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Original Article

Haplotypes of *RHO* polymorphisms and susceptibility to age-related macular degeneration

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Abstract: Objective: To investigate whether haplotypes of rhodopsin (*RHO*) polymorphisms including rs7984, rs2855552, rs2855557 and rs2410 were associated with age-related macular degeneration (AMD) risk in Chinese Han population. Methods: Genotypes of rs7984, rs2855552, rs2855557 and rs2410 were detected with polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) in 186 cases and 196 healthy controls. Then, the haplotypes were established with Haploview 4.2 software. And the effects of clinical charactersitics on the frequency of GTTG haplotype were also analyzed. Odds ratios (ORs) with 95% confidence interval (95% CI) were utilized to assess the relationship of haplotypes and genotypes of *RHO* polymorphisms with susceptibility to AMD. Results: Genotype distribution of all polymorphisms in control group were all in agreement with Hardy-Weinberg equilibrium (HWE) (P>0.05). In the analysis, we found that mutant alleles of rs7984 and rs2855557 were both associated with increased risk of AMD. For genotype analysis, rs7984 AA and rs2855557AA, rs2410GG genotypes all could increase the risk for AMD (OR=1.905, 95% CI=1.143-3.174; OR=2.226, 95% CI=1.261-3.932; OR=2.073, 95% CI=1.105-3.888). However, rs2855552 showed no effects on the onset of AMD. Compared with GTTA, the haplotypes of GGTG, ATAA and GTTG were all related with AMD susceptibility. Further analysis suggested that age, hypertension and hyperlipidemia history play important roles in the frequency alteration of GTTG haplotype. Conclusion: *RHO* polymorphisms (rs7984, rs2855557 and rs2410) and haplotypes may confer remarkable susceptibility to AMD. Further investigation showed that gene and environmental factors may work together in the pathogenesis of AMD.

Keywords: Rhodopsin, age-related macular degeneration, haplotype, polymorphism

Introduction

Age-related macular degeneration (AMD) is the most important eye diseases which can lead to blind in western developed countries currently, and its morbidity increases year by year with the aggravation of aging of population [1-4]. In America, there are nearly 10 million Americans suffer from AMD and the morbidity of AMD ranges from 7% to 10% among the elderly aged over 75 [5, 6]. AMD, characterized by the clinical feature of drusen sedimentation in the macular region and nearby retinas between retinal pigmented epithelium and choroidea, contributes to the progressive damage of macular area and further causes the impairment of the central visual field [7]. It has been demonstrated that pathogenesis of AMD involves many genes, of which rhodopsin (*RHO*) gene draw more attention [8].

The 6952 bp-length humans *RHO* gene locates on chromosome 3q21-24, consists of 4 introns and 5 exons and encodes *RHO* protein, which is one of the receptors of G binding protein related to human diseases [9]. *RHO* gene was first described in the literature as a causative gene for adRP by Dryja et al [10, 11]. Mutations in *RHO* gene often lead to abnormal pathophysiology of human vision. Molecular biology studies have identified a variety of polymorphisms sites of *RHO* gene, changes of which can regulate or alter the expression of *RHO* gene [6, 10]. Moreover, it has been reported that mutations

Table 1. Primer sequence of *RHO* polymorphisms

Polymorphism	MAF	Site	Primer	Length	T_m (°C)
rs2410	0.489	Exon 5	Forward: 5'-AGCCATCCCACCAGGAGCAG-3'	293 bp	65
			Reverse: 5'-CTCCTGGTGGGATGGCTGTG-3'		
rs7984	0.411	Exon 1	Forward: 5'-GCTTAGGAGGGGGAGGTCAC-3'	238 bp	60
			Reverse: 5'-GTGACCTCCCCCTCCTAAGC-3'		
rs2855552	0.256	Intron 1	Forward: 5'-TGCAAAGCTGGGTGACGGGG-3'	200 bp	68
			Reverse: 5'-AAGGCTGGCAGAGAGACGCT-3'		
rs2855557	0.422	Intron 4	Forward: 5'-GCATTTCCAGCAAGCCCTCC-3'	217 bp	64
			Reverse: 5'-TGGAGGGCTTGCTGGAAATG-3'		

in *RHO* gene account for about 25% of adRP cases in Caucasians [12]. Besides, conformational changes of *RHO* gene may lead to the hyperpolarization of the rod cells, which play a vital role in vision [13]. However, the function mechanism of *ROH* mutation in the pathogenesis of AMD is still unclear.

