TABLE 3. Haplotype Frequencies of Tag Single Nucleotide Polymorphisms of the Toll-like Receptor 4 Gene Compared with Previous Study

	This Study										Previous Study			
Tag SNPs rs10759930, rs11536889, rs7037117, and rs7045953	POAG (n = 184)	*P* Value	Overall *P* Value (POAG)	NTG (n = 365)	*P* Value	Overall *P* Value (NTG)	XFG (n = 109)	*P* Value	Overall *P* Value (XFG)	Control (n = 216)	NTG (n = 250)	Control (n=318)	*P* Value	Overall *P* Value (NTG)
TGAA	0.311	.000072	.00097	0.360	.003	.057	0.362	.036	0.134	0.448	0.350	0.402	.070	.044
TCAA	0.228	.882		0.242	.465		0.229	.863		0.223	0.226	0.247	.41	
CGAA	0.208	.030		0.164	.519		0.173	.439		0.150	0.166	0.159	.75	
CGGA	0.125	.406		0.137	.126		0.146	.141		0.107	0.154	0.102	.0090	
CGGG	0.090	.080		0.074	.280		0.067	.664		0.058	0.096	0.077	.26	
Tag SNPs rs10759930 and rs7037117														
TA	0.539	.00014	.0017	0.603	.020	.085	0.591	.044	0.201	0.674	0.575	0.649	.0044	.010
CG	0.216	.063		0.219	.023		0.219	.086		0.164	0.249	0.179	.21	
CA	0.238	.0073		0.178	.524		0.185	.479		0.162	0.173	0.169		

NTG = normal-tension glaucoma; POAG = primary open-angle glaucoma; SNP = single nucleotide polymorphism; XFG = exfoliation glaucoma.

TABLE 4. Haplotype Frequencies of Tag Single Nucleotide Polymorphisms of the Toll-like Receptor 4 Gene Between Primary Open-Angle, Normal-Tension, and Exfoliation Glaucoma and Control Subjects

Tag SNPs rs10759930, rs1927914, rs1927911, and rs2149356	POAG (n = 184)	*P* Value	Overall *P* (POAG)	NTG (n = 365)	*P* Value	Overall *P* (NTG)	XFG (n = 109)	*P* Value	Overall *P* (XFG)	Control (n = 216)
TTCC	0.516	.000014	.00078	0.589	.009	0.018	0.573	.020	.014	.667
CCTA	0.418	.00033		0.384	.003		0.395	.012		.296
CCCC	0.005	.138		0.008	.211		NA	NA		.016

NTG = normal-tension glaucoma; POAG = primary open-angle glaucoma; XFG = exfoliation glaucoma.

rs7045953) were studied earlier.[21] The results showed that NTG and XFG were not statistically significant (overall $P = .057$, $P = .134$), but POAG was statistically significant (overall $P = .00097$; Table 3). The tag SNPs, rs10759930 and rs7037117, used in that study were similar with these haplotypes (Table 3).

Other haplotypes, rs10759930, rs1927914, rs1927911, and rs2149356, had higher statistical significance (overall $P = .00078$ in POAG; overall $P = .018$ in NTG, and overall $P = .014$ in XFG; Table 4).

DISCUSSION

• **TOLL-LIKE RECEPTOR 4 POLYMORPHISMS IN PRIMARY OPEN-ANGLE GLAUCOMA, NORMAL-TENSION GLAUCOMA, AND EXFOLIATION GLAUCOMA SUBJECTS:** Shibuya and associates showed that rs7037117, located in the 3′-untranslated region of *TLR4*, was most strongly associated with NTG.[21] Compared to earlier reports, the intragenic SNP rs2149356 could be more associated with NTG and also with POAG and XFG in this study. The statistics of all 8 genotypes showed that *TLR4* had approximately the same tendency for all corresponded allele frequencies (Table 2). The haplotypes rs10759930, rs1927914, rs1927911, and rs2149356 had the higher statistically significant values in both groups (overall $P = .00078$, $P = .018$, and $P = .014$, respectively; Table 4). On the other hand, this haplotype was shown to be not significant, and even in the original study,[21] it was statistically marginal ($P = .044$ for 4 SNPs and $P = .010$ for 2 SNPs). Thus, these haplotypes and/or SNPs are valuable for screening for glaucoma in the Japanese.

Subjects enrolled in this study and those reported by Shibuya and associates[21] were from across Japan; however, the subjects from our study were predominantly from northern Japan. The difference in the heterogeneity may explain the slight differences between the 2 studies. An association between the SNPs and POAG and XFG was not expected before this study because the IOP has a predominant effect on these diseases. So it is interesting that *TLR4* would be associated with those phenotypes of POAG and XFG, and the risk associations were stronger in POAG than in NTG. Recently, Suh, and associates showed that *TLR4* gene polymorphisms do not associate significantly with NTG in a Korean population,[22] but they did not examine it in POAG and XFG subjects. It should be evaluated in various types of glaucoma in different populations.

• **FUNCTION OF *TLR4* GENE:** Innate immunity produces antimicrobial peptides against many kinds of pathogens in the host defense system, and these induce adaptive immunity secondarily. Together, they play important roles in the total immune system.[24] Targeting TLR signaling has implications in the control of infection, vaccine design, desensitization to allergens, and downregulation of inflammation. *TLR4*-deficient mice were reported to have an upregulation of NADPH oxidase (Nox3), which increased the oxidative stress.[25] Although the function of the 8 SNPs on the *TLR4* gene was not examined, rs10759930 and rs1927914 exist within the 5′ untranslated region, rs1927911, rs12377632, and rs2149356 exist within introns, and rs11536889, rs7037117, and rs7045953 exist within the 3′ untranslated region. There is a possibility that these SNPs influence the stability of the mRNA and expression of the *TLR4* gene because rs11536889 exists near exon3.

TLR4 is expressed in the conjunctiva, cornea, iris, ciliary body, choroid, retina, and retinal pigment epithelium. In the retina, changes in the glial cells may be associated with glaucoma, especially NTG, which is not so dependent on the IOP. Widespread chronic stress is evident in the retina and optic nerve head by the strong upregulation of the HSPs in glaucomatous eyes.[26] Recently, an upregulation of toll-like receptors TLR2, TLR3, and TLR4 was found in human glaucoma donor eyes, which is consistent with the strongly increased level of expression of HSPs.[27] Immunohistochemical analyses supported an upregulated expression of TLRs in both microglia and astrocytes in glaucomatous retinas. It has been postulated that changes in the microenvironment of injured axons will alter the glycosaminoglycan composition in the lamina cribrosa, and this may account for the increased vulnerability of the remaining axons to sustain further damage independent of the IOP.

The significance of these findings in POAG more than NTG raises further speculation. Chronic stress could influence the aqueous humor and may adversely affect the outflow structure to increase resistance to outflow, with alterations of the trabecular meshwork and intrascleral channels and collapse of the Schlemm canal. There is a possibility that *TLR4* might have an effect on the alterations of the aqueous humor dynamics and injury to the glaucomatous retina in eyes with POAG and XFG.

• **AUTOIMMUNE DISEASES, CHRONIC INFLAMMATION, AND GLAUCOMA:** To date, the genes of the TLR family have not been candidates as genetic modifiers of glaucoma susceptibility, but they have been implicated in other autoimmune diseases and allergic diseases, including rheumatoid arthritis[28,29] and bronchical asthma.[30,31] It is interesting that the net *TLR4* sequence variants and the *TLR4* signaling network would affect not only the development of NTG but also POAG and XFG. Chronic infection by certain bacteria and viruses may play a role in inflammation.[32] More specifically, chronic infection by *Helicobater pyori* may induce a persistent systemic and vascular inflammation and endothelial dysfunction.[33] The results of one study showed that the specific IgG antibody levels of *H. pylori* were significantly increased in the aqueous humor and serum of patients with POAG and

XFG.[34] In addition, the titer of *H. pylori* antibody in the aqueous humor might reflect the severity of glaucomatous damage in POAG patients. We hypothesized that some types of chronic infection and/or inflammation can lead to the development of glaucoma especially POAG.

In conclusion, we have identified *TLR4* SNPs as genetic susceptibility alleles for POAG, NTG, and XFG in the Japanese population. Our findings would support the idea that changes in the regulation of TLR signaling in human glaucoma may be associated with innate and adaptive immune responses. Further investigations on different ethnic populations, and on the structure and function of the *TLR4* protein, would be helpful in understanding the pathogenesis of POAG, NTG, and XFG.

ALL AUTHORS HAVE COMPLETED AND SUBMITTED THE ICMJE FORM FOR DISCLOSURE OF POTENTIAL CONFLICTS OF interest and none were reported. This study was supported in part by a Grant-In-Aid for Scientific Research from the Ministry of Education, Science, and Culture of the Japanese Government (NF; C-22591928), by grant from the Ministry of Health, Labor and Welfare of Japan to N.F., and by a grant from the Japan-China Medical Association, Japan, to D.S. Involved in conception and design of the study (Y.T., D.S., Y.M., N.F.); data collection (Y.T., D.S., Ai.S., To.F., N.Y., Ta.F., H.A., H.I., X.Z., At.S., Y.O., K.N.), analysis (Y.T., D.S., N.F.), and interpretation of data (T.Y., T.N., N.F.); and preparation, review, and approval of the manuscript (K.N., T.N., N.F.). The purpose and procedures of the experiment were explained to all patients, and informed consent was obtained. The procedures used conformed to the tenets of the Declaration of Helsinki, and this study was approved by the Tohoku University Institutional Review Board prospectively. Informed consent for participation in this research was obtained from all patients.

Y. Takano and D. Shi contributed equally to this work.

The authors thank Duco Hamasaki, Professor Emeritus, Bascom Palmer Eye Institute, University of Miami, Miami, Florida, for his critical comments and valuable assistance.

