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An association between the Calpastatin (CAST) gene and keratoconus

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Abstract

Purpose—Keratoconus is a genetically heterogeneous corneal dystrophy. Previously, we performed two genome-wide linkage scans in a four generation autosomal dominant pedigree and repeatedly mapped a keratoconus locus to a genomic region located on chromosome 5q overlapping the gene encoding the inhibitor of calpains, calpastatin (CAST). To test whether variants in CAST gene are involved in genetic susceptibility to keratoconus we performed genetic testing of polymorphic markers in CAST gene in family and case-control panels of patients with keratoconus.

Methods—We genotyped SNPs (Single Nucleotide Polymorphisms) located in CAST gene in 262 patients in 40 Caucasian keratoconus families and in a Caucasian case-control panel with 304 cases and 518 controls. Generalized estimating equation models accounting for familial correlations implemented in GWA program were used for association testing in families. Logistic regression models implemented in PLINK were performed to test associations in case-control samples.

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Conflicts of Interest

The authors have no conflict of interest in any materials discussed in this article.

Results—Genetic testing of first set of seven SNPs in familial samples revealed two tentative nominally significant markers (rs4869307 $p=0.03$; rs27654: $p=0.07$). Additional genotyping of twelve tightly spaced SNPs identified CAST SNP rs4434401 to be associated with keratoconus in both familial and case-control panels with p values of 0.005 and 0.05, respectively; and with combined meta p value of familial and case-control cohorts of 0.002, or, after Bonferroni correction, 0.04.

Conclusions—Linkage analysis and genetic association support involvement of CAST gene in the genetic susceptibility to keratoconus. In-silico analysis of CAST expression suggests differential regulation of calpain/calpastatin system in cornea as a potential mechanism of functional defect.

Keywords

Linkage analysis; genetic association; common variation; keratoconus; calpains; calpastatin

Introduction

Keratoconus (KC, MIM #148300) is a non-inflammatory corneal dystrophy characterized by progressive corneal thinning and resulting in moderate to marked visual impairment ¹. While spectacles lenses may be used for vision correction in the early stages, corneal transplantation is often required for visual rehabilitation in severe cases. The prevalence of keratoconus has been estimated between about 1:1200 ² and 1 in 2,000 ³ in Caucasian population. It is a primary indication for cornea transplantation in Western developed countries ⁴.

Symptoms of keratoconus usually manifest in the teens and the progression slows after age 30, arresting around the fourth decade of life ^{1,5}. There is accumulating evidence supporting a genetic basis for this disease, which include multi-generation pedigrees with keratoconus ^{1,6}; reports of probability for siblings of keratoconus patients to develop keratoconus was found to be in the range of 17–76% ⁷; and the twin studies which demonstrate a 54% concordance rate among monozygotic twins ^{8,9}. Most reported keratoconus cases are sporadic; while 6–23% cases are reported have a positive family history ^{1,10}. In such cases, both autosomal dominant and recessive patterns of inheritance have been described ^{11,12}.

Genome wide linkage analysis of affected pedigrees has demonstrated evidence of disease susceptibility genes mapping to several putative chromosomal loci, including: 16q22.3-q23.1, identified in 20 Finnish small pedigrees ¹³; 20q12, detected in an isolated population of Tasmania, Australia ¹⁴; 15q22.23-q24.2, identified in a large Northern Irish family ¹⁵, recently attributed to the mutation in microRNA (miR-184) gene ¹⁶; 3p14-q13, identified in an Italian two-generation autosomal dominant pedigree ¹⁷ and 5q21 by two studies ^{18,19}. These extensive linkage studies provide further support for a genetic basis for keratoconus while clearly demonstrating its genetically complex nature.