In current study, we selected 4 polymorphisms sites (rs2410, rs7984, rs2855552 and rs2855557) in *RHO* gene and analyzed the relationship of each SNP and haplotypes with AMD susceptibility in Chinese Han population.

Materials and methods

Subjects

We conducted a multicentric case-control study through respectively selecting a certain number of AMD cases and controls from hospitals during January, 2008 to May, 2010. The inclusion and exclusion criteria for cases and controls were established. The inclusion criteria for cases were as follows: ① aged over 50; ② diagnosed as AMD by fluorescein fundus angiography (FFA) or optical coherence tomography (OCT); ③ with no other retinal diseases; ④ signed informed consent. The cases would be excluded if they meet the following items: ① high myopia (≥6.00D); ② polypoidal choroidal vasculopathy (PCV); ③ macular dystrophia; ④ lesions of central serous retinal choroid; ⑤ vein occlusion; ⑥ diabetic retinopathy; ⑦ uveitis; ⑧ other diseases affecting functions of retinal photoreceptor cells.

The inclusion criteria for controls included: ① aged over 50 (age gap between two groups was no more than five years); ② not suffering from AMD with fundus examination. And the individuals with severe physical defects or chronic diseases were excluded. Meanwhile, the participants who did not comply with the survey and blood test were also precluded from the study.

DNA extraction and primer design

DNA was extracted by the method of phenol/chloroform extraction. Then the DNA was purified with glass milk precipitation and stored at -20°C. Primers were design by Premer 5.0 software and synthesized by GenScript (Nanjing) Co., Ltd. The sequence of primers was showed in **Table 1**.

PCR reaction

PCR reaction mixture included ddH_2O 40.5 μl, 10×PCR buffer (with 1.5 mmol/l Mg^{2+}) 5 μl, 10 mmol/L dNTP 1 μl, Taq polymerase 5 μl (Promega Company, USA), forward and reverse primer respectively 1 μl (20 μmol/l), template DNA 1 μl (500 mg). PCR amplification was performed under the following condition: initial degeneration for 2 min at 94°C, 35 cycles of degeneration for 40 s at 94°C, annealing for 40 s (annealing temperature: 60°C for rs2410; 55°C for rs7984; 63°C for rs2855552; 59°C for rs2855557) and extension for 40 s at 72°C, finally extension for 10 min at 72°C. PCR products were purified by Multiscreen-PCR purification plate (Millipore Company, USA).

Genotype determination

Restricted fragment length polymorphisms (RFLP) was used to determine genotypes of rs2410, rs7984, rs2855552 and rs2855557. The purified PCR products were respectively mixed with, restriction enzymes of Apa I, BamH I, BsaHI and BsmFI (New England Biolabs Beverly, Ma, USA). 20 μl digestion mixture included DNA 1 μg, incision enzyme 5 U, corresponding 10×Buffer 2 μl and supplemental ddH_2O. Then the mixture was treated with water

Table 2. General data and correlation between *RHO* polymorphisms and AMD risk

Item	Case	Control	*P* value	OR (95% CI)
Age (x±s)	63.53±9.7	62.95±8.6	0.716	
Gender				
Men	97	110	0.473	
Women	89	86		
rs7984				
GG	63	70	-	1.00
AG	51	84	0.112	0.675 (0.415-1.098)
AA	72	42	0.013	1.905 (1.143-3.174)
G	177	224	-	1.00
A	195	168	0.008	1.469 (1.104-1.954)
rs2855552				
TT	98 (52.7)	104	-	1.00
GT	79 (42.5)	82	0.917	1.022 (0.676-1.547)
GG	9 (4.8)	10	0.924	0.955 (0.372-2.450)
T	275 (73.9)	290	-	1.00
G	97 (26.1)	102	0.986	1.003 (0.726-1.386)
rs2855557				
TT	53	59	-	1.00
AT	71	106	0.228	0.746 (0.463-1.202)
AA	62	31	0.005	2.226 (1.261-3.932)
T	177	224	-	1.00
A	195	168	0.008	1.469 (1.104-1.954)
rs2410				
AA	82	93	-	1.00
AG	71	84	0.849	0.959 (0.621-1.479)
GG	33	19	0.035	1.970 (1.041-3.727)
A	235	270	-	1.00
G	137	122	0.096	1.290 (0.956-1.742)