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SUPPLEMENTAL TABLE. Primer Sequences for Toll-like Receptor 4 Gene Amplification Used in This Study

	Forward Primer	Reverse Primer	Annealing Temperature(C)
rs10759930	gtacagggtgtttgggagga	catggaccaatgctcttgtg	63
rs1927914	tgatgaggattgaaaatgtgga	acaaaatggtccctcacagc	60
rs1927911	ttaaatactccatatcattggggagac	gagagcattcagaaattagatgg	62
rs12377632	tggtatttggctttctgttcc	aaggtttctggggcaagttt	56
rs2149356	ccttggatcaagtttagccatt	tttccacaaaactcgctcct	60
rs11536889	ccctgtacccttctcactgc	gtttctgaggaggctggatg	62
rs7037117	ttaacccttcccacctttc	agagtttgggacctgctcaa	60
rs7045953	ttcccatgttccctcatttc	ggggcaaaagagaaactcct	59

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PLOS ONE

Association of *HK2* and *NCK2* with Normal Tension Glaucoma in the Japanese Population

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Abstract

Although family studies and genome-wide association studies have shown that genetic factors play a role in glaucoma, it has been difficult to identify the specific genetic variants involved. We tested 669 single nucleotide polymorphisms (SNPs) from the region of chromosome 2 that includes the GLC1B glaucoma locus for association with primary open-angle glaucoma (POAG) and normal tension glaucoma (NTG) in the Japanese population. We performed a two-stage case-control study. The first cohort consisted of 123 POAG cases, 121 NTG cases and 120 controls: the second cohort consisted of 187 POAG cases, 286 NTG cases, and 271 controls. Out of six SNPs showing significant association with POAG in the first round screening, seven SNPs were tested in the second round. Rs678350 in the *HK2* gene coding sequence showed significant allelic ($p = 0.0027$ in Stage Two, 2.7XE-4 in meta-analysis) association with POAG, and significant allelic ($p = 4.7$XE-4 in Stage Two, 1.0XE-5 in meta-analysis) association with NTG. Although alleles in the *TMEM182* gene did not show significant association with glaucoma in the second round, subjects with the A/A allele in *TMEM182* rs869833 showed worse visual field mean deviation ($p = 0.01$). Even though rs2033008 in the *NCK2* gene coding sequence did not show significant association in the first round, it had previously shown association with NTG so it was tested for association with NTG in round 2 ($p = 0.0053$ in Stage Two). Immunohistochemistry showed that both *HK2* and *NCK2* are expressed in the retinal ganglion cell layer. Once multi-testing was taken into account, only *HK2* showed significant association with POAG and NTG in Stage Two. Our data also support previous reports of *NCK2* association with NTG, and raise questions about what role *TMEM182* might play in phenotypic variability. Our data suggest that *HK2* may play an important role in NTG in the Japanese population.

Citation: Shi D, Funayama T, Mashima Y, Takano Y, Shimizu A, et al. (2013) Association of *HK2* and *NCK2* with Normal Tension Glaucoma in the Japanese Population. PLoS ONE 8(1): e54115. doi:10.1371/journal.pone.0054115

Editor: Rajiv R. Mohan, University of Missouri-Columbia, United States of America

Received August 16, 2012; **Accepted** December 6, 2012; **Published** January 22, 2013

Funding: This study was supported in part by a Grant-In-Aid for Scientific Research from the Ministry of Education, Science, and Culture of the Japanese Government (C-22591928), Tokyo, Japan (NF); by a grant from the Ministry of Health, Labor and Welfare of Japan (NF); by Tohoku Medical Megabank Project (NF); by a grant from the Japan-China Medical Association (DS); by a grant R01-EY011671 from the National Eye Institute at the National Institutes of Health (JER), Bethesda, MD, USA; and a grant from Research to Prevent Blindness, New York, NY, USA (JER). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Glaucoma is a complex, heterogeneous disease characterized by a progressive degeneration of the optic nerve fibers, and is the second highest cause of blindness worldwide affecting approximately 70 million people [1]. The most common type of open-angle glaucoma, primary open-angle glaucoma (POAG), is associated with elevated intraocular pressure (IOP) [2], and another less-common subgroup of open-angle glaucoma, called low-tension glaucoma (LTG) [3,4] or normal tension glaucoma (NTG) is associated with IOP that does not rise outside of the normal range [5]. The prevalence of NTG is reported to be higher among the Japanese than among Caucasians [6,7]. This is an important medical and public health problem because simple screening programs based on detection of elevated IOP are not effective in a population where NTG is highly prevalent. Thus, an accurate diagnostic test for presymptomatic detection of individuals at risk for glaucoma, especially NTG in Japan, is urgently needed.

Open-angle glaucoma is a genetically heterogeneous disorder attributed to the interaction of multiple genes and environmental factors [8,9]. More than 15 POAG loci have been identified by linkage, and five open-angle glaucoma genes located within those loci have been identified [10,11]. More recently, genome-wide association studies (GWAS) using high-density single nucleotide polymorphism (SNP) arrays have been used to identify genetic risk factors involved in the common, complex forms of open-angle glaucoma that do not show classical Mendelian inheritance

patterns. Burdon et al. identified susceptibility loci at TMCO1 and CDKN2B-AS1 that contribute to severe forms of glaucoma [12]. Ramdas et al. used meta-analysis of data from six separate studies to find significant evidence that three common variants of CDKN2B, ATOH7 and SIX1 are associated with POAG [13]. Wiggs et al. found significant evidence that genetic variants in CDKN2B-AS1 and a gene desert on 8q22 are associated with optic nerve damage in glaucoma [14].

Based on a linkage study involving 6 Caucasian families in the UK, the *GLC1B* locus for adult-onset open-angle glaucoma was identified at chromosome 2cen–q13 [15]. The patients in these families had clinical characteristics of low to moderate IOP, disease onset in their late 40 s, and a good response to medical therapy, and those phenotypes mimic the majority of Japanese NTG cases. Thus, the screening of the gene around GLC1B locus may be useful for diagnosis of POAG and NTG in the general Japanese population.

The purpose of this study was to screen for candidate genes for POAG and NTG on chromosome 2, around the GLC1B (glaucoma 1, open angle, B) locus in unrelated Japanese patients, using high density SNP scanning and case-control association. Here we report one gene that shows significant association with POAG and NTG, support for a previously reported association with NTG, and a gene for which genotype is predictive of severity of mean deviation on the visual field test.

Results

A Two-stage Case-control Study of SNPs on Chromosome2

To identify a gene associated with glaucoma we did a high-density scan of the region around GLC1B on chromosome 2 by screening 669 SNPs on chromosome 2 in a two-stage case-control study design (Figure 1). We were especially interested in whether any SNPs that fall within genes in the GLC1B region might be associated with POAG or NTG (Table 1). Among genes from this region we found fourteen SNPs that show significant evidence of association with POAG, and nine SNPs that show significant evidence of association with NTG. Four of the SNP alleles which show significant evidence of association are identical between the POAG and NTG subjects.

We identified six SNPs in Stage One that showed evidence of association with POAG (rs1239066, rs1529385, rs869833, rs960011, rs1027003) and two SNPs (rs678350, rs2033008) that showed association with NTG (Figure 2 and Table 2). The SNPs rs869833 and rs960011 are located within the *TMEM182* gene, which contains 5 exons and 229 amino acids. The amino acid sequence of *TMEM182* predicts an evolutionarily-conserved novel transmembrane protein, which consists of four putative membrane-spanning regions indicative of an integral membrane topology. The SNP rs678350 is located within the *Hexokinase 2 (HK2)* gene which contains 18 exons and 917 amino acids. The SNP rs678350 exists on intron1 of *HK2* gene. The *HK2* gene produces a protein product localizes on the outer membrane of mitochondria and plays an important role in intracellular glucose metabolism by catalyzing the conversion of glucose to glucose-6-phosphate. There are no known genes closely neighboring the SNP rs1239066 and rs1027003. We have checked the SNPs near rs1529385 within LOC129293 and TMSB10 and did not find get any positive polymorphisms.

We selected three genes for second stage mutation screening. *TMEM182* and *HK2* genes were selected because they contain SNPs that showed significant evidence of association in Stage One in this study. Even though SNPs in the *NCK2* gene showed significant evidence of association ($p = 0.014$), the *NCK2* gene was selected for second stage mutation screening based of the previous report that it is associated with NTG [16].

The SNP rs1239066 showed significant evidence of association in both Stage One and Stage Two screenings. In the meta-analysis it showed significant evidence of association (POAG, NTG; $P = 0.001$, 0.005), as did rs1027003 (POAG; $P = 0,010$) (Table 2).

HK2 Variants Detected in this Study

The SNP rs678350 in the *HK2* gene coding sequence showed significant allelic ($p = 0.0027$ in Stage Two, 2.7XE-4 in meta-analysis) association with POAG, and significant allelic ($p = 4.7$XE-4 in Stage Two, 1.0XE-5 in meta-analysis) association

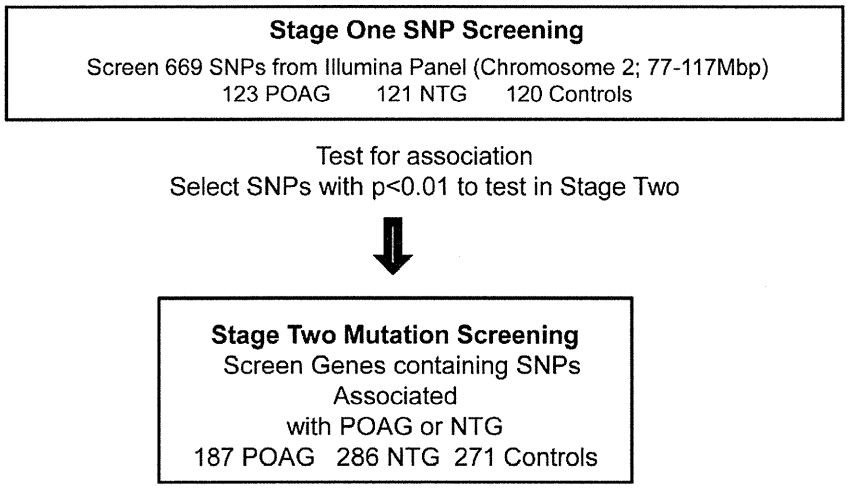

Figure 1. Experimental Study Design. The study used a first stage of SNP screening in one population to identify SNPs and genes to be tested in a second population through SNP association testing and mutation screening of genes containing SNPs associated with glaucoma. Stage Two tested SNPs for association in a second population and did mutation screening in that second population in genes containing SNPs that showed significant association with POAG or NTG ($p<0.01$) in Stage One of our study or that had been previously reported to show significant evidence of association [16].
doi:10.1371/journal.pone.0054115.g001

Table 1. Stage one Test of SNPs in GLC1B-Region Genes for Association with POAG or NTG.

