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Research paper

Evaluating the association between calpastatin (CAST) gene and keratoconus in the Han Chinese population

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ABSTRACT

Purpose: To investigate whether calpastatin (*CAST*) gene polymorphisms are in association with keratoconus (KC) in Han Chinese population.

Methods: Four SNPs (rs4434401, rs7704167, rs26504, and rs10053056) in *CAST* gene were genotyped in 120 unrelated Han Chinese KC patients and 305 age and gender matched healthy controls, using TaqMan SNP genotyping method. PLINK and LDmatrix software was used for data analysis.

Results: SNP rs4434401, whose contribution to KC susceptibility has been established in Caucasians, still kept its effect in our population. The C allele frequency of rs4434401 was markedly higher in cases (27.7%) than in the controls (20.7%, $P = 0.03654$, OR = 1.47, 95%CI = 1.02–2.11). The genotype distribution of rs4434401 showed marginal difference between KC cases and controls. The allelic and genotype frequencies of other three tested SNPs showed no significant difference between cases and controls.

Conclusion: We confirmed previous report that SNP(s) in CAST gene conferred risk for KC susceptibility in Han Chinese population, suggesting the potential contribution of *CAST* gene to KC development.

1. Introduction

Keratoconus (KC) is a degenerative corneal disorder featured by progressive thinning and cone-shaped protrusion of the cornea, which eventually causes impaired vision, irregular astigmatism and cornea scarring (Rabinowitz, 1998). The estimated prevalence of KC is reported to be 54.4 per 100,000 and the approximated incidence of KC is 1 in 2000 individuals in the general population (Jonas et al., 2009). Previous epidemiology studies suggested that Asians have a four times of increment in incidence of KC, indicating substantial effects of population differences underlying the disease (Georgiou et al., 2004). Given the limited availability of medical treatments, KC is one of the three top indications for corneal transplantation in the U.S. and worldwide.

KC has a complex etiology, with genetic, behavioral, and environmental factors all contributing to the disease (Abu-Amero et al., 2014). In particular, there is growing evidence showing a strong genetic component of KC. For example, the probability for relatives of KC patients to develop KC ranged from 17% to 76% (Rabinowitz et al., 1992), and a 54% concordance rate among monozygotic twins was reported by twins studies (Bechara et al., 1996). To date, many strides have been made to elucidate the genetic architecture of KC, and recent genome-wide association studies (GWAS) and genome-wide linkage studies (GWLS) have made markedly progress to identify genetic variations that are closely associated with this disease. Single nucleotide polymorphisms (SNPs) located in the following genes have been implicated, including *HGF* (Dudakova et al., 2015), *RAB3GAP1* (Li et al., 2012), *LOX* (Li et al., 2013), *DOCK9* (Czugala et al., 2012), *CAST* (Li et al., 2013), *VSX1* (Burdon and Vincent, 2013), *ZNF469* (Lu et al., 2013), *WNT10A* (Cuellar-Partida et al., 2015), *IL1A*, *IL1B* (Wang et al., 2016), *SOD1* (Moschos et al., 2015), and several central corneal thickness (CCT) associated loci like *COL5A1*, *COL4A3*, *COL4A4*, *FNDC3B*, *FOXO1*, and *MPDZ-NF1B* (Lu et al., 2013) and so on. Some of these loci have been independently replicated in other populations, including in Han Chinese (Wang et al., 2016; Hao et al., 2015; Wang et al., 2013), although great heterogeneity might exist across different populations.

CAST gene encodes calpastatin, the inhibitor of calpains, which influences many cellular activities, such as proliferation, apoptosis, migration and so on. Previous initial linkage studies narrowed down a KC locus to a genomic region located at 5q14.3-q21.1, which

Abbreviations: CAST, calpastatin; KC, keratoconus; SNP, Single nucleotide polymorphisms; GWAS, genome-wide association studies; GWLS, genome-wide linkage studies; CCT, central corneal thickness; MAF, minor allele frequency; OR, odds ratio; CI, confidence interval; HWE, Hardy-Weinberg equilibrium; CEU, Caucasians; CHB, Han Chinese in Beijing; EAS, East Asians; 1000G, 1000 genomes project; eQTLs, expression quantitative trait loci; GTEx, Genotype-Tissue Expression; LD, linkage disequilibrium

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overlapped the *CAST* gene. Following comprehensive linkage and association analysis confirmed the contribution of the *CAST* gene to KC susceptibility. SNP rs4434401 that located in the intron of *CAST* conferred to KC susceptibility in both family and case-control panels in Caucasians (CEU) (Li et al., 2013). Another SNP rs10053056 also showed association with KC. However, considering the potential heterogeneity existed across populations, whether these SNPs are associated with KC in other populations remains unknown. It is also possible that other SNPs at the same locus may contribute to KC susceptibility, so that an in-depth investigation on this region is required. Expression quantitative trait loci (eQTLs) are genomic loci that affect gene expression. Previous reports have demonstrated that disease-associated SNPs are more likely to be eQTLs (Nicolae et al., 2010), indicating that SNPs for eQTLs might be potential candidates for fine-mapping and further replication.

Herein, we performed a replication study on several SNPs in *CAST* gene, to investigate their contribution to KC susceptibility in an independent Han Chinese population.