Table 3. Haplotypes associated with AMD risk

Haplotype 1-2-3-4	Case (2n=372)	Control (2n=392)	*P* value	OR (95% CI)
GTTA	40	102	-	1.00
GGTG	97	102	0.000	2.425 (1.532-3.839)
ATAA	195	168	0.000	2.960 (1.945-4.504)
GTTG	40	20	0.000	5.100 (2.664-9.764)

1: rs7984; 2: rs2855552; 3: rs2855557; 4: rs2410.

bath at 37°C and 65°C for 12 h sequentially. The digestion products were detected by 2% agarose el electrophoresis (Agarose-1000; Gibco BRL, Rockville, MD, USA).

Haplotype construction

In the analysis, we found that rs2410, rs7984, rs2855552 and rs2855557 show up a block within *RHO* gene. Then we analyzed their haplotype species with Haploview 4.2 software.

Statistical analysis

Chi-square test was utilized to check the distributional differences of age, smoking, drinking and allele between case and control groups and then verify whether genotype frequencies were consistent with Hardy-Weinberg equilibrium (HWE). MannWhitney-U test was used to compare the difference of age distribution between groups. Association of polymorphisms, haplotypes and the risk of AMD were estimated by odds ratio (OR) with 95% confidence interval (95% CI) which was obtained after the adjustment of age, gender, smoking and drinking by unconditional logistic regression. All the statistical tests were bilateral probability test and conducted with SPSS 11.5 software. It was considered to be significant difference when *P* value is smaller than 0.05.

Results

Correlation of genotypes and AMD susceptibility

The four polymorphisms sites (rs7984, rs2855552, rs2855557 and rs2410) existed in all samples. In the analysis of genotype distribution, we found that genotype distribution of each polymorphism sites were consistent with HWE (*P*= 0.08, 0.22, 0.14 and 0.10). Compared with control group, mutant allele frequencies of rs7984 and rs2855557 were much higher than corresponding wild allele, which suggested that the mutant alleles were related with the occurrence of AMD (OR=1.469, 95% CI=1.104-1.954; OR=1.469, 95% CI=1.104-1.954). For genotype analysis, rs7984 AA and rs2855557AA, rs2410GG genotypes all showed strong effects on the onset of AMD (OR=1.905, 95% CI=1.143-3.174; OR= 2.226, 95% CI=1.261-3.932; OR=2.073, 95% CI=1.105-3.888). There was no significant difference neither in allele frequency of

Table 4. GTTG haplotype associated with AMD risk

Factor	GTTG haplotype		*P* value	OR (95% CI)
	Case (2n=372)	Control (2n=392)		
Total	40/372	20/392	0.000	5.100 (2.664-9.764)
Gender				
Men	24/194	10/220	-	1.00
Women	16/178	10/172	0.461	0.667 (0.226-1.965)
Age				
≤60	10/178	10/184	-	1.00
61~70	10/132	6/152	0.453	1.667 (0.437-6.358)
≥71	20/62	3/56	0.008	6.667 (1.492-29.787)
Smoking				
No	21/212	11/228	-	1.00
Yes	19/160	9/164	0.855	1.106 (0.376-3.248)
Drinking				
No	17/198	12/208	-	1.00
Yes	13/174	8/184	0.815	1.147 (0.363-3.621)
Hypertension				
No	11/188	14/200	-	1.00
Yes	29/184	6/192	0.002	1.152 (1.887-20.053)
Hyperlipidemia				
No	11/202	12/210	-	1.00
Yes	29/170	8/182	0.015	3.955 (1.275-12.269)

rs2855552 polymorphisms (*P*=0.917) nor in genotype frequency of it (*P*=0.986) between two groups (**Table 2**).

Association of haplotypes and AMD risk

As shown in **Table 3**, there are four haplotypes combination: GTTA, GGTG, ATAA and GTTG. And the results indicated that the haplotypes were all significantly associated with increased risk for AMD (OR=2.425, 95% CI=1.532-3.839; OR=2.960, 95% CI=1.945-4.504; OR=5.100, 95% CI=2.664-9.764).

Correlation analysis of GTTG haplotype and clinical characteristics

Then we investigated the relationship of clinical characteristics with GTTG hapotype (**Table 4**). For the individuals older than 71, the frequency of GTTG hapotype was much higher in case group (*P*=0.008). In addition, hypertension (*P*=0.005) and hyperlipidemia history also showed effects on the frequency of GTTG hapotype (*P*=0.002, 0.015). Above results suggested that gene and environmental factor may work together in the pathogenesis of AMD.

