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Confirmation and further mapping of the GLC3C locus in primary congenital glaucoma

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1. ABSTRACT

We investigated the relationship between primary congenital glaucoma (PCG) in the Chinese Han population and its candidate locus GLC3C. 152 nuclear families (patients with normal parents) without carrying the *CYP1B1* mutation were enrolled. Fluorescence Labeled Multiplex-PCR was used to genotype 12 short tandem repeats (STRs) within GLC3C region and transmission disequilibrium test (TDT) was used to analyze the association between PCG and these STR markers. Sixteen haplotype tag single nucleotide polymorphisms (htSNPs) were chosen from the location where the TDT tests showed positive results. Matrix-assisted laser desorption/ionization Time-of-flight (MALDI-TOF) mass spectrometry was used to perform SNP genotyping Haplotypes constructed from these SNPs were analyzed. The TDT results of STRs in the GLC3C area indicated that D14S279, D14S555 and D14S74 have significant transmission disequilibrium signals ($p = 0.0210$, 0.0096 and 0.0034), with a genetic distance of 0.006cM among them. Significant transmission disequilibrium ($P=0.0010$) occurred between the haplotype TAACG of rs2111701- rs4020123- rs4903696- rs11159318- rs177216 and the disease. Detection of disease causing genes within this region needs further study.

2. INTRODUCTION

Glaucoma is the second leading cause of vision loss in the world. About 67 million people suffer from glaucoma around the world and it is estimated that 5.2 million to 6.7 million of them have lost their eyesight (1). Of particular interest, Primary Congenital Glaucoma (PCG) severely limits an infant's visual development, which could result in life-long visual disability and may be classified as a major birth defect (2). The attack rate of PCG in newborns has distinct racial and regional differences. The incidence of PCG in newborns in western countries is 1:10000 (1:5000□1:22000) on average, with maximum occurrence in gypsies of Slovakia, which has been reported as 1:1250, and it is 1:10000 in Chinese Han newborns.

Among all the PCG cases, only 10-12% have family histories of this disease and most of the remainder cases are sporadic. It is reported that sporadic cases are closely related to heredity as well (3), and there is heterogeneity among different cases. Most of the inherited forms of glaucoma are presented as autosomal recessive with penetrance of 40-100% (3-5). Minority families exhibit autosomal pseudooverdominance inheritance, while the current viewpoint tends to imply it is polygenetic or multifactorial inheritance (6).

At present, according to family-based linkage analysis, three major loci were identified to be related to the onset of PCG, GLC3A (2p21) (7), GLC3B (1p36.2-p36.1) (8) and GLC3C (l4q24.3) (9). The mutation rate of gene *CYP1B1* (11-13) located at the GLC3A site differs dramatically between different patient groups (10), which is 90-100% in Arabs and Gypsies (11), 30-50% in Indonesia and Morocco (12-15), and 20% in Japan (16). The previous research including 116 sporadic cases in the Chinese Han population by this research group shows that only 17.2% patients are caused by the GLC3A mutation (17), which indicates that there must be other disease causing genes present in PCG patients in Chinese Han population. Locus GLC3B was identified from linkage analysis on four families with no relationship to *CYP1B1* genes in 1996, localized in lp36.2-p36.1, inside a region of about 3cM (18). There are 16 genes specifically expressed by trabecular meshwork in this area. The third candidate area, GLC3C, was identified by a sperate PCG family of multi-generational autosomal recessive inheritance, localized in 14q24.3, with a genetic distance of about 2.9cM (10). Recently, a study was conducted on two large PCG families of consanguineous marriage in Pakistan identified the causal gene LTBP2 in this region (21, 22). LTBP2 is a potential transforming growth factor binding protein, upstream of GLC3C (22).

Currently, the main methods for causal gene location include family-based linkage analysis and population-based association study. These two methods have their own advantages; however they also have certain limitations. Population association study depends on the linkage disequilibrium between the genetic marker and the disease causing gene, and is subject to the influence of population stratification, which can result in false positive or false negative results. Linkage analysis can be used to seek out relatively large areas and is not subject to the influence of population stratification, however its location precision is dependent on the size of the family and its structure.