Genomic Information			POAG			NTG			Control
rs number	Location	Gene Symbol	MAF	Odds ratio (CI)	p value*	MAF	Odds ratio (CI)	p value*	MAF
rs741788	2p13	*DCTN1*	0.455	1.47 (1.02–2.12)	0.038	0.455	1.47 (1.02–2.11)	0.040	0.363
rs909177		*DCTN1*	0.455	1.44 (1.00–2.08)	0.047	0.463	1.49 (1.03–2.14)	0.032	0.367
rs740277		*DCTN1*	0.455	1.44 (1.00–2.08)	0.047	0.463	1.49 (1.03–2.14)	0.032	0.367
rs678350	2p13	*HK2*	0.333	1.50 (1.01–2.23)	0.043	0.371	1.77 (1.19–2.62)	0.004	0.250
rs651071		*HK2*	0.199	0.60 (0.40–0.92)	0.018	0.256	0.84 (0.56–1.25)	0.383	0.292
rs1807090		*HK2*	0.268	1.59 (1.03–2.44)	0.034	0.238	1.35 (0.87–2.10)	0.181	0.188
rs1239066	2p12		0.293	3.04 (1.95–4.72)	0.009	0.269	1.55 (1.01–2.38)	0.045	0.192
rs53915	2p12-p11.1	*CTNNA2*	0.199	0.64 (0.42–0.98)	0.039	0.260	0.91 (0.61–1.36)	0.641	0.279
rs1529385	2p11.2	*LOC129293 (C2orf89)*	0.053	0.39 (0.20–0.77)	0.005	0.087	0.66 (0.37–1.20)	0.173	0.125
rs1053561	2p11.2	*TGOLN2*	0.114	0.53 (0.32–0.88)	0.012	0.178	0.89 (0.56–1.40)	0.609	0.196
rs1562322	2p11.2	*LOC51255 (RNF181)*	0.321	0.88 (0.61–1.29)	0.520	0.256	0.64 (0.43–0.95)	0.027	0.349
rs3024831	2p12-p11.2	*SFTPB*	0.250	0.92 (0.61–1.38)	0.675	0.190	0.65 (0.42–0.99)	0.045	0.267
rs6875	2q11.2	*RW1 (TMEM131)*	0.008	0.21 (0.05–0.98)	0.030	0.025	0.66 (0.23–1.88)	0.431	0.038
rs1982336		*RW1 (TMEM131)*	0.008	0.23 (0.05–1.05)	0.027	0.029	0.75 (0.28–2.05)	0.576	0.038
rs718159		*RW1 (TMEM131)*	0.008	0.21 (0.05–0.98)	0.028	0.029	0.76 (0.28–2.07)	0.587	0.038
rs222	2q11.2	*INPP4A*	0.183	0.69 (0.44–1.06)	0.091	0.161	0.59 (0.38–0.93)	0.021	0.246
rs1530028	2q11.2	*FLJ45273 (LONRF2)*	0.228	0.66 (0.44–0.99)	0.045	0.306	0.99 (0.67–1.46)	0.952	0.308
rs1030902	2q11.2	*ALS2*	0.225	0.65 (0.44–0.98)	0.039	0.314	1.03 (0.70–1.51)	0.892	0.308
rs1369482	2q11.2	*NPAS2*	0.244	0.65 (0.43–0.96)	0.030	0.298	0.85 (0.58–1.24)	0.398	0.333
rs871656	2q12	*IL1R1*	0.337	0.70 (0.49–1.01)	0.058	0.322	0.66 (0.45–0.95)	0.025	0.421
rs878539	2q11.2	*SLC9A2 (NHE2)*	0.463	1.52 (1.06–2.18)	0.024	0.422	1.28 (0.89–1.85)	0.185	0.363
rs869833	2q12.1	*TMEM182*	0.467	1.66 (1.15–2.40)	0.006	0.376	1.14 (0.79–1.65)	0.490	0.346
rs960011		*TMEM182*	0.415	0.57 (0.40–0.82)	0.001	0.512	0.85 (0.60–1.21)	0.232	0.554**
rs2033008	2q12	*NCK2*	0.293	0.76 (0.51–1.11)	0.147	0.252	0.62 (0.42–0.91)	0.015	0.354
rs1027003	2q12		0.110	3.16 (1.46–6.88)	0.002	0.058	1.58 (0.67–3.71)	0.295	0.038
rs1474220	2q12.3	*GCC2*	0.106	0.59 (0.35–1.00)	0.050	0.136	0.79 (0.48–1.30)	0.353	0.167
rs899259	2q13	*EDAR*	0.098	0.66 (0.38–1.14)	0.134	0.075	0.49 (0.28–0.90)	0.019	0.142
rs1509414	2q13	*BENE(MALL)*	0.037	0.40 (0.18–0.88)	0.020	0.041	0.45 (0.21–0.98)	0.039	0.096
rs1567366	2q13	*NPHP1*	0.561***	1.34 (0.94–1.92)	0.105	0.576***	1.44 (1.01–2.07)	0.045	0.488
rs2119112	2q14.2	*MARCO*	0.110	0.57 (0.34–0.95)	0.029	0.107	0.55 (0.33–0.93)	0.025	0.179

*chi-square test.
**minor allele frequency in stage 2 control was 0.494.
***minor allele in control was major allele in POAG and NTG subjects.
MAF; minor allele frequency, CI; confidence interval.
doi:10.1371/journal.pone.0054115.t001

with NTG (Table 2). The rs678350 showed also a significant difference in genotype frequency (p = 0.0046 and 0.0039) in the POAG and NTG groups (Table 3). In the second round, we screened the *HK2* coding sequence and intron-exon boundaries for mutations in POAG and NTG patients. After direct sequencing, we found 2 coding SNPs; p.Gln142His (A/T at the third nucleotide; rs2229621) in exon 4 and p.Arg844Lys (G/A at the second nucleotide; rs2229629) in exon 17. The allelic frequency of the p.Gln142His (A/T) variant was significantly higher in the NTG group than in the control group (p = 0.025), but it was not higher in the POAG group than in the control group (p = 0.181). The genotype frequency of the p.Gln142His (A/T) variant (dominant model) was significantly higher in the NTG group than in the control group (p = 0.019) but we did not find evidence that the frequency in the POAG group was different from the control group (p = 0.179). There was no evidence of a significant difference between POAG and NTG for p.Arg844Lys. No other mutation was found. We tested the LD block and found no linkage disequilibrium between SNPs rs678350 and rs2229621 (D′ = 0.08). We tested the correlation between the phenotypes POAG or NTG and the genotypes screened in the second stage screening of HK2, and found no association with any phenotypes including age at diagnosis, maximum IOP under medication, and MD value of the visual field (Table 4). None of the polymorphisms showed deviation from Hardy–Weinberg equilibrium ($P<0.05$).

NCK2 Variants Detected in this Study

The SNP rs2033008 in the *NCK2* gene showed a significant difference in allelic frequency (p = 0.015 in Stage One, p = 0.0053 in Stage Two, and 2.2XE-4 in meta-analysis) between controls and NTG, but not between control and POAG status (p = 0.147 in Stage One, 0.35 in Stage Two, 0.12 in meta-analysis) (Table 2 and