In addition to the linkage analysis, association testing and mutation analysis of candidate genes have been widely used to identify keratoconus susceptibility genes. However, to date, only a limited number of candidate genes/or regions for keratoconus have been reported, such as VSM1 ^{20–23}, HGF ²⁴, and most recently, LOX ²⁵. In addition, functional defects consequences of these associated variants have yet to be determined ^{20–23}. Genome-wide association study undertaken by our group also identified a novel region, overlapping candidate gene RAB3GAP1, which contributes to the genetic susceptibility of keratoconus ²⁶.

Our group has identified a large four generation Caucasian pedigree and mapped the responsible gene to a novel 8.2 MB (mega bases or million base pairs) genomic region located at 5q14.3-q21.1, containing more than 50 known or predicted genes²⁷. Recently, we performed additional genotyping of tightly spaced SNPs (Single Nucleotide Polymorphisms) in the linkage region which allowed us to narrow the region down to approximately 5MB (96 MB-100 MB)²⁸. Gene coding for Calpastatin, an endogenous inhibitor of calpains (CAST, OMIM 114090) was located in the 5q15 region overlapping the original linkage region, and is still located under this narrowed linkage peak, thus making it a candidate gene for keratoconus.

In the present study we tested tightly spaced SNPs located in the CAST gene in two independent family based and case-control based Caucasian cohorts and found genetic evidence indicating potential involvement of CAST gene in both familial and sporadic keratoconus.

Materials and Methods

Study Panels

Keratoconus families—Family members of keratoconus cases diagnosed at the Cornea Genetic Eye Institute were recruited to perform family-based studies. A total of 262 individuals from 40 families consisting of 131 keratoconus patients and 131 unaffected family members were obtained for this study. This panel was ascertained through a clinically affected keratoconus proband as a part of the longitudinal videokeratography and genetic study of keratoconus at the Cedars-Sinai Medical Center in Los Angeles, California.

Case-control cohort—An independent group of keratoconus cases and controls was recruited as a part of the collaborative replication study²⁶. 232 independent keratoconus cases were recruited at the Cornea Genetic Eye Institute; 26 cases at the Jules Stein Eye Institute; and 46 cases at University Hospitals Eye Institute. This replication panel consisted of a total of 304 keratoconus cases and 518 normal controls who were also recruited at Cedars-Sinai Medical Center in Los Angeles.

Controls used for this study were recruited at Cedars-Sinai Medical Center and mostly consisted of spouses of patients enrolled into the keratoconus genetic research study or spouses of individuals presenting for LASIK surgery screening, and unaffected family members of keratoconus patients. Absence of keratoconus in these individuals was determined based on clinical examination and videokeratography. Some Caucasian subjects recruited locally for other inflammatory diseases (non-ocular diseases) were also included as population controls.

Diagnosis

The diagnosis of KC was based on clinical examination and confirmed with videokeratography. All family members underwent clinical examinations including slit-lamp biomicroscopy, cycloplegic retinoscopy, and fundus evaluations. The slit-lamp biomicroscope was used to examine whether there was stromal corneal thinning, Vogt's striae, or a Fleischer ring. The retinoscopy examination was performed with a fully dilated pupil (20 minutes after phenylephrine 2.5% and cyclopentolate 1% drops had been instilled in the eye) to determine the presence or absence of retro illumination signs of keratoconus, such as the oil droplet sign and scissoring of the red reflex. Videokeratography evaluation was also performed on each eye using the Topographic Modeling System. Subjects were assigned as having KC if they had at least one clinical sign of KC and a confirmatory videokeratography map with an asymmetric bowtie with skewed radial axis above and

below the horizontal meridian (AB/SRAX) pattern¹. Only patients with isolated keratoconus were recruited, any affected individual or family members with any other corneal genetic disease, including forme fruste keratoconus, or disease known to be associated with keratoconus were excluded.

Institutional Review Board approval was obtained at Cedars-Sinai Medical Center. Written informed consents were obtained from all subjects after an explanation of the nature and possible consequences of study participation, including participation in this study and the establishment of a permanent lymphoblastoid cell lines. All study procedures were within the tenets of the Declaration of Helsinki.