Discussion

In western countries, macular degeneration is the main cause of blindness among populations aged over 50. The number of blind cases with macular degeneration is lager than the total number of those with glaucoma, cataract and diabetes retinopathy [14, 15]. In China, the morbidity of macular degeneration is 6.04%-11.19% in people aged 60 to 69, and the incidence trends to increase with the rapid aging of population [16, 17]. Macular degeneration has been identified as one of the most serious eye diseases in international ophthalmology. The herapeutic treatment for macular degeneration at present are urgently needed to improve.

RHO gene encodes *RHO* protein, a transmembrane protein, contains three functional domains, that is, extracellular domain, transmembrane domain and cytoplasmic domain [18]. *RHO* protein expresses specifically in rod photoreceptor cells and plays a crucial role in the transformation process of optical signal to nervous impulse. Once activated by the photon, the protein can result in cascade reactions in the transduction process of vision signal and thus promote the production of vision signal [11, 19, 20]. Katagiri et al., have investigated the genetic and clinical features of patients with rhodopsin (*RHO*) mutations and found *RHO* mutations can precise predicte disease severity [21]. Until now, the relationship of haplotypes of *RHO* and AMD susceptibilityhave not been studied.

Our study demonstrated that rs7984, rs2855552 rs2855557 and rs2410 in *RHO* promoter region were prevalent in Chinese Han population. And the analysis showed that rs7984 AA and rs2855557 AA, rs2410 GG genotypes were

all related with AMD susceptibility. However, only mutant alleles of rs7984 and rs2855557 were associated with risk of AMD.

As respect to genetic factors for diseases, not only genetic polymorphisms individually result in the occurrence and development of diseases, but also certain interactions among them show effects on the disease [22]. Haplotypes exactly reflect genetic inter-relation and study on haplotypes is helpful for discovering multiple genetic polymorphisms involved in diseases [23, 24]. Our study established haplotypes for *RHO* polymorphisms sites and analyzed the correlations between haplotypes of *RHO* polymorphisms and susceptibility to AMD. Based on the results, the four haplotypes composed of rs7984, rs2855552, rs2855557 and rs2410 were all associated with AMD susceptibility. Then we analyzed the effects of clinical characteristics on the frequency of GTTG haplotype. As a result, age, hypertension and hyperlipidemia all could alter the frequency of GTTG haplotype.

In conclusion, haplotypes formed by *RHO* rs7984, rs2855552, rs2855557 and rs2410 polymorphisms sites alone or combination with environmental factors may be likely to influence the susceptibility of AMD. Nonetheless, how *RHO* polymorphisms and their haplotypes regulate serum levels and expression of *RHO* and how they participate in the occurrence and development of AMD is needed to be further investigated in subsequent studies.

Disclosure of conflict of interest

None.

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References

[1] Schaumberg DA, Hankinson SE, Guo Q, Rimm E and Hunter DJ. A prospective study of 2 major age-related macular degeneration susceptibility alleles and interactions with modifiable risk factors. Arch Ophthalmol 2007; 125: 55-62.

[2] Kondo N, Honda S, Ishibashi K, Tsukahara Y and Negi A. LOC387715/HTRA1 variants in polypoidal choroidal vasculopathy and age-related macular degeneration in a Japanese population. Am J Ophthalmol 2007; 144: 608-612.

[3] Xu Y, Guan N, Xu J, Yang X, Ma K, Zhou H, Zhang F, Snellingen T, Jiao Y, Liu X, Wang N and Liu N. Association of CFH, LOC387715, and HTRA1 polymorphisms with exudative age-related macular degeneration in a northern Chinese population. Mol Vis 2008; 14: 1373-1381.

[4] Yang Z, Camp NJ, Sun H, Tong Z, Gibbs D, Cameron DJ, Chen H, Zhao Y, Pearson E, Li X, Chien J, Dewan A, Harmon J, Bernstein PS, Shridhar V, Zabriskie NA, Hoh J, Howes K and Zhang K. A variant of the HTRA1 gene increases susceptibility to age-related macular degeneration. Science 2006; 314: 992-993.

[5] Ferris FL 3rd. Senile macular degeneration: review of epidemiologic features. Am J Epidemiol 1983; 118: 132-151.