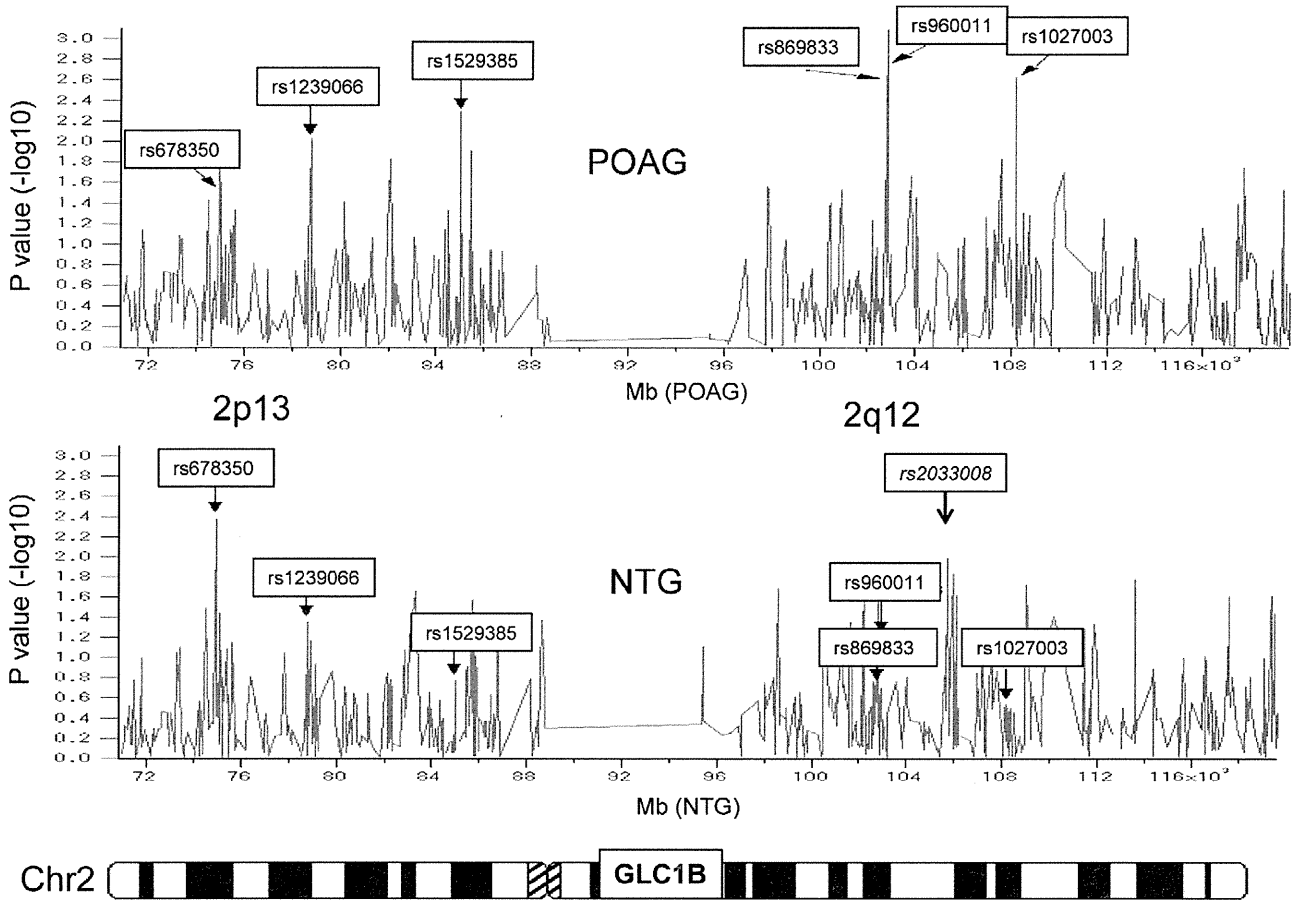

Figure 2. High-density scan of the GLC1B region on chromosome 2 to identify candidate glaucoma genes. Six SNPs that showed significant evidence of association with POAG or NTG ($p<0.01$) and the previously-reported candidate *NCK2* gene are shown. Vertical line shows p value (−log10), and horizontal line shows chromosomal location (kb).
doi:10.1371/journal.pone.0054115.g002

Table 5). The odds ratio for association with NTG supports a model in which *NCK2* is associated with NTG; OR = 0.69 (0.53–0.89), but the odds ratio for association with POAG was not significant; 0.87 (0.65–1.16) in Stage Two. Only this rs2033008 polymorphism in Stage One showed deviation from Hardy–Weinberg equilibrium ($p = 0.030$).

We screened the sequence of the *NCK2* coding sequence and intron-exon boundaries for mutations in POAG and NTG patients and found 1 synonymous coding base change: Thr14Thr (ACC >ACT) in one NTG subject. Although SNP rs2033008 showed significant association but the Thr14Thr variant showed no statistical difference in allele frequency between NTG and normal subjects ($p = 0.33$). The Thr14Thr heterozygotes (A/A) in the NTG subjects have the worse Mean Deviation value of the visual field compared with human reference sequence (T/T) ($p = 0.05$) (Table 6).

The single NTG subject with the Thr14Thr variant was a 50 year old woman whose father also had NTG. Her age at diagnosis was 40 years old. Her initial IOP was 15 mmHg in each eye. The mean deviation (MD) of the visual field test was −3.87 dB in the right eye, and −1.97 dB in the left eye, reflecting a mild NTG phenotype.

TMEM182 Variants Detected in this Study

We found no mutations in *TMEM182* coding sequence and intron-exon boundaries, for mutations in POAG and NTG patients. After finding association for SNPs rs869833 and rs960011 in the POAG and NTG subjects in Stage One, we did not find this association confirmed in Stage Two SNP testing (Table 7). When we tested for correlation between Stage Two case endophenotypes and TMEM182 genotype, we found association with the MD value of the visual field in POAG subjects. The POAG subjects homozygous for the A/A allele of SNP rs869833 have worse Mean Deviation value of the visual field test compared with those who carry the G/G genotype in Stage Two subjects ($p = 0.01$) (Table 8).

Immunohistochemistory of the *HK2* and *NCK2*

Representative immunohistochemistry (IHC) photographs with *Hk2*, *Nck2*, astrocyte marker (GFAP) and retinal ganglion cell marker (C38) on the retinas of untreated mice were shown (Fig. 3, A, B). *Hk2* and *Nck2* were strongly immunoreactive in the ganglion cell layer. C38 signals co-localized with *Hk2* or *Nck2* in the ganglion cell layer, as indicated by arrows (Fig. 3 A, B). *Hk2* expression is only located in the ganglion cell layer. *Nck* is expressed in the ganglion cell layer, inner nuclear layer, and outer plexiform layer, with the highest level of expression in the ganglion cell layer.

Discussion

HK2 in POAG and NTG subjects

The rs678350 in the *HK2* gene coding sequence showed significant allelic ($p = 0.043$ in Stage One, $p = 0.0027$ in Stage

Table 2. Stage One and Stage Two Association Test Results.

Stage One Screening

		POAG			NTG			CNTL
SNP	**Minor Allele**	**MAF**	**Odds ratio**	**p value***	**MAF**	**Odds ratio**	**p value***	**MAF**
rs1239066	C	0.293	3.04 (1.95–4.72)	0.009	0.269	1.55 (1.01–2.38)	0.045	0.192
rs1529385	T	0.053	0.39 (0.20–0.77)	0.005	0.087	0.66 (0.37–1.20)	0.173	0.125
rs869833	G	0.467	1.66 (1.15–2.40)	0.006	0.376	1.14 (0.79–1.65)	0.490	0.346
rs960011	T	0.415	0.57 (0.40–0.82)	0.001	0.512	0.85 (0.60–1.21)	0.232	0.554
rs1027003	G	0.110	3.16 (1.46–6.88)	0.002	0.058	1.58 (0.67–3.71)	0.295	0.038
rs678350	G	0.333	1.50 (1.01–2.23)	0.043	0.371	1.77 (1.19–2.62)	0.004	0.250
rs2033008	A	0.293	0.76 (0.51–1.11)	0.147	0.252	0.62 (0.42–0.91)	0.015	0.354

Stage Two Screening

		POAG			NTG			CNTL
SNP	**Minor Allele**	**MAF**	**Odds ratio**	**p value***	**MAF**	**Odds ratio**	**p value***	**MAF**
rs1239066	C	0.246	1.70 (1.09–2.65)	0.019	0.240	1.64 (1.06–2.51)	0.024	0.161
rs1529385	T	0.122	1.42 (0.73–2.77)	0.305	0.072	0.79 (0.37–1.68)	0.543	0.089
rs869833	G	0.409	0.98 (0.75–1.28)	0.855	0.421	1.03 (0.81–1.30)	0.834	0.415
rs960011	T	0.497	1.13 (0.86–1.47)	0.363	0.481	1.06 (0.84–1.34)	0.641	0.467
rs1027003	G	0.049	1.10 (0.42–2.90)	0.840	0.047	1.04 (0.30–2.83)	0.944	0.045
rs678350	G	0.334	1.58 (1.18–2.11)	0.0027	0.337	1.60 (1.23–2.08)	4.7XE-4	0.242
rs2033008	A	0.297	0.87 (0.65–1.16)	0.348	0.250	0.69 (0.53–0.89)	0.0053	0.327

Meta-analysis

		POAG			NTG			CNTL
SNP	**Minor Allele**	**MAF**	**Odds ratio**	**p value***	**MAF**	**Odds ratio**	**p value***	**MAF**
rs1239066	C	0.264	1.65 (1.22–2.23)	0.001	0.248	1.52 (1.14–2.04)	0.005	0.178
rs1529385	T	0.082	0.73 (0.46–1.16)	0.20	0.081	0.71 (0.45–1.14)	0.16	0.109
rs869833	G	0.432	1.17 (0.95–1.45)	0.16	0.408	1.06 (0.87–1.30)	0.54	0.393
rs960011	T	0.465	0.89 (0.72–1.10)	0.28	0.490	0.99 (0.81–1.20)	0.92	0.494
rs1027003	G	0.084	2.17 (1.20–3.92)	0.010	0.053	1.32 (0.69–2.53)	0.42	0.041
rs678350	G	0.334	1.56 (1.23–1.96)	2.7XE-4	0.347	1.65 (1.32–2.05)	1.0XE-5	0.244
rs2033008	A	0.295	0.83 (0.66–1.04)	0.12	0.251	0.66 (0.53–0.82)	2.2XE-4	0.335

MAF; Minor allele frequency, CNTL; Control.
*Fisher's exact test.
doi:10.1371/journal.pone.0054115.t002

Table 3. Stage Two *HK2* SNPs Allele Frequencies in Japanese POAG, NTG and Control Subjects.

	Allele frequency				Genotype			
rs678350	**A**	**G**	**Odds ratio (CI)**	**p value***	**A/A**	**A/G**	**G/G**	**p value***
POAG	0.666	0.334	1.58 (1.18–2.11)	0.0027	82/187	85/187	20/187	0.0046
NTG	0.663	0.337	1.60 (1.23–2.08)	4.7XE-4	133/286	113/286	40/286	0.0039
Control	0.758	0.242			161/271	89/271	21/271	
	Allele frequency				**Genotype**			
rs2229621/Q142H	**A**	**T**	**Odds ratio (CI)**	**p value***	**A/A**	**A/T**	**T/T**	**p value***
POAG	0.738	0.262	1.24 (0.92–1.69)	0.181	102/187	72/187	13/187	0.179
NTG	0.719	0.281	1.36 (1.04–1.79)	0.025	146/286	119/286	21/286	0.019
Control	0.777	0.223			165/271	91/271	15/271	

*Fisher's exact test; dominant model.
G/G or T/T is mutant homozygote, A/G or A/T is heterozygote, and A/A is wild homozygote.
doi:10.1371/journal.pone.0054115.t003

Two, 2.7XE-4 in meta-analysis association with POAG, and significant allelic (p = 0.004 in Stage One, p = 4.7XE-4 in Stage Two, 1.0XE-5 in meta-analysis) association with NTG (Table 2). The rs678350 polymorphism showed a significant case-control difference in genotype frequency ($p = 0.0046$ and 0.0039) in the POAG and NTG groups (Table 3). However, there was no association of this SNP with glaucoma endophenotypes including age at diagnosis, maximum intra ocular pressure under medication, and MD value of the visual field. So the *HK2* gene may contribute to disease susceptibility to POAG and NTG, but may not account for all of the phenotypic variability between individuals whose glaucoma results from variants in this gene. Our association findings suggest that the *HK2* gene that contains this polymorphism might play a role in POAG and NTG in the Japanese population, but it remains to be seen whether rs678350 is actually causative, perhaps through altering transcription or splicing, or whether another allele(s) in this gene or its regulatory region might actually be causing the disease. There remains a possibility that the p.Gln142His (A/T) SNP in *HK2* may play a role in disease pathology, but our study can only show association, not causation. Because none of our subjects come from the original families used to map the GLC1B locus, we can only draw conclusions regarding the possible role of this gene in the Japanese population, but this finding raises questions about whether this could be the GLC1B gene.