DNA Collection

DNA was extracted from either blood samples or EBV transformed lymphoblastoid cell lines following a standard protocol from Puregene Gentra Systems, Inc. (Minneapolis, MN). DNA concentration was measured by a NanoDrop spectrophotometer (NanoDrop Technologies, Rockland, DE) with one micro liter volume and the DNA quality was estimated by the absorbance ratio of OD₂₆₀/OD₂₈₀. DNA sample was diluted in TE buffer (pH 8.0) and was stored at -20°C.

Genotyping

Initial set of CAST SNPs—A total of eight SNPs (rs9314177, rs17086561, rs4869307, rs151904, rs10515244, rs27654, rs3214019 and rs27765, Table 1) located in the introns of CAST gene were identified and genotyped using TaqMan allelic discrimination technology (Applied Biosystems Inc., Foster City, CA) in the family sample. The allelic discrimination assay was performed on an ABI 7000 Sequence Detection System with a reaction volume of 10µl, containing 1× TaqMan Universal PCR Master Mix, 2 pmole of each of two probes and 9 pmole of each of two PCR primers and 100 ng of sample DNA using PCR cycling conditions as suggested by the manufacturer. Ten percent of samples were duplicated for genotyping quality control.

Additional set of SNPs for fine-mapping and replication—Using the software Tagger as implemented in Haploview^{29,30} and data from the CEPH population in the International HapMap project, release 2^{31,32}, we designed a panel of fourteen SNPs in the CAST gene and genotyped them using the custom iSelect Infinium beadchip technology³³ (Illumina Inc. San Diego CA) in both family and case-control samples. Twelve SNPs were retained for analysis following clustering quality control (Table 2). The average genotyping rate for samples genotyped on the iSelect platform and passing quality control was 99.98%. Genotyping concordance among 20 pairs of blind duplicates 100%. Two tentatively associated SNPs (rs4869307 and rs27654) identified in the family panel were also genotyped in case-control panel. SNP rs27654 was added to the iSelect beadchip described above. SNP rs4869307 was genotyped using pre-designed TaqMan® genotyping assay (Applied Biosystems Inc.) with allelic discrimination on ABI 7900 Real-Time PCR System (Applied Biosystems Inc.).

Statistical analysis

Testing for genetic association in familial and case-control panels—We tested each individual SNP for association with keratoconus in both family panel and case-control panels using specific association tests designed for family and case-control studies, respectively. Association tests controlling for familial correlations by Generalized Estimating Equation (GEE) model were performed in families using the GWAF package³⁴. The association analysis of a single SNP for case-control panel was performed by the PLINK program, v1.07³⁵ (<http://pngu.mgh.harvard.edu/purcell/plink/>). Odds ratios (OR)

and their standard errors (SE) were calculated using SNP data under logistic regression models.

Meta-analysis—Meta-analysis of all SNPs obtained from the logistic regression models from family cohort and replication cohort was calculated using an inverse-variance weighting by PLINK ³⁵. We applied significance threshold of 2×10^{-3} based on Bonferroni correction for number of SNPs tested. Regional association plots were generated by LocusZoom (<http://cgs.sph.umich.edu/locuszoom/>) ³⁶.

Results

SNPs in CAST gene are associated with keratoconus in a family panel

We analyzed seven SNPs (rs9314177, rs4869307, rs151904, rs10515244, rs27654, rs3214019 and rs27765) in the introns of CAST gene and genotyped them in a family-based keratoconus cohort containing 262 Caucasian individuals in 40 pedigrees. Additionally selected eighth SNP rs17086561 was not polymorphic and was excluded from the analysis. We used GWAF computer package ³⁴ to test genotype–phenotype association with family data, and identified two SNPs (rs4869307 and rs27654) associated with keratoconus with nominally significant p values $p=0.03$ and 0.07 , respectively (Table 1). These SNPs are located 64 KB apart.