Due to most forms of PCG present as autosomal recessive inheritance and the elimination of consanguineous marriage in China, fewer large families with PCG in the Chinese Han population are available for genetic research. In this situation, nuclear families were chosen as the subjects of this study. According to *CYP1B1* gene mutation screening, 152 nuclear families that did not carry any *CYP1B1* gene mutations were chosen for analysis. After excluding the *CYP1B1* gene mutation, the study was focused on observing the relationship between the GLC3C region and PCG. A Transmission Disequilibrium Test (TDT) (23-24) was used to verify the linkage relationship between PCG and this genomic area. Both theoretical and practical examples have proven that TDT has a stronger efficacy in locating complex trait-related candidate gene sites (25-26). In addition, TDT has the advantage of less influenced by sampling bias, such as population stratification, sample stratification, etc.

3. MATERIALS AND METHODS

3.1. Sample diagnosis and collection

3.1.1. Clinical examination

Slit lamps and surgical microscopes were used to observe the anterior segment, gonioscopes were used to observe the structure of chamber angle, and adirect ophthalmoscopes were used to observe the ocular fundus. Tono-PEN® was used to measure the intraocular pressure prior to or during surgery.

3.1.2.Case diagnosis

Diagnostic standard of PCG: Onset age is less than three years; cornea has accretion or edema, intraocular pressure is greater than 21mmHg and cup disk ratio is enlarged or dissymmetrical between two eyes. Diseases such as congenital macrocornea, apriority, Fuch's dystrophy, ocular trauma/birth trauma, intro-ocular placeholder, and other congenital anomalies of secondary glaucoma, such as Aniridia, Neuroinomatosis, Sturge-Weber Symptom Complex, Axenfeld-Rieger Complex, Peter's Anomaly, Iridocorneal Endothelial Syndrome (ICE), etc, were excluded.

3.1.3. Sample collection

1-3mL of peripheral blood was obtained from each patient with PCG and their parents using an ACD anticoagulative tube. Samples were stored in a -20°C freezer. All research subjects were from Chinese Han population. All the PCG patients were pre-screened *CYP1B1* gene mutation in a former study (the results are reported separately). 152 nuclear families (patients and their parents) were enrolled. There was no consanguineous marriage in any of the families in our study. Parents of the patients were free of any hereditary eye-disease. All of the research subjects agreed to participate the study and signed consent forms regarding the donation of a sample for scientific research, as approved by the Ethics Committee of the Eye and ENT hospital, Fudan University.

3.2. Genotyping

3.2.1. Short tandem repeat (STR) genotyping

15 STRs were selected from the GLC3C region (Table 1). Fluorescence Labeled Multiplex-PCR was used to perform STR amplification. The amplified products were loaded and results were recorded on an ABI 3730xl DNA analyzer (Applied Biosystems, Foster, CA, USA).

3.2.2. Single nucleotide polymorphism (SNP) genotyping

Based on the results of STRs analysis, 17 haplotype tag SNPs (htSNPs) were identified within 40kb upstream and 40kb downstream of the positive STRs area. These markers were subjected to further accurate the region of transmission disequilibrium (Table 2). Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) was used to complete the genotyping for htSNPs by using a time-of-flight mass-based biochip system (MassARRAY™) platform (27-29). As a result of the analysis, 16 htSNPs were successfully genotyped.