The HK2 gene product plays an important role in intracellular glucose metabolism by catalyzing the conversion of glucose to glucose-6-phosphate. The *HK2* gene localizes to the outer membrane of mitochondria. Since reduced glucose-6-phosphate content in muscle has been demonstrated in pre-non-insulin-dependent diabetes mellitus (pre-NIDDM) and NIDDM subjects, *HK2* was investigated as a promising candidate gene for noninsulin-dependent diabetes mellitus (NIDDM; OMIM125853) [17,18]; however, those studies concluded that mutations of the *HK2* gene, including a common p.Gln142His polymorphism is not a major etiologic factor for NIDDM in the Finnish [17,18,19,20], British [19], and Danish [20] populations. In brain, mitochondrial-hexokinase activity plays a key antioxidant role protecting against oxidative stress (ROS) [21], and complements the classical antioxidant enzymes that protect against oxidative stress [22]. Hexokinase antagonizes the release of mitochondrial cytochrome C activation of Akt, which is recognized as a potent inhibitor of apoptosis. *HK2* is probably associated with an anti-oxidative reaction and inhibition of apoptosis through Bax/Bak-mediated cytochrome *c* release [23]. Leber's hereditary optic neuropathy (LHON) -associated mitochondrial DNA mutations were found in Japanese patients with POAG [24], so it is reasonable to consider a gene whose product plays a role in mitochondria as a candidate gene for other phenotypes involving optic neuropathy.

NCK2 genes in POAG and NTG subjects

The *NCK2* gene, which was previously reported to be associated with NTG [16], encodes a member of the NCK family of adaptor proteins, and the adaptor protein which associates with tyrosine-phosphorylated growth factor receptors of their cellular substrates. SH2/SH3 domain-containing adapter proteins, such as the NCK family, play a major role in regulating tyrosine kinase signaling [25]. Previously, microsatellite marker D2S176 within the GLC1B locus showed significant association with NTG in the Japanese population, and D2S176 is located 24 kb from the *NCK2* gene [16]. Brain-derived neurotrophic factor (BDNF) binds to and activates the TrkB tyrosine kinase receptor to regulate cell differentiation and survival in the nervous system. BDNF

Table 4. Correlation between the POAG or NTG Endophenotypes and *HK2* SNPs Screened in Stage Two.

Endophenotype	Age at diagnosis (y.o.)				Maximum IOP* (mmHg)				MD value of the visual field (dB)			
rs678350 genotype	**A/A**	**A/G**	**G/G**	**p value****	**A/A**	**A/G**	**G/G**	**p value****	**A/A**	**A/G**	**G/G**	**p value****
POAG	61.6	55.3	59.0	0.83	24.0	23.6	21.9	0.56	−14.82	−15.64	−11.94	0.53
NTG	57.5	54.9	56.9	0.99	17.1	16.0	17.6	0.64	−10.72	−12.10	−7.14	0.37
rs2229621 genotype	**A/A**	**A/T**	**T/T**	**p value****	**A/A**	**A/T**	**T/T**	**p value****	**A/A**	**A/T**	**T/T**	**p value****
POAG	58.5	58.0	55.5	0.93	24.4	22.9	25.5	0.94	−16.42	−11.94	−17.65	0.65
NTG	57.8	55.2	57.4	0.91	16.1	17.5	16.9	0.63	−11.93	−10.34	−11.16	0.95

*IOP; intraocular pressure (under medication).
**Dunnett's test.
G/G or T/T is mutant homozygote, A/G or A/T is heterozygote, and A/A is wild homozygote.
doi:10.1371/journal.pone.0054115.t004

stimulation promotes interaction of *Nck2* with TrkB in cortical neuron [26]. And BDNF signaling in glia is known to play important roles in neural protection and regeneration, particularly in conversion of Muller glia to photoreceptors [27]. In our study, it is interesting that the *NCK2* variant rs2033008 showed a significantly difference from the control population in the NTG group, where the disease pathology seems to be focused on the retinal ganglion cells and the optic nerve, but not in the POAG group, where a substantial disease component localizes to the anterior chamber of the eye (Table 5). Thus although our study falls short of achieving a level of significance needed to identify *NCK2* de novo as a glaucoma gene and this polymorphism showed deviation from Hardy–Weinberg equilibrium ($p = 0.030$ in Stage One), our data do support the prior finding of significant allele frequency differences between NTG cases and normal controls in the Japanese population [16]. It is unclear whether this deviation from Hardy-Weinberg equilibrium in Stage One might represent the absence of some alleles from this population because they are associated with diagnoses specifically excluded from this study, such as ocular hypertension.

Immunohistochemistory of the *Hk2* and *Nck2*

Although KH2 and NCK2 had previously been detected in retina, more precise localization to specific cell types is needed to begin understanding how the gene products might play a role in disease pathology. In the representative IHC photographs, antibody against *Hk2* was strongly immunoreactive in the ganglion cell layer (GCL). The *Hk2* protein localizes to the outer membrane of mitochondria, and interestingly the *Hk2* protein appears in the GCL. *Nck2*, which interacts with BDNF, is expressed in ganglion cell layer (GCL), inner nuclear layer (INL) and outer plexiform layer (OPL), and most expressed in GCL. This localization makes it highly conceivable that the *Hk2 gene products* could each play a role in glaucoma, and there is possibility that *Nck2* could have relationship with glaucoma.

TMEM182 in POAG and NTG

SNPs rs869833 and rs960011 in the TMEM182 gene showed significant association with POAG and NTG in Stage One that was not confirmed in the second stage. The primary amino acid sequence of *TMEM182* predicts an evolutionarily-conserved novel transmembrane protein, which consists of four putative membrane-spanning regions indicative of an integral membrane topology. The *TMEM182* protein sequence lacks homologies with previously-defined protein families. However, the pro-inflammatory cytokine TNFα down-regulates *TMEM182* transcript expression in adipocytes [28]. Its transcript expresses in white adipose tissues, heart, muscle, and lower relative levels of *TMEM182* transcript are found in kidney, testis, and brain. Identification of the intracellular signaling pathway involved in the TNFα-mediated decrease might be one clue offering insights into association between POAG and *TMEM182* function. Failure to confirm the association with POAG in the second stage could be attributable to clinical heterogeneity, but this result still needs to be confirmed in a second population. Nakano et al. demonstrated heterogeneity in the Japanese POAG population when their genome-wide association study of 1,575 Japanese POAG and normal subjects identified significant evidence of association with 6 SNPs on Chromosome 1, 10, 12 [29]. They did not report evidence of association with SNPs on Chromosome 2.

Table 5. Stage Two *NCK2* SNP rs2033008 Allele Frequencies in Japanese POAG, NTG and Controls Subjects.

	Allele frequency				Genotype			
	T	**A**	**Odds ratio (CI)**	**p value***	**T/T**	**T/A**	**A/A**	**p value****
POAG	0.703	0.297	0.87 (0.65–1.16)	0.35	89/187	85/187	13/187	0.069
NTG	0.750	0.250	0.69 (0.53–0.89)	0.0053	159/286	111/286	16/286	0.0056
Control	0.673	0.327			130/271	105/271	36/271	

*Fisher's exact test,
**Chi-square test.
A/A is mutant homozygote, T/A is heterozygote, and T/T is wild homozygote.
doi:10.1371/journal.pone.0054115.t005

Table 6. Correlation between the POAG or NTG Endophenotypes and *NCK2* SNP rs2033008 Screened in Stage Two.

Endophenotype	Age at diagnosis (y.o.)				Maximum IOP* (mmHg)				MD value of the visual field (dB)			
Genotype	T/T	T/A	A/A	p value**	T/T	T/A	A/A	p value**	T/T	T/A	A/A	p value**
POAG	55.8	61.0	54.2	0.94	23.7	24.2	25.0	0.87	−15.38	−14.94	−12.28	0.63
NTG	56.8	56.0	52.2	0.70	16.7	17.0	16.0	0.79	−9.83	−10.95	−16.28	0.05

*IOP; intraocular pressure (under medication).
**Dunnett's test.
A/A is mutant homozygote, T/A is heterozygote, and T/T is wild homozygote.
doi:10.1371/journal.pone.0054115.t006

Thus these SNPs on Chr2 might be the variants for which our study is not well-powered, or clinical heterogeneity might be complicating our ability to detect the association in our limited sample size.

In our data set, *HK2* shows the strongest evidence of association with NTG in the Japanese population out of all of the genes that have SNPs represented on the screening panel we used.

Even when a single simple Mendelian locus causes a disease, variants in other genes may contribute to phenotypic variability, and phenotypic complexity along with locus and allele heterogeneity can complicate the problem of identifying the underlying causes of the disease. Our findings raise questions about whether additional genes in this region may be contributing to phenotypic heterogeneity within the NTG and POAG populations. The MD values of the visual field in these studies indicate middle to advanced stages of the disease, with the range of values possibly resulting from a combination of genetic complexity and genetic heterogeneity.

On the other hand, we also have to consider the importance of apparent gene deserts. The SNP rs1239066 shows significant evidence of association in both Stage One and Stage Two screenings, and in the meta-analysis (POAG, NTG; P = 0.001, 0.005). Significant evidence was also found for rs1027003 (POAG; P = 0,010) (Table 2), but neither one is in the immediate vicinity of a known gene. Wiggs et al. found significant evidence that genetic variants in a gene desert on 8q22 are associated with optic nerve damage in glaucoma [14]. So additional follow up studies will need to explore this gene desert region to determine whether any functional sequences there are playing a role in the disease.