Fine-mapping and replication using tagging SNPs

To perform a more comprehensive testing of CAST gene, we used tagging approach to identify SNPs with the highest potential to capture the greatest amount of common variation ³⁰. These optimized tag SNPs and two tentatively associated SNPs identified in the family panel were genotyped in both family panel and in case-control panel of 822 samples (304 cases vs. 518 controls). In addition, we selected SNPs with the highest potential of successful genotyping on Illumina's iSelect platform.

We identified several SNPs with a nominal association in the family panel; however, only one SNP (rs4434401) was associated in both family panel and in the case-control samples with p values of 0.005 and 0.05, respectively (Table 2). Meta-analysis after combining two cohorts identified an association at rs4434401 with a p value of 0.002 (Figure 1). The association remained significant after Bonferroni correction with $p=0.04$.

Discussion

CAST (Calpastatin) gene is located at 5q15 chromosomal region under the linkage peak identified in the genome wide scan of a four generation keratoconus family ²⁷. It is still located in the refined linkage region identified in a second linkage scan using tightly spaced SNPs ²⁸. We found that it contains SNP rs4434401 positively associated with keratoconus in both familial and case-control panels, with statistically significant Bonferroni corrected meta p value of 0.04. These results provide initial evidence for CAST gene involvement in keratoconus susceptibility. We are currently performing sequencing of CAST gene to identify coding variants associated with keratoconus risk alleles.

Calpastatin is a natural endogenous highly specific inhibitor of calpains, nonlysosomal intracellular proteases ³⁷. The calpains represent a well-conserved family of calcium-dependent cysteine proteases. The mammalian calpains include two ubiquitous isoforms, calpain 1 (μ -calpain) and calpain 2 (m-calpain), and additional tissue-specific proteins, such as lens-specific calpains Lp82 and Lp85 ^{38,39} and corneal epithelium-specific variant of

calpain 3 Cn94⁴⁰. These proteins show broad substrate specificity and influence many aspects of cell physiology including migration, proliferation and apoptosis⁴¹.

Animal studies suggest that calpain/calpastatin system is present in the different parts of the eye, including cornea. Studies undertaken in rats have demonstrated a calpain/calpastatin system in corneal epithelium, where it is speculated to play a role in epithelial cell turnover and wound healing⁴². Immunohistochemical localization of calpains and calpastatin in the rabbit eye revealed presence of μ -calpain, m-calpain and calpastatin in corneal epithelial keratinocytes, endothelial cells and in corneal fibroblasts keratocytes, albeit at variable levels⁴³. These results suggest that functions of calpain/calpastatin system vary from being involved in cytoskeleton modifications which occur in association with changes in cell shape and migratory activity and in mitotic activity during proliferation in the epithelium and in secretory processes in the other non-proliferating cells.

Mammalian calpastatin constitutes a part of calpain-calpastatin system, which is ubiquitously present in the majority of tissues; however, it also known to have multiple tissue-specific isoforms produced mostly by alternative splicing of the N-terminal exons^{44,45}. To get an insight on the calpastatin's expression in human eye tissues including cornea, we performed extensive examination of human expressed sequences located in the NCBI Unigene database (<http://www.ncbi.nlm.nih.gov/unigene/>) and in the Eyebrowse site (<http://eyebrowse.cit.nih.gov/>), which displays ESTs (Expressed Sequence Tags) obtained from cDNA clones from eye tissues derived from NEIBank and other sources. We identified a number of ESTs matching to the CAST gene in mixed fetal and adult eye tissue as well as in different parts of the eye such as trabecular meshwork, pterygm, lens, and cornea (Table 3). Corneal expression was limited to a single EST (Expression Sequence Tag) from corneal epithelial cells, a single EST from corneal stroma and, remarkably, five ESTs from the unamplified human keratoconus cornea library constructed by our group in collaboration with National Eye Institute⁴⁶. Based on sequence alignments between ESTs and known CAST transcripts located in the Reference RNA sequences (refseq_rna) database, we identified ESTs matching CAST transcript variant 2 (GenBank NM_173060.3), 6 (GenBank NM_001042440.2), and 11 (GenBank NM_001190442.1) which encode isoforms b, f, and m, respectively (Supplemental Figure 1 A, B), as well as non-coding transcript 12 (GenBank NR_033798.1) to be expressed in normal and keratoconus corneal tissue (Table 3, Supplemental Figure 1A). Interestingly, we also identified two ESTs derived from keratoconus corneal tissue to be uniquely matched to CAST transcript variant 2 (isoform b, Supplemental Figure 1B). Transcript variants 2, 11 and 12 have all been shown to be shorter than the longest CAST isoform f encoded by the longest CAST transcript variant 6 (Supplemental Figure 1A). Variant 2 differs in the 5' UTR, lacks a portion of the 5' coding region, initiates translation at a downstream start codon, and lacks in-frame exon compared to variant 6. Variant 11 has multiple differences in the coding region but maintains the reading frame, compared to variant 6. Variant 12 has multiple differences compared to variant 6 and identified in the Gene NCBI database as non-coding.