Table 1. STR genetic marker and primer information

STRs	Physical location (bp, v35.1)	PCR product length(bp)	Primer sequence
D14S61	75404993	197-227	F:5'-CCTGCTAAAAGTCAAGTGGG-3'
			R:5'-AATGGCGTATCAGAGAGGAA-3'
D14S53	75992089	135-155	F:5'-CAACAAGAGCGAAACTCGC-3'
			R:5'-GAAGACTCAAGATATAGCAG-3'
D14S983	76590260	222-270	F:5'-TGGACTGGTTAGCCTCAGTG-3'
			R:5'-GCATCAACTGGCTTCCAATC-3'
D14S254	77448481	123-159	F:5'-TGGCTCGCTGAGTACCAC-3'
			R:5'-GGCCTTGAAGACCTACGG-3'
D14S74	77728132	291-313	F:5'-CCTGTACCACTACCTGAGTTGAGT-3'
			R:5'-CTTTGGCTGCCCGAAA-3'
D14S263	76399206	148-158	F:5'-GCCTTGAAATCTTGGGTTATCTTTG-3'
			R:5'-CTCTAGGACTTGGCGAATGGTTG-3'
D14S279	76508300	196-212	F:5'-CTGGTGACCTCAGCATGTATTACTC-3'
			R:5'-CAGGTATTCCCAATATGCAGCC-3'
D14S1020	77561818	228-268	F:5'-GCCTTTACAGAGGGACTCATC-3'
			R:5'-TCTACTGGGAGCTAGGGCAC-3'
D14S59	77142282	99-109	F:5'-CTTTTGCTTTCCCAGGAGG-3'
			R:5'-GTGCTGATGCAAGAAGGTGA-3'
D14S539	29141585	320	F:5'-GAGTAAGACCCTGTCAACA-3'
			R:5'-TCTGTTAACCTCTGGTGCT-3'
D14S555	77592932	211	F:5'-GGCAACAGAACCAGACTCT-3'
			R:5'-TCCTACCTTTCGTTGTACTG-3'
D14S1008	78971571	112-126	F:5'-TGGCAAGGATGTAGAACACT-3'
			R:5'-CTTAGCCTGGGTGACAGAG-3'
D14S287	78630860	240-254	F:5'-GCTTAGGAGNTCAGTTACATTCA-3'
			R:5'-GGTAGAGTTGAAAGCAATAGAGA-3'
D14S42	76200151	115-133	F:5'-GGTCACTGATCGCCTTTGAA-3'
			R:5'-GGAGGTTGTGAACACCTTGA-3'
D14S606	81323692	265-282	F:5'-AGGGGTCAGACTCTGTTAAGC-3'
			R:5'-GTCTTCAATGTTTTAACTTTGTGG-3'
D14S1000	81175672	107-133	F:5'-TGGATTGTATTGCCAACTG-3'
			R:5'-TGTGTACCCAAGCATAAGTAGG-3'

bp: base pair, F: forward, R: reverse

Table 2. Selected SNP near positive STR

STR	Haplotype block	Physical location (v35.1)	SNP serial number	Reaction block
D14S279	1	76466863	rs7146614	w1
	2	76477572	rs177246	w1
	2	76484375	rs177237	fail
	3	76496965	rs1955578	w2
	3	76497839	rs2111701	w1
	3	76503179	rs4020123	w1
	3	76506401	rs4903696	w1
	3	76511884	rs11159318	w1
	3	76519727	rs177216	w1
	3	76524947	rs3850370	w2
	4	76542207	rs4556722	w1
	4	76546555	rs2124	w1
	4	76547806	rs8021191	w1
D14S555	1	77551640	rs12323794	w1
	1	77556398	rs10139445	w1
	1	77562072	rs2370901	w1
	2	77619164	rs2061920	w1
D14S74	1	77717279	rs7153957	w1
	1	77717704	rs2219846	w1
	1	77737084	rs12432262	w1

3.3. Data analysis

3.3.1.Transmission disequilibrium test (TDT)

TDT is a type of linkage analysis which can detect linkage in regions with linkage disequilibrium and also detect if linkage exists by comparing the ratio of transmission and non-transmission of an allele (23-24). A non-transmission allele was used as an internal control for the transmission allele and can theoretically eliminate the influence of sample stratification. According to the mathematical model, null hypothesis (H_0) assumes the probability for heterozygous parents to transmit any allele to an infant is equivalent, i.e. 50%, when the restructuring of a genetic marker and the disease site is completely random; therefore there is no linked relationship among them. Conversely, if a genetic marker and the disease site are closely linked, ($H_{1)}$ the specific allele has more likelihood to be transmitted by the heterozygous parents to their affected offspring.

12 out of the 15 STRs within the GLC3C area were typed successfully in 152 nuclear families. GeneMapper3.7 software (Applied Biosystems) was used to analyze the STR genotyping results. TDT was used to analyze the transmission condition of the alleles of each

Table 3. Transmission disequilibrium test results of STR typing

STR marker	Chisq	Global-p	Sex-Averaged(cM)	NCBIv35.1(bp)
D14S61	15.33	0.2237	86.29	75,404,993
D14S53	13.43	0.0978	86.292	75,992,089
D14S42	10.71	0.2959	86.293	76,200,151
D14S263	9.41	0.1519	87.36	76,399,206
D14S279	14.77	0.0221	87.361	76,508300
D14S983	7.96	0.7172	87.362	76,590,260
D14S59	8.87	0.2625	87.363	77,142,282
D14S1020	4.23	0.9362	87.365	77,561,818
D14S555	13.37	0.0096	87.366	77,592,932
D14S74	22.96	0.0034	87.367	77,728,132
D14S287	4.47	0.9236	89.19	78,630,860
D14S606	2.89	0.7163	91.621	81,323,692

In the above three STRs located within the transmission disequilibrium area (mentioned in bold), 16 SNPs which have genotyped successfully have no unbalanced signal in transmission (TDT test result refers to Table 4).