Glaucoma is a complex disease, and it involves genetic variants that confer moderate to low effect sizes (e.g., OR = 1.2–1.5). The OR which was identified in the first stage with the P value cutoff of 0.01 was 1.68. This cutoff value was a bit strict to exclude the false positives.

Further investigations of the structure and function of the *HK2, NCK2* and *TMEM182* proteins would be helpful in understanding the pathogenesis of POAG and NTG. Our data suggest that *HK2* may play an important role in NTG in the Japanese population; although our data suggest that *HK2* might be the GLC1B gene, a firm conclusion on the subject awaits screening of members of the families originally used to map the GLC1B locus.

Patients and Methods

Ethics statement

This study was approved by the Institutional Review Board of Tohoku University, Keio University, Tokyo Metropolitan Police Hospital, Niigata University, Ideta Eye Hospital, and all procedures were conducted in accordance with the Declaration of Helsinki. All participants provided written informed consent after an explanation of the purpose and procedures to publish these case details.

Patient Recruitment and Characteristics

The samples used in the first screening (Stage One) were collected in Keio University hospitals, Tokyo Metropolitan Police Hospital, Niigata University, and Ideta Eye Hospital, and the

Table 7. Stage Two *TMEM182* SNP Allele Frequencies in Japanese POAG, NTG and Control Subjects.

	Allele frequency			Genotype			
rs869833	A	G	p value*	A/A	A/G	G/G	p value**
POAG	0.590	0.410	0.855	61/187	99/187	27/187	0.095
NTG	0.579	0.421	0.834	94/286	143/286	49/286	0.269
Control	0.585	0.415		100/271	117/271	54/271	
	Allele frequency			**Genotype**			
rs960011	**C**	**T**	**p value***	**C/C**	**C/T**	**T/T**	**p value****
POAG	0.503	0.497	0.363	45/187	98/187	44/187	0.479
NTG	0.520	0.480	0.641	76/286	145/286	65/286	0.782
Control	0.533	0.467		79/271	131/271	61/271	

*Fisher's exact test,
**Chi-square test.
G/G or T/T is mutant homozygote, A/G or C/T is heterozygote, and A/A or C/C is wild homozygote.
doi:10.1371/journal.pone.0054115.t007

Table 8. Correlation between the POAG or NTG Endophenotypes and *TMEM182* SNPs Screened in Stage Two.

Endophenotype	Age at diagnosis (y.o.)				Maximum IOP* (mmHg)				MD value of the visual field (dB)			
rs869833 Genotype	**A/A**	**A/G**	**G/G**	**p value****	**A/A**	**A/G**	**G/G**	**p value****	**A/A**	**A/G**	**G/G**	**p value****
POAG	54.8	61.1	56.0	0.95	24.7	23.8	23.3	0.80	−17.5	−15.0	−9.95	0.01
NTG	55.8	55.7	59.9	0.40	16.9	16.9	16.4	0.84	−12.60	−9.62	−12.21	0.98
rs960011Genotype	**C/C**	**C/T**	**T/T**	**p value****	**C/C**	**C/T**	**T/T**	**p value****	**C/C**	**C/T**	**T/T**	**p value****
POAG	55.5	57.3	62.0	0.29	24.8	24.4	23.2	0.73	−14.2	−18.5	−9.44	0.37
NTG	53.8	56.4	60.1	0.14	17.1	17.3	15.7	0.16	−10.77	−11.02	−11.76	0.88

*IOP; intra ocular pressure (under medication),
**Dunnett's test.
G/G or T/T is mutant homozygote, A/G or C/T is heterozygote, and A/A or C/C is wild homozygote.
doi:10.1371/journal.pone.0054115.t008

samples used in the second screening (Stage Two) were collected at Tohoku University.

Routine ophthalmic examinations were performed on all subjects. Individuals were included as POAG cases if they fulfilled the following inclusion criteria: 1) applanation IOP greater than 22 mm Hg in each eye; 2) spherical equivalent more than −8 diopter; 3) glaucomatous cupping in each eye including cup-to-disc ratio greater than 0.7; 4) visual field loss measured by Goldmann perimetry or Humphrey automated field analyzer (Carl Zeiss Meditec, Dublin, CA) in Stage One. The severity of the visual field defects was scored from 1 to 5 according to previously reported criteria ref. The data obtained by two types of perimetry were combined using a five-point scale: 1, no alterations; 2, early defects; 3, moderate defects; 4, severe defects; and 5, light perception only or no light perception. The first four groups on this severity scale followed Kozaki's classification based on Goldmann perimetry or the classification was based on results of Humphrey automated field analyzer [30,31]. Kozaki's classification is widely used in Japan. In Stage Two, all of the visual field loss were measured by Humphrey automated field analyzer according to Anderson-Pattela classification [30] consistent with the glaucomatous cupping in at least one eye; and 5) open anterior chamber angles; and exclusion of secondary causes (e.g., trauma, uveitis, or steroid-induced glaucoma). The criteria for NTG were the same as for POAG except that NTG subjects showed applanation IOP less than 22 mm Hg in both eyes at each examination. Baseline clinical parameters including age, gender, spherical equivalent base line visual acuity (VA), IOP measured by Goldmann applanation tonometry were recorded at the time of first diagnosis of POAG or NTG in each patient. Mean deviation (MD) values indicative of visual field damage were obtained by the Swedish interactive threshold algorithm (SITA)-standard strategy of the 30-2 program of HFA (Carl Zeiss Meditec, Dublin, Californuia, USA). MD was used on reliable visual field test results (<20% fixation errors, <33% false-positive results, and <33% false-negative results). Control subjects had these characteristics: IOP less than 22 mm Hg, normal optic discs, and no family history of glaucoma. To decrease the chance of enrolling individuals with pre-symp-

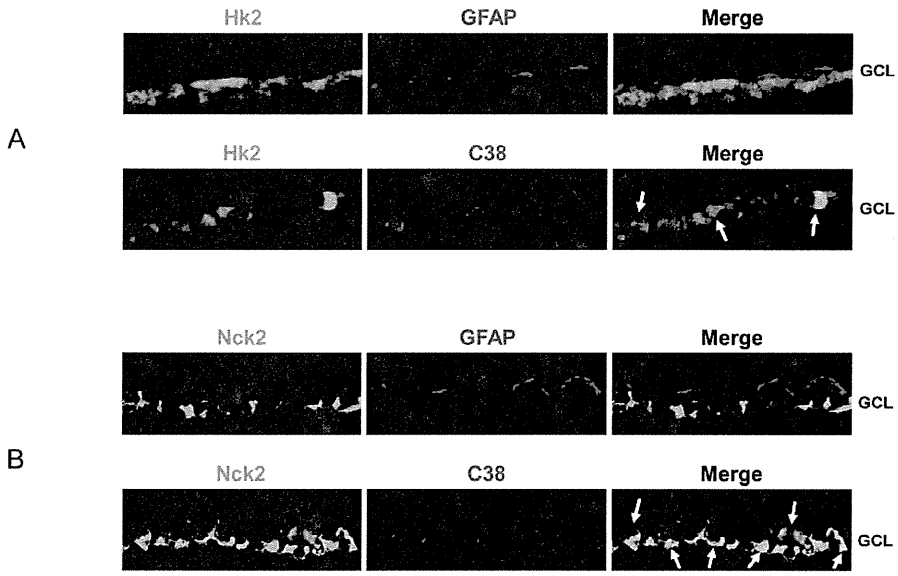

Figure 3. *Hk2* and *Nck2* Immunohistochemistory. Representative IHC photographs showing Hk2, Nck2, astrocyte maker (GFAP) and RGC marker (C38) in the retinas of untreated mice. Arrows indicated the co-localization area.
doi:10.1371/journal.pone.0054115.g003

tomatic glaucoma, we limited this group to individuals older than 60 years.

Two Stage Screening Protocol

The two-stage screening strategy is shown in Figure 1. The first stage screened used 669 SNPs from the GLC1B region, using the Illumina panel #8 (Chromosome 2; 77–117 Mbp) (Illumina, San Diego, CA, USA) carried by Illumina (San Diego, CA, USA), and each SNP was tested for association with POAG or NTG. The first stage used a cohort of 368 unrelated Japanese individuals: 123 POAG cases (63 men and 60 women), 121 NTG cases (61 men and 60 women) and 120 normal subjects (61 men and 59 women). Mean age of POAG cases was 56.9±11.5 years. Mean age of NTG cases was 54.0±12.3 years. Mean age of controls was 70.3±10.2 years. The visual field scores were 2.8±1.0 in POAG cases and 2.7±0.9 in NTG cases (Table 9). Single-nucleotide polymorphisms (SNPs) with a call rate <90% were excluded from the analysis. All of the polymorphisms showed no deviation from Hardy–Weinberg equilibrium ($P>0.05$) except rs2033008 in Stage One ($p = 0.030$).

The second stage screened SNPs that showed significant evidence for association in the first round in this study ($p<0.01$) and in the NCK2 gene, which previously showed association with NTG [16]. The odds ratio which was identified in the first stage with the P value cutoff of 0.01 was 1.68. Second stage screening was carried out using sequencing of DNA PCR amplified by polymerase chain reaction from genomic DNA samples from a population of 473 unrelated Japanese individuals, including 187 POAG cases (119 men and 68 women), 286 cases NTG (139 men and 147 women), and 271 control subjects (145 men and 126 women). Mean age of POAG cases was 57.8±12.0 years. Mean age of NTG cases was 56.4±13.3years. Mean age of controls was 69.7±9.3 years. Maximum intra ocular pressure under medication were 23.5±5.3 mmHg in POAG subjects and 16.8±2.4 mmHg in NTG subjects. Mean deviation (MD) value of the visual field test was −15.0±9.0 dB in POAG cases and −11.0±7.1 dB in NTG cases (Table 9).

Sample Preparation

Genomic DNA was extracted from leukocytes of the peripheral blood. It was purified by the Qiagen QIAamp Blood Kit (Qiagen, Valencia, CA, USA).