In contrast to cornea, in the lens tissue we identified three ESTs non-uniquely mapped to variants 2, 6, and 11 but also two ESTs with no perfect match to any of CAST transcripts in refseq_rna database (Table 3). Interestingly, blastn search of EST database using lens EST BU735062.1 identified significant match to two testis ESTs similar to testis calpastatin (GenBank Q15786). These results suggest tissue specific differences in nature of calpastatin regulation of calpains in the different parts of the eye.

Abnormalities in regulation of the calpain-calpastatin proteolytic system, including due to the genetic defects, are associated with several human disorders including cancer⁴⁷,

Alzheimer's disease⁴⁸, myocardial infarctions⁴⁹, diabetes⁵⁰, muscular dystrophies⁵¹, including muscular dystrophy LGMD2A⁵², and cataract formation⁵³.

The calpastatin to calpain ratio is an important factor in regulating calpain-induced proteolysis in tissues; however, because of additional important regulatory mechanism the effects are not always the same. High levels of calpastatin are implicated in lack of appreciable calpain-induced proteolysis in an experimental cataract model in primate and human lenses⁵⁴. Overexpression of calpastatin has been shown to significantly improve necrotic processes in the dystrophic muscle of mdx mice thus proving that inhibition of calpains might provide a therapeutic option for treatment of progressive muscular dystrophy DMD⁵⁵. It will be of great interest to assess expression levels and activity of different isoforms of calpastatin in cornea tissue of keratoconus patients and controls carrying risk allele of rs4434401 to determine whether this process is affected in the pathogenesis leading to corneal defects associated with keratoconus. This could open a possibility of developing non-surgical therapeutic option based on manipulation of the calpain-calpastatin system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Regional plot of meta p values of SNPs in CAST gene. Position of the positively associated SNP rs4434401 is defined by purple diamond. Other SNPs are represented by circles of different colors corresponding to their r^2 (measure of linkage disequilibrium) with rs4434401.

Table 1

Results of the initial association testing of randomly selected SNPs in CAST gene in families with keratoconus. MAF (Minor Allele Frequency) in cases (F_A) and controls (F_U) was presented separately.

SNP	Position	Minor allele	F_A	F_U	OR	P
rs9314177	96,028,860	A	0.33	0.35	0.89	0.52
rs4869307	96,028,968	A	0.19	0.27	0.67	0.03
rs151904	96,066,652	G	0.30	0.33	0.88	0.52
rs10515244	96,078,337	G	0.13	0.13	1.01	0.97
rs27654	96,093,222	A	0.31	0.37	0.78	0.07
rs3214019	96,103,110	G	0.05	0.03	1.84	0.15
rs27765	96,103,589	A	0.08	0.08	1.01	0.98

Table 2

Results of the follow up association testing with tagging SNPs in CAST gene in family and case-control panels. MAF (Minor Allele Frequency) in cases (F_A) and controls (F_U) was presented separately.