Table 4. Transmission distortion test result of single SNP

No.	SNP	Advantage transmission	Transmission: non-transmission	Chi-square value	p value
1	rs7146614	A	52:43	0.853	0.3558
2	rs177246	A	45:29	3.459	0.0629
3	rs2111701	C	61:51	0.893	0.3447
4	rs4020123	G	64:54	0.847	0.3573
5	rs4903696	C	54:40	2.085	0.1487
6	rs11159318	T	60:50	0.909	0.3404
7	rs177216	A	50:35	2.647	0.1037
8	rs4556722	A	69:51	2.7	0.1003
9	rs2124	A	58:53	0.225	0.6351
10	rs8021191	T	58:53	0.225	0.6351
11	rs12323794	C	31:30	0.016	0.8981
12	rs10139445	A	13:9	0.727	0.3938
13	rs2370901	T	43:40	0.108	0.7419
14	rs7153957	A	58:55	0.08	0.7778
15	rs2219846	G	61:53	0.561	0.4537
16	rs12432262	C	52:50	0.039	0.843

STR. 17 htSNPs were selected in the positive area, indicated by the results of STR analysis. This same method was used to test the transmission disequilibrium of the 16 successfully typed SNPs in the nuclear families. Unphased 3.0.13 software was used to analyse the TDT for STRs (30-31), and Haploview 4.1 software was used to analyse the TDT for SNPs.

3.3.2. Haplotype analysis

In order to observe if there is an advantageous haplotype in the diseased samples, we analyzed haplotypes constructed from 16 SNPs which had been typed successfully. The Haploview 4.1 software package was used to analyze linkage disequilibrium block and association. A chi-square test was used to evaluate the linkage intensity between each haplotype and patient, and a permutation test was used to correct multiple tests.

4. RESULTS

The TDT tests for single STR showed that D14S279, D14S555 and D14S74 had statistical significance (p values = 0.0221, 0.0096, and 0.0034, respectively) (Table 3). The genetic distance of these three STRs is about 0.006cM, which indicates a strong linkage region for transmission in the patients with PCG.

In our data, the heterozygosity of a single SNP is quite low. As a result, none of the SNPs showed statistical significant associations (Talbe 4). Then haplotypes were constructed based on the genotyping data of 16 SNPs, and the transmission disequilibrium of each haplotype block was analyzed inside each nuclear family. (Figure 2 and Table 5) These results showed that the TAACG haplotype of rs2111701- rs4020123- rs4903696- rs11159318- rs177216 was significantly (p □ 0.0010) associated with the disease. After 10000 iterations of the Permutation test, this haplotype remained significant (p □ 0.0135) (Table 6)

5. DISCUSSION

At present, STR or SNP markers have mainly been used in applying the principle of linkage disequilibrium to locate a disease-related gene. To improve the effectiveness of analysis of linkage disequilibrium in researching nuclear families of PCG, we combined the usage of these two different types of genetic markers, STR and SNP. On the basis of results of transmission disequilibrium by using STRs, we further supplemented our research by using tag SNPs to conduct verification, perform a more elaborate analysis, and ascertain the signals linked with the phenotype of glaucoma in a smaller region.

To reduce the required number of SNPs for genotyping, tag SNPs within the candidate region were selected. The efficiency of genotyping data can be dramatically improved by using the enormous amount of SNP data generated from the international HapMap project, combined with linkage disequilibrium blocks. When the linkage disequilibrium area is large, the information within the region can usually be delegated by a small number of SNPs. The markers necessary in obtaining a majority or all