Mutation Screening

Mutation screening was carried out in genes that contained SNPs that showed significant evidence of association in the first stage (HK2 and TMEM182) plus the previously-reported NCK2 gene [16]. All of the exons of the *HK2*, *NCK2*, *TMSB10* and *TMEM182* genes, and positive SNPs were amplified by a polymerase chain reaction (PCR) using 0.5 μM concentration of primers in an amplification mixture (25 μl) containing 0.2 mM dNTPs and 0.5 U Ex Taq polymerase (Takara Bio, Shiga, Japan) with 30 ng template DNA. Oligonucleotides for amplification and sequencing were selected using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi/ provided in the public domain by the Massachusetts Institute of Technology, Cambridge, MA). Primers for amplification and sequencing of coding sequence were placed in introns far enough from the intron/exon junctions to allow for visualization of the splice site sequences. The PCR fragments were purified with ExoSAP-IT (USB, Cleveland, Ohio, USA), sequenced by the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Perkin-Elmer, Foster City, CA, USA) on an automated DNA sequencer (ABI PRISM™ 3100 Genetic Analyzer, Perkin-Elmer, Waltham, MA, USA).

Statistical Analysis

The significance of association was determined by contingency table analysis using Fisher's exact test or Chi-square test, depending on cell counts. In estimation of genotype-phenotype correlation, we used Dunnett's test to compare group means of those carrying the mutant variant being tested against the group means of those carrying the normal, reference sequence. Odds ratios (approximating to relative risk) were calculated as a measure of the association between the allele frequency and the phenotype of POAG/NTG, estimated using the SNPAlyze program version 7.0 (Dynacom, Yokohama, Japan). Hardy–Weinberg equilibrium was analyzed using gene frequencies obtained by simple gene counting and the chi-square test with Yates' correction for comparing observed and expected values.

Immunohistochemistory of the *HK2* and *NCK2*

Murine retinas were fixed with 4% PFA at 4°C overnight and then cryoprotected in phosphate buffered saline (PBS) with 20% sucrose. Cryosections (thickness 10 μm) were mounted on the slides and incubated with blocking buffer (10% goat serum, 0.5% gelatin, 3% BSA and 0.2% Tween 20 in PBS). Next, they were incubated with primary antibodies at 4°C overnight. Primary

Table 9. Clinical Characteristics of Subjects studied in Stage One and Two Screening.

Endophenotype		Age at diagnosis (y.o.)	Maximum IOP* (mmHg)	The Visual Field Score**
Stage One	POAG	56.9±11.4	25.3±5.6	2.8±1.0
	NTG	54.0±12.2	16.0±2.3	2.7±0.9
	Control	70.3±10.2	13.9±2.2	
Stage Two	POAG	57.8±12.0	23.5±5.3	−15.0±9.0 (dB)
	NTG	56.4±13.3	16.8±2.4	−11.0±7.1 (dB)
	Control	69.7±9.3	13.9±2.2	

*IOP; intra ocular pressure (under medication).

**The Visual Field Score was evaluated by Humphrey MD value or Goldmann perimetry (Stage One) and Humphrey MD value (Stage Two).

In Stage One, the severity of the visual field defects was scored from 1 to 5 according to previously reported criteria. The data obtained by two types of perimetry were combined using a five-point scale: 1, no alterations; 2, early defects; 3, moderate defects; 4, severe defects; and 5, light perception only or no light perception. The first four groups on this severity scale followed Kozaki's classification based on Goldmann perimetry or the classification was based on results of visual field perimetry (Humphrey Field Analyzer; Carl Zeiss Meditec, Dublin, CA). Kozaki's classification is widely used in Japan.

doi:10.1371/journal.pone.0054115.t009

antibodies used were Mouse anti-Glial Fibrillary Acidic Protein (GFAP) (1:200; MAB360; Chemicon, Millipore, MA, USA), mouse anti-C38 (1:200; provided by Dr. Jun Kosaka), rabbit anti-NCK2 (1:200; ab14590; Abcam), or rabbit anti-HK2 (1:200; 2867S; Cell Signaling Technology, MA, USA). The sections were washed three times with PBST (PBS containing 0.2% Tween 20) and then incubated with secondary goat anti-rabbit IgG antibody (1:200; A11008 Invitrogen, Carlsbad, CA, USA) tagged with Alexa 488 or goat anti-mouse IgG A11030; Invitrogen, Carlsbad, CA, USA) tagged with Alexa 546 for 1 hour. The slides were washed three times and mounted with Vectashield mounting medium (H1000; Vector, Burlingame, CA).

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Acknowledgments

The authors thank Dr. Duco I. Hamasaki for editing the manuscript, and thank Dr. Nariyuki Yamada and Prof. Makoto Tamai for experimental suggestions and support. We are grateful to Dr. Jun Kosaka for providing antibody mouse anti-C38.

Author Contributions

Conceived and designed the experiments: T. Funayama YM KN NF. Performed the experiments: DS T. Funayama YT AS KY MM AM TN NF. Analyzed the data: DS T. Funayama YM JER NF. Contributed reagents/materials/analysis tools: T. Funayama YT AS NY T. Fukuchi HA HI TN. Wrote the paper: DS KY JER NF.

4. ChIP-seq解析をはじめる人のために
—ENCODEのChIP-seq解析ガイドライン

舟山 亮

次世代DNAシークエンサーを用いたChIP-seq解析は，ゲノム上のタンパク質結合部位を網羅的に同定する手法である．細胞周期を同調した細胞からクロマチンサンプルを調製すれば，細胞周期の進行に伴う結合パターンの変化を明らかにすることもできる．しかし，実験計画を立てる際には，配列の読み取り量や対照サンプルの設定など，考慮すべき項目が多くある．本稿では，ENCODEが定めたChIP-seq解析のガイドラインを参考に，ChIP-seq実験のポイントを解説したい．

はじめに

ChIP-seq（chromatin immunoprecipitation followed by high-throughput DNA sequencing）解析は，ゲノム上のタンパク質の分布を明らかにするための手法として広く利用されている[1]．しかし，実験プロトコルやデータの評価方法に関する統一的な指針がないため，報告されているChIP-seqデータには品質のばらつきがあり，これが複数のデータを用いた統合的なメタ解析を困難にしていた．そこで，ENCODE（エンコード，The Encyclopedia of DNA Elements）計画コンソーシアムは，ChIP-seq実験のガイドラインを作成し，これに基づいて1,000以上のChIP-seq実験を行って，ヒトゲノムにコードされている機能要素を明らかにしてきた．本稿では，ENCODE計画の背景と成果を概観した後，ENCODEが定めたChIP-seq解析のガイドラインについて解説する．

1 ENCODE計画

2003年にヒトゲノム計画が完了した結果，30億塩基対のゲノム配列と20,000～25,000のタンパク質コード遺伝子が明らかになった[2]．しかしながら，細

[キーワード&略語]
ChIP-seq, ENCODE, 次世代シークエンス，クロマチン

CAGE：cap analysis of gene expression
ChIP-seq：chromatin immunoprecipitation followed by high-throughput DNA sequencing
ENCODE：The Encyclopedia of DNA Elements
IDR：irreproducible discovery rate
NHGRI：National Human Genome Research Institute（アメリカ国立ヒトゲノム研究所）
NRF：non-redundant fraction
UCSC：University of California Santa Cruz（カリフォルニア大学サンタクルーズ校）

ChIP-seq guidelines used by ENCODE

Ryo Funayama：Division of Cell Proliferation, United Centers for Advanced Research and Translational Medicine, Tohoku University Graduate School of Medicine（東北大学大学院医学系研究科附属創生応用医学研究センター細胞増殖制御分野）

胞が遺伝情報をどのように読み取っているのか，遺伝情報がどのようにして疾患を生み出すのかを明らかにするには，タンパク質コード遺伝子だけでなく，ノンコーディング遺伝子や転写の制御領域など，ヒトゲノムにコードされているすべての機能要素を明らかにすることが重要であると考えられた．

この目的を達成するために組織されたのがENCODE計画コンソーシアムである[3]．アメリカ国立ヒトゲノム研究所（NHGRI）が立ち上げたENCODEには，アメリカ，日本，イギリス，スペイン，シンガポールの5カ国が参加している．日本から参加した理化学研究所のオミックス基盤研究領域は，CAGE（cap analysis of gene expression）法を利用した遺伝子の転写開始点の同定に貢献した[4]．

2004年に発足したENCODEは，はじめにヒトゲノムの1％を対象にパイロット解析を実施し，機能要素の同定が可能であることを2007年に報告した[5]．その後，さらに5年間をかけて全ゲノム領域の解析を進めて，2012年にヒトゲノムの80％の領域に機能があることを報告した[6]．ENCODEの成果は，Nature誌，Genome Research誌，Genome Biology誌に合計30報の論文として発表されている．また，ENCODEのシークエンス解析データは，UCSC（University of California Santa Cruz）のゲノムブラウザ（http://genome.ucsc.edu/ENCODE/）を通して閲覧，ダウンロードすることができる[7][8]．これからChIP-seq解析を計画する人は，まず，ENCODEのデータベースに目的タンパク質が含まれていないか検索してみよう．

2 ChIP-seq解析の流れ

ENCODEでは転写因子やヒストン翻訳後修飾の分布を決定するために，1,000以上のChIP-seq解析を行っている．解析では，まず，標的タンパク質に対する特異抗体を用いてタンパク質-DNA複合体を免疫沈降する（**図1**）．沈降物からDNAを精製し，末端にアダプターDNAを付加したライブラリを調製した後，次世代シークエンサーにより塩基配列を決定する．読み取った配列（リードまたはタグという※1）は参照ゲノムにマッピングされ，任意のゲノム領域にマッピングされたリード数を数えることにより，タンパク質結合部位を決定する（ピークコール）．