2. Materials and methods

2.1. Subjects

Totally, 120 Chinese KC patients with no records of family history and 305 age and gender matched healthy controls were included in this hospital-based case-control study. KC patients were recruited from ophthalmology clinic at the Eye and ENT Hospital of Fudan University from October 2015 to March 2017. All of them were self-reported Han Chinese ethnicity and were living in East China. The patients were diagnosed on the basis of the following clinical examination (Vogt's striae, corneal stromal thinning, Munson's sign, Fleischer's ring, refractive errors and signs of videokeratography). The control subjects were also recruited from Eye and ENT Hospital of Fudan University. They were accidentally injured with no ocular disease. All participants gave written informed consent forms. The whole research progress was approved by the Ethics Committee of Eye and ENT Hospital of Fudan University and was performed following the declaration of Helsinki.

2.2. DNA isolation

Genomic DNA was isolated from the peripheral blood with the QIAGEN FlexiGene DNA kit (Qiagen, Hamburg, Germany) following the manufacturer's procedure. DNA concentration was examined using a NanoDrop spectrophotometer (NanoDrop Technologies, DE). Qualified DNA samples were diluted with TE buffer (pH 8.0) and were stored at −20 °C.

2.3. SNP genotyping

A total of 4 SNPs (rs4434401, rs7704167, rs26504, and rs10053056) located in *CAST* gene were selected for replication, using a TaqMan SNP genotyping method with Applied Biosystems Assays-on-Demand probes and primers (Catalog nos. C_26382038_10 for rs4434401, C_29091758_10 for rs7704167, C_26382064_10 for rs26504, and C_3056784_10 for rs10053056).

2.4. Association analysis

PLINK software (http://zzz.bwh.harvard.edu/plink/) was applied to perform the statistical analyses. The validation of frequency of each SNP in case and control subjects was examined for deviation from the Hardy-Weinberg equilibrium (HWE) using an exact test. A *Chi*-squared test was adopted to compare allelic frequency of each SNP between cases and controls. Odds ratios (ORs) and their 95% confidence intervals (CIs) adjusted for gender and age were calculated using SNP data under logistic regression model. Statistical significance was declared as $P < 0.05$. We used the LDmatrix software (https://analysistools.nci.nih.gov/LDlink/?tab=ldmatrix) for analysis of linkage disequilibrium.

Table 1
Characteristics of KC cases and controls.

Feature	Cases (n = 120)	Controls (n = 305)
Gender (female/male)	29/91	100/205
Average age (years)[a]	22.77 ± 5.69	27.56 ± 3.89
Age range (years)	13–45	15–33
Disease onset age (years)[a]	20.96 ± 5.08	NA
Visual activity[a]	OS:0.61 ± 0.25 OD:0.35 ± 0.26	NA

[a] Data was shown as mean ± S.D.

3. Results

A total of 120 Chinese KC cases and 305 gender and age matched healthy controls were included in this study. As shown in Table 1, the average age of the KC cases was 22.77 ± 5.69 years, and the percentage of males was 75.8%. The average age of the control subjects was 27.56 ± 3.89 years, and 67.2% of them were males, similar to that of the case group.

SNP rs4434401 and rs10053056 were selected for replication in Han Chinese population, given their confirmed association in Caucasians. In addition, we conducted eQTL analysis to select other candidate SNPs for following replication. A total of 89 single-tissue eQTLs for *CAST* in whole blood was retrieved from the Genotype-Tissue Expression (GTEx) Portal (Release V7, dbGaP Accession phs000424.v7.p2, on July 20, 2017) (Consortium, 2013). After examining the linkage disequilibrium (LD) patterns among these eQTLs in Han Chinese population (1000 Genomes Project Phase 3), we found many of them were aggregated in certain LD blocks (Table S1). Then, twenty-four of them were filtered out since they were nonpolymorphic or with low minor allele frequency (MAF < 5%) in East Asians (EAS), and another twelve eQTLs were filtered out due to their marginal *P* values (with an arbitrary cutoff of $P < 1.0 \times 10^{-10}$). In addition, the MAF of SNP rs149319 showed no difference between KC cases and controls in previous CEU study, so SNP rs149319 and other ten SNPs in high LD with it were removed. Therefore, the most outstanding eQTLs in each individual LD block (rs7704167 and rs26504.), were also subjected to genotyping (Table 2), and the LD patterns among the tested SNPs were presented in Fig. 1 (shown as r^2 values in EAS or CEU). The detailed filtering process and the LD patterns among all the 89 eQTLs were shown in Table S1.

The average genotyping call rate for each interrogated SNP was 95.2%, and all of the SNPs were in HWE in the control group (Table 2). The basic association results of these SNPs were shown in Table 3. Only the minor allele C of SNP rs44344401 was substantially associated with KC risk in Han Chinese population ($P = 0.03654$, OR = 1.47, 95% CI = 1.02–2.11). In addition, the genotype distribution of rs4434401 revealed marginal difference between KC cases and controls. Under the dominant model, genotype (TT&CT) of rs4434401 showed an OR of 1.51 (95%CI = 0.97–2.37, $P = 0.06915$, shown in Table 4). The allelic and genotype frequencies of other three tested SNPs did not show significant difference between cases and controls.

4. Discussion

Previous epidemiologic studies documented a wide prevalence range of KC around the world. Rates as low as 0.0003% in Russians and as high as 2.5% in Asians have been reported, suggesting a very high prevalence in Asian countries (Chan et al., 2002; Kok et al., 2012). Although a number of susceptibility loci have been identified in KC, many of these studies were performed in European countries. In Han Chinese population, only three replication studies were conducted. The