Table 5. Results of the haplotype TDT within each haplotype block

Block	Haplotype	Frequency	Transmission: non-transmission	Chi-square value	p Value
Block 1	GA	0.543	66.7 : 59.4	0.418	0.5179
	AA	0.264	52.4 : 43.4	0.845	0.3580
	GT	0.193	29.0 : 45.3	3.561	0.0591
Block 2	CGATG	0.479	65.0 : 58.0	0.398	0.5279
	TACCA	0.221	49.0 : 35.0	2.333	0.1266
	TAACG	0.158	18.0 : 43.8	10.746	0.0010
	TAATG	0.049	14.0 : 10.2	0.584	0.4447
	CAATG	0.046	11.0 : 11.0	0	1.0000
	CGCCG	0.023	7.0 : 4.0	0.818	0.3657
	TACCG	0.022	2.0 : 5.0	1.286	0.2568
Block 3	AAT	0.487	69.3 : 50.4	2.978	0.0844
	CGC	0.262	50.4 : 54.4	0.157	0.6919
	CAT	0.212	33.7 : 47.6	2.369	0.1238
	CGT	0.014	2.9 : 3.0	0.001	0.9791
	CAC	0.012	3.0 : 3.0	0	0.9991
	AGC	0.011	2.6 : 2.6	0.001	0.9818
Block 4	AT	0.764	43.1 : 40.0	0.114	0.7352
	GC	0.167	30.0 : 31.0	0.016	0.8981
	AC	0.069	16.0 : 18.1	0.127	0.7213
Block 5	AAA	0.564	55.0 : 61.0	0.310	0.5774
	GGC	0.315	52.0 : 48.0	0.160	0.6892
	AGA	0.066	20.0 : 9.0	4.172	0.0411
	GGA	0.050	8.0 : 15.0	2.129	0.1445

Table 6. Haplotype transmission distortion test result after 10^4 iterations of the permutation test

Haplotype	Chi-square value	p value
Block 2: TAACG	10.746	0.0135*
Block 5: AGA	4.172	0.5272
Block 1: GT	3.561	0.6666
Block 3: AAT	2.978	0.7783
Block 3: CAT	2.369	0.9211
Block 2: TACCA	2.333	0.9276
Block 5: GGA	2.129	0.9502
Block 2: TACCG	1.286	0.9967
Block 1: GA	0.418	1.0000
Block 1: AA	0.845	1.0000
Block 2: CGATG	0.398	1.0000
Block 2: TAATG	0.584	1.0000
Block 2: CAATG	0	1.0000
Block 2: CGCCG	0.818	1.0000
Block 3: CGC	0.157	1.0000
Block 3: CGT	0.001	1.0000
Block 3: CAC	0	1.0000
Block 3: AGC	0.001	1.0000
Block 4: AT	0.114	1.0000
Block 4: GC	0.016	1.0000
Block 4: AC	0.127	1.0000
Block 5: AAA	0.31	1.0000
Block 5: GGC	0.16	1.0000

of the information of linkage disequilibrium are called htSNP (37). By typing a small number of tag SNPs, all or most of the information regarding linkage disequilibrium in the region, including the frequency of common haplotypes, can be obtained. Thus, there is no need to analyze all of the common variances within the region or the entire genome. Using htSNP, the region and phenotype association studies can be conducted effectively (38). There are a variety of statistical methods (36, 38) used for the definition of htSNP. In this study, haplotype blocks were determined and haplotypes were constructed based on the HapMap Phase I data of Chinese Han Population by using Gabriel's method (confidence intervals) (39). In practice, it was found that haplotype blocks and htSNP are not identical under different methods. Therefore, in order to obtain more accurate positioning results, functional validation, larger sample size studies and multiple repetitions within different populations, as well as cellular and animal models, are necessary.

Analysis of the results of STRs, especially htSNPs data, indicates that the area of haplotype TAACG inside GLLC3C or nearby regions carries a pathogenic mutation related to PCG. This region contains a 22kb interval of high-risk haplotype (as defined by htSNPs) and has no known genes but only one pseudogene, FRDAP. FRDAP has a coding sequence by functional prediction; there is no evidence of transcription or function-related information so far. However, it is possible that markers in this region are in linkage disequilibrium with the deleterious mutation on the causable gene, which is not necessarily nearby. Further studies will be needed to evaluate the association of this region and discover the real PCG causing gene.

Figure 1. Typical primary congenital glaucoma.

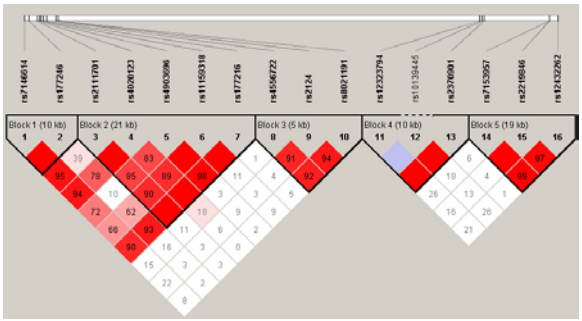

Figure 2. SNP haplotype map construction. 16 htSNPs form 5 major haplotype blocks. The TAACG of the second haplotype block has stronger transmission distortion in PCG triple families.

6. ACKNOWLEDGMENTS

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