[6] Friedman DS, O'Colmain BJ, Munoz B, Tomany SC, McCarty C, de Jong PT, Nemesure B, Mitchell P and Kempen J. Prevalence of age-related macular degeneration in the United States. Arch Ophthalmol 2004; 122: 564-572.

[7] Klein R, Klein BE and Linton KL. Prevalence of age-related maculopathy. The Beaver Dam Eye Study. Ophthalmology 1992; 99: 933-943.

[8] Stenkamp RE, Teller DC and Palczewski K. Rhodopsin: a structural primer for G-protein coupled receptors. Arch Pharm (Weinheim) 2005; 338: 209-216.

[9] Dryja TP, McEvoy JA, McGee TL and Berson EL. Novel rhodopsin mutations Gly114Val and Gln184Pro in dominant retinitis pigmentosa. Invest Ophthalmol Vis Sci 2000; 41: 3124-3127.

[10] Dryja TP, McGee TL, Hahn LB, Cowley GS, Olsson JE, Reichel E, Sandberg MA and Berson EL. Mutations within the rhodopsin gene in patients with autosomal dominant retinitis pigmentosa. N Engl J Med 1990; 323: 1302-1307.

[11] Dryja TP, McGee TL, Reichel E, Hahn LB, Cowley GS, Yandell DW, Sandberg MA and Berson EL. A point mutation of the rhodopsin gene in one form of retinitis pigmentosa. Nature 1990; 343: 364-366.

[12] Hartong DT, Berson EL and Dryja TP. Retinitis pigmentosa. Lancet 2006; 368: 1795-1809.

[13] Gilbert C, Rahi J, Eckstein M and Foster A. Hereditary disease as a cause of childhood blindness: regional variation. Results of blind school studies undertaken in countries of Latin America, Asia and Africa. Ophthalmic Genet 1995; 16: 1-10.

[14] Zhu W, Deng Y, Jie K, Luo D, Liu Z, Yu L, Zeng E and Wan F. Detection of single nucleotide poly-

morphisms by PCR conformation-difference gel electrophoresis. Biotechnol Lett 2013; 35: 515-522.
[15] Klein R, Peto T, Bird A and Vannewkirk MR. The epidemiology of age-related macular degeneration. Am J Ophthalmol 2004; 137: 486-495.
[16] Hyman LG, Lilienfeld AM, Ferris FL 3rd and Fine SL. Senile macular degeneration: a case-control study. Am J Epidemiol 1983; 118: 213-227.
[17] Ambati J, Ambati BK, Yoo SH, Ianchulev S and Adamis AP. Age-related macular degeneration: etiology, pathogenesis, and therapeutic strategies. Surv Ophthalmol 2003; 48: 257-293.
[18] Natarajan S. Retinitis pigmentosa: a brief overview. Indian J Ophthalmol 2011; 59: 343-346.
[19] Zhang XL, Liu M, Meng XH, Fu WL, Yin ZQ, Huang JF and Zhang X. Mutational analysis of the rhodopsin gene in Chinese ADRP families by conformation sensitive gel electrophoresis. Life Sci 2006; 78: 1494-1498.
[20] Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, Yamamoto M and Miyano M. Crystal structure of rhodopsin: A G protein-coupled receptor. Science 2000; 289: 739-745.
[21] Katagiri S, Hayashi T, Akahori M, Itabashi T, Nishino J, Yoshitake K, Furuno M, Ikeo K, Okada T, Tsuneoka H and Iwata T. RHO Mutations (p. W126L and p.A346P) in Two Japanese Families with Autosomal Dominant Retinitis Pigmentosa. J Ophthalmol 2014; 2014: 210947.
[22] Kim KJ, Lee HJ, Park MH, Cha SH, Kim KS, Kim HT, Kimm K, Oh B and Lee JY. SNP identification, linkage disequilibrium, and haplotype analysis for a 200-kb genomic region in a Korean population. Genomics 2006; 88: 535-540.
[23] Ke X, Durrant C, Morris AP, Hunt S, Bentley DR, Deloukas P and Cardon LR. Efficiency and consistency of haplotype tagging of dense SNP maps in multiple samples. Hum Mol Genet 2004; 13: 2557-2565.
[24] Stumpf MP. Haplotype diversity and SNP frequency dependence in the description of genetic variation. Eur J Hum Genet 2004; 12: 469-477.