SNP	Position	MA	Families				Case-control				Meta analysis	
			F_A	F_U	OR	p	F_A	F_U	OR	p	OR	p
rs7733671	96,000,269	A	0.22	0.21	1.01	0.98	0.26	0.24	1.16	0.20	1.14	0.22
rs1421912	96,000,795	G	0.52	0.42	1.79	0.006	0.45	0.51	0.80	0.03	0.94	0.51
rs17086512	96,015,057	C	0.24	0.33	0.54	0.008	0.23	0.22	1.09	0.48	0.96	0.71
rs149319	96,025,249	A	0.24	0.24	0.93	0.79	0.32	0.28	1.22	0.07	1.18	0.09
rs4869307 *	96,028,968	A	0.19	0.27	0.67	0.03	0.20	0.19	1.12	0.68	0.78	0.10
rs4434401	96,039,025	T	0.52	0.40	1.92	0.005	0.53	0.48	1.23	0.05	1.32	0.002
rs10053056	96,043,420	T	0.54	0.43	1.81	0.008	0.47	0.43	1.17	0.14	1.25	0.01
rs149313	96,070,205	T	0.49	0.49	1.02	0.94	0.50	0.48	1.08	0.44	1.07	0.44
rs151835	96,070,982	A	0.35	0.34	1.08	0.78	0.33	0.29	1.17	0.15	1.15	0.15
rs754615	96,086,334	C	0.37	0.29	1.67	0.03	0.39	0.41	0.90	0.31	0.99	0.95
rs27654 *	96,093,222	A	0.31	0.37	0.78	0.07	0.33	0.28	1.28	0.03	1.05	0.60
rs27037	96,094,694	T	0.31	0.35	0.82	0.35	0.27	0.30	0.85	0.16	0.85	0.11
rs7356594	96,096,189	T	0.32	0.25	1.57	0.04	0.28	0.31	0.84	0.13	0.96	0.68
rs25862	96,104,835	A	0.49	0.36	2.01	0.003	0.47	0.48	0.94	0.51	1.05	0.57

SNP with significant association in both panels indicated in bold.

SNPs previously tested in family panel with no association in case-control panel are marked by *.

Table 3

Results of Nucleotide BLAST (blastn) searches of ESTs located in the Unigene database through Reference RNA sequences (refseq_rna) database. Organism Homo sapiens (taxid:9606) is selected.

GenBank EST	Eye Tissue	Matching CAST transcript variants
CV571754.1	Keratoconus Cornea	2, 6, 11
CV575316.1	Keratoconus Cornea	2, 6, 11, 12
CV575678.1	Keratoconus Cornea	2
CV570418.1	Keratoconus Cornea	2
CV573496.1	Keratoconus Cornea	2, 6
AA057646.1	Corneal Stroma	2, 6
BY999384.1	Corneal Epithelial Cells	2, 6, 11
BF726263.1	Lens	None *
BM675238.1	Lens	2, 6
BM685059.1	Lens	2, 6, 11
BM696435.1	Lens	2, 11
BU735062.1	Lens	None **
CD678651.1	Trabecular Meshwork	2, 6, 11
DR423722.1	Pterygm	2, 6, 11
DR423743.1	Pterygm	2, 6, 11
BM676369.1	Fetal Eyes	2, 6, 11
BM680808.1	Fetal Eyes	2, 6, 11
BM724289.1	Fetal Eyes	2, 6, 11
CK301125.1	Fetal Eyes	2, 6, 11
BU738777.1	Fetal Eyes	2, 6, 11
BM714053.1	Mixed Fetal/ Adult Eyes	2, 6
BM726189.1	Mixed Fetal/ Adult Eyes	2, 6, 11
BM932029.1	Mixed Fetal/ Adult Eyes	2, 6, 11
BM713247.1	Mixed Eye Tissue	2, 6, 11

* Sequence alignment indicates potentially novel lens-specific splice variant

** Sequence alignment indicates different potentially novel lens-specific splice variant