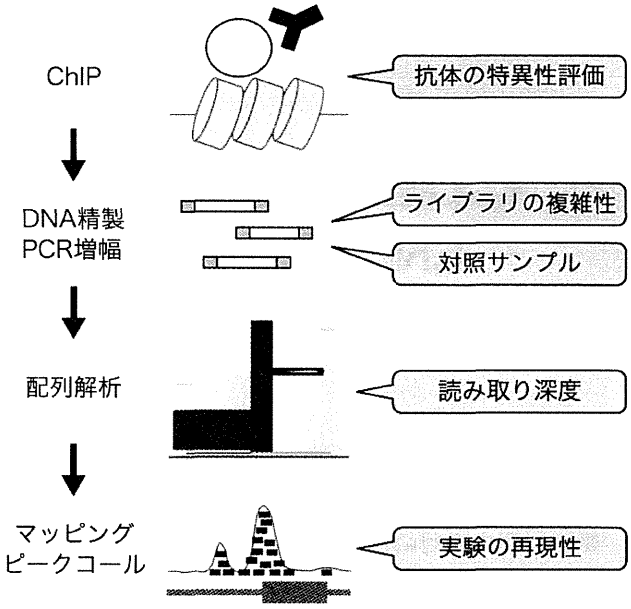

図1　ChIP-seq解析の流れとENCODEガイドライン
ChIP-seq解析の4つのステップを示した．ChIPでは5～100 ngのDNAを回収し，シークエンスライブラリを調製する．ENCODEが作成したChIP-seqガイドラインのポイントを吹き出しで示した

ChIP-seqの場合，次世代シークエンサーで配列を読み取るときは，長いリード長を読み取る必要はない．35塩基程度を読み取れば，大部分のリードはゲノムの1カ所にマップできる．読み取り方式は，DNA断片の片端を読み取るシングルエンド方式より，両端を読み取るペアエンド方式の方が，解析に使用できる有効リード数が多くなることが報告されている[9]．

タンパク質結合部位を高解像度で決定するには，150～500 bp（ヌクレオソーム1～3個分）のクロマチン断片を解析するのがよい．また，免疫沈降物をエキソヌクレアーゼで処理し，タンパク質結合部位のDNAをピンポイントで配列解析することにより，タンパク質結合部位を高解像度で決定できる手法（Exo-seq）も開発されている[10][11]．

※1　リード・タグ
DNAシークエンサーで読み取った配列のこと．読み取った配列の数をリード数，またはタグ数といい，配列の長さをリード長という（タグ長という言葉はあまり使われない）．

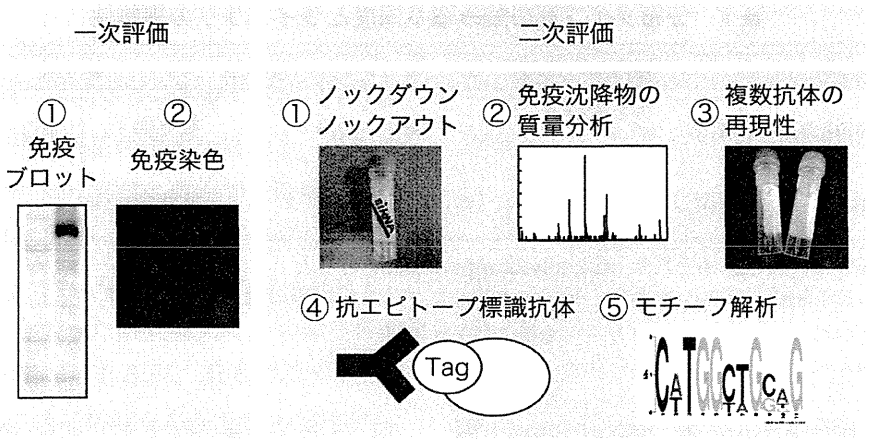

図2　ChIP-seq解析に使用する抗体の評価方法
一次評価と二次評価の中から，それぞれ1つ以上の実験を行い，抗体を評価する．免疫ブロットを行うと，抗体がどれくらい非特異的な結合を示すのかがわかる．免疫染色を行うと，抗体が核内抗原と反応しているかどうかがわかる．抗エピトープ標識抗体を使用する場合は，標識タンパク質の発現量に注意する必要がある．過剰な発現により標的タンパク質の結合部位が変化する可能性があるためである．また，DNAモチーフ解析は，先行研究により結合親和性の高いモチーフ配列が明らかになっている場合にのみ有効である

3 ENCODEのChIP-seqガイドライン

ENCODE計画は，ChIP-seqデータの品質のばらつきを最小限に抑え，複数のデータを用いたメタ解析を可能にするために，実験方法の基準を定めたガイドラインを作成した[12]（**図1**）．ここでは，このガイドラインの中から，実験計画の立案の際に特に考慮するべきポイントを解説する．

1）抗体の特異性の評価

ChIP実験の成否は免疫沈降に使用する抗体の品質によって決まると言って過言でない．ENCODEは，抗体の特異性を評価する方法を定め，それらを一次評価と二次評価の2つに分類した（**図2**）．抗体の評価は，一次評価と二次評価のそれぞれから1つ以上の実験を選択して行う．一次評価は，免疫ブロットまたは免疫染色からなる．二次評価は，次の5つの実験からなる．①ノックダウンまたはノックアウトサンプルを用いた特異性の検証，②質量分析による免疫沈降物の解析，③複数の抗体を用いた再現性の確認，④抗エピトープ標識抗体を用いたChIP-seq解析，⑤タンパク質結合部位のDNAモチーフ解析．

これらの実験結果は，抗体がChIP解析に適していることを保証するものではない．また，ENCODEは，各実験結果の評価基準については言及していない．それは，免疫ブロットで非特異的なバンドが多く観察されたとしても，ChIP条件下では標的タンパク質を特異的に認識できる可能性があるからである．重要なのは，抗体の特異性に関する情報を，ChIP-seq解析結果と一緒に提示することである．なお，市販されている227の抗転写因子抗体を評価した結果，ENCODEの基準を満たし，ChIP-seqに使用できた抗体は44個（約20％）だったという[12]．

2）読み取り深度とライブラリの複雑性

タンパク質結合部位を統計学的に高い信頼性をもって検出するためには，信頼性を与えるのに十分な数のリード数を読み取る必要がある．したがって，標的タンパク質がゲノム上でどのように分布するかを予想し，解析に必要なリード数を見積もることが重要である．予想といっても，分布が次の2つのパターンのどちらに該当するかを大雑把に予想する程度でよい．A）一般的な転写因子やヒストンH3リシン4トリメチル（H3K4me3）修飾のようにゲノムに限局するパターン（Point-source），B）ヒストンH3リシン9トリメチル修飾（H3K9me3）のようにゲノムの広い領域にわたって局在するパターン（Broad-source）．

ENCODEが採用した読み取り深度を**表1**にまとめ

表1　分布タイプ別の読み取り深度とライブラリの複雑性

分布のタイプ	読み取り深度	ライブラリの複雑性	タンパク質の例
Point-source因子	＞1,000万 ユニークリード※1	NRF＞0.8※2	転写因子，H3K4me3
Broad-source因子	＞2,000万 ユニークリード	NRF＞0.8	H3K9me3

※1：ユニークリード＝uniquely mapped reads のこと
※2：NRF＝(ユニークリードがマップしたゲノムの位置の数)／(ユニークリードの数)
1,000万ユニークリードを読み取った場合

た．Point-source因子は1,000万，Broad-source因子は2,000万のuniquely mapped reads※2（本稿では，ユニークリードという）の読み取りを基準としている．ただし，ヒストンH3リシン27トリメチル(H3K27me3）修飾のように，広範に分布する領域と限局して分布する領域とが混在するパターンも存在する[13]（Mixed-source）．リード数が少ないと検出できるタンパク質結合部位の解像度が低下するので，Mixed-source因子の分布を解析する場合は，少なくとも1億以上のリードを取得することを推奨したい(Hosogane et al. 投稿中)．

ライブラリは，多様なDNA断片によって構成された，複雑性の高いものが好ましい．例えば，免疫沈降されたDNA量が極端に少ないと（5 ng以下），PCR反応の過程で一部のDNA断片がライブラリの大部分を占め，複雑性が低下する．ENCODEは，「ユニークリードがマップしたゲノムの位置の数」／「ユニークリードの数」を複雑性の指標（non-redundant fraction：NRF）と定義し，NRF＞0.8の複雑さを基準としている（**表1**）．複雑性の低いライブラリを次世代シークエンサーで解析すると，読み取りの品質（quality value）が低下することがあるので，複雑性の低いサンプルと高いサンプルを混合して読み取るなどの工夫が必要である．

3）対照サンプルと実験の再現性

ChIP-seqはサンプル調製の際にPCR反応を行うので，PCRで増幅されやすいDNA領域が濃縮されると，それがタンパク質結合部位として誤って検出されてしまう可能性がある．そこで，通常，免疫沈降していないクロマチンサンプル（input DNA），または，コントロールイムノグロブリンを用いて免疫沈降したサンプル（control IgG）のどちらかを陰性対照として解析することで，標的タンパク質の結合領域が濃縮されたのか，PCRで増幅されやすい領域が濃縮されたのかを区別する．

解析計画を立てる際，陰性対照サンプルをどれくらい読み取るか議論することがしばしばある．ENCODEでは上記2つのコントロールサンプルのいずれかを，少なくとも目的サンプルと同程度のリード数読み取っている．すなわち，転写因子のChIP-seqの場合，陰性対照サンプルを少なくとも1,000万リードは読み取る．リード数が極端に少ないと，PCRバイアスの影響を正しく評価できないためである．また，control IgGサンプルはDNA量がきわめて少ないため，複雑性の低いライブラリができやすい．このような複雑性の低いデータを用いて目的サンプルのデータを補正すると，新たなバイアスを生じる原因となるので注意したい．

ENCODEのデータの大部分は，2つ以上のbiological replicateのサンプルを解析し，結果の再現性を検証している．例えば，培養細胞を用いて転写因子のChIP-seq解析を行う場合，2つの異なる培養皿からそれぞれクロマチンサンプルを調製して実験し，転写因子結合部位のピークコールを行う．その後，2つのデータがどの程度一致しているかを，irreproducible discovery rate（IDR）解析とよばれる統計学手法により評価する[14]．サンプル数が多いとシークエンス解析のコストが増加してしまうが，抗体の特異性に不安がある場合はぜひ再現性を検証しておきたい．

※2　Uniquely mapped read
ゲノムの1カ所にだけマッピングされるリードのこと．2カ所以上にマッピングされるリードはゲノムのリピート領域に由来することがあり，解析に使用しないことが多いため，これらを区別して表現する．