARC*, were excluded as glaucoma genes, suggesting that genes that are biologically relevant to glaucoma are not necessarily genetic components of the disease. With the exclusion of *SPARC*, the causal gene at GLC1M remains to be identified. At the GLC1N locus, although our data strongly suggest *MEGF11* as the causal gene, the remaining genes at this locus should be screened out to confirm its role. Furthermore, mutations in known genes, including *MEGF11* identified in this study, account for only a small fraction of overall POAG. Therefore, further large-scale sequence analyses are warranted to identify new genes for POAG. With the advent of the next-generation sequencing platform, sequencing capacity has been greatly enhanced. Moreover, exome-sequencing has been used successfully in pinpointing the genes for some Mendelian disorders, such as Freeman-Sheldon syndrome (Ng et al. 2009) and Miller syndrome (Ng et al. 2010). Such technologies will speed up the identification of POAG genes. In particular, to apply the high-throughput sequencing technology in the screening of all candidate genes at the linkage loci will facilitate the discovery of the causal genes.

Regarding susceptibility genes, the *TLR4* rs7037117 higher-risk genotype GG has a PAR of 6.42% for HTG. The risk genotype AG of rs4236601 at the *CAV1/CAV2* gene region has a PAR of 2.71% for HTG and 0.87% for NTG. The TT+CT genotypes at rs1533428 have a PAR of 42.7% and 30.3% for late-onset HTG and NTG, respectively. These findings suggest that, while the three susceptibility genes altogether may explain a large portion of genetic risk of POAG, other associated genes are yet to be identified. The

genome-wide association approach is powerful in the identification of common susceptibility variants for complex diseases. The GWA study in Caucasians has identified rs4236601 at *CAV1/CAV2* for POAG. Further GWA studies in POAG will probably lead to the identification of new susceptibility genetic markers for the disease.

6.2.2 Unraveling the roles of the genes and mutations

In this study a putative gene, *MEGF11*, for POAG was identified, supported by the identification of a group of putative mutations, including c.1090delT, IVS17+2insT, IVS17-2A>G, IVS17-4C>G and c.2472A>C. Two putative mutations Gly157Ala and Ala182Val were detected in *NTF4*. However, it is premature to conclude that *MEGF11* and *NTF4* are causative genes for POAG in a lack of functional investigation. Moreover, the gene tagged by rs1533428 at 2p16.3 remains unannotated. Therefore, functional characterizations (e.g., gene expression profiles in the eye, normal properties of the proteins, mutation analysis, generation of transgenic animal models, etc.) of the genes and variants are warranted to unravel their biological implication in the disease, and to identify the disease pathways in which they are involved. This in return will lead to the identification of more disease genes in the same or related pathways, further elucidating the genetic architecture of POAG.

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Electronic-Database Information

The URLs for data and tools in this thesis are as following:

Alternative Splicing Database	http://www.ebi.ac.uk/asd-srv/wb.cgi/
Automated Splice Site Analyses	https://splice.uwo.ca/
BioGPS	http://biogps.gnf.org/
dbSNP database	http://www.ncbi.nlm.nih.gov/projects/SNP/
Ensembl genome browser	http://www.ensembl.org/Homo_sapiens/
GLIDERS	http://mather.well.ox.ac.uk/GLIDERS/
Haploview	http://www.broadinstitute.org/haploview/
International HapMap project	http://hapmap.ncbi.nlm.nih.gov/
NCBI Map Viewer	http://www.ncbi.nlm.nih.gov/projects/mapview/
Online Mendelian Inheritance in Man	http://www.ncbi.nlm.nih.gov/omim/
PLINK	http://pngu.mgh.harvard.edu/purcell/plink/
PolyPhen	http://genetics.bwh.harvard.edu/pph/
Primer3	http://frodo.wi.mit.edu/primer3/
SIFT	http://sift.jcvi.org/
SNPSpD	http://gump.qimr.edu.au/general/daleN/SNPSpD/
WHAP	http://pngu.mgh.harvard.edu/~purcell/whap/