Quantitative trait loci associated with murine central corneal thickness

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Lively GD, Koehn D, Hedberg-Buenz A, Wang K, Anderson MG. Quantitative trait loci associated with murine central corneal thickness. Physiol Genomics 42: 281–286, 2010. First published April 27, 2010; doi:10.1152/physiolgenomics.00140.2009.—The cornea is a specialized transparent tissue responsible for refracting light, serving as a protective barrier, and lending structural support to eye shape. Given its importance, the cornea exhibits a surprising amount of phenotypic variability in some traits, including central corneal thickness (CCT). More than a mere anatomic curiosity, differences in CCT have recently been associated with risk for glaucoma. Although multiple lines of evidence support a strong role for heredity in regulating CCT, the responsible genes remain unknown. To better understand the genetic basis of CCT variability, we conducted a genomewide quantitative trait locus (QTL) analysis with (C57BLKS/J × SJL/J) F2 mice. This experiment identified a locus, Cctg1 (central corneal thickness QTL 1) on chromosome 7 (Chr 7; peak, 105 Mb), that is significantly associated with CCT. To independently test the biological significance of these results, (C57BLKS/J × NZB/BINJ) F2 mice were generated and analyzed for associations with Chr 7. This experiment identified a significant association at 131 Mb. Furthermore, low-generation congenic mice in which the Chr 7 QTL interval from the SJL strain was transferred onto the KS background had CCT values significantly higher than inbred KS mice. These results demonstrate that the genetic dependence of CCT in mice is a multigenic trait, which in these contexts is significantly regulated by a region on Chr 7. Future identification of the genes for these QTL will provide improved understanding of the processes regulating CCT and the pathophysiology of glaucoma.

GLAUCOMA is a group of degenerative optic neuropathies estimated to affect over 60 million people worldwide (42). Risk factors for glaucoma involve a mixture of physiological and hereditary factors, including intraocular pressure, age, race, and family history (25). Recently, central corneal thickness (CCT) has become recognized as an additional risk factor that must also be considered. The Ocular Hypertension Treatment Study, a large prospective study of people with ocular hypertension, found that participants with a CCT of ≤555 μm had a threefold greater risk of developing primary open-angle glaucoma compared with participants who had a CCT of >588 μm (4, 5, 15, 29). Similar findings have been reported in several additional studies (30, 36, 37). Adding to the negative consequences associated with decreased CCT, there is a strong ethnic contribution to CCT (1, 8, 11, 16, 27, 38, 49). For example, African-Americans on average have statistically thinner CCT than Caucasians (26, 50). Thus studies of CCT represent an opportunity to gain new knowledge of mechanisms contributing to glaucoma risk and address an important health disparity issue.

The biological basis for the association between CCT and glaucoma risk remains unknown. The cornea is not involved in regulation of aqueous humor dynamics and intraocular pressure, or for that matter any of the known clinical features of glaucoma pathophysiology. Thus the strong influence of CCT is quite surprising. In the absence of a specific hypothesis based on existing knowledge, one approach for uncovering the relationship of CCT to glaucoma is through phenotype-driven genetics. In humans, ethnic (1, 8, 11, 16, 27, 38, 49), familial (2, 10), and twin (45) studies all provide evidence suggesting that CCT is strongly influenced by heredity. Likewise, several studies have recently found that CCT is a genetic background-dependent trait among inbred strains of mice (33, 39, 41, 46). Among mice 100–120 days of age, mean CCT ranges from 89.2 ± 6.6 μm in C57BLKS/J (KS) mice to 123.8 ± 6.2 μm in SJL/J (SIL) mice, with a near continuous variation in other strains between these extremes (33). Thus genetic studies should be able to be employed to identify loci regulating CCT and, ultimately, to study the molecular mechanisms linking CCT to glaucoma risk.

Here inbred mice with strain-specific differences in CCT were utilized to study the heredity of CCT. There are very few inbred strains of mice that develop glaucoma, and none of these has known differences in CCT (21, 35). Therefore, it is not currently possible to study the heredity of both traits simultaneously. We propose a sequential strategy, first identifying loci influencing CCT and subsequently assessing their potential glaucoma relevance. Thus the present goal was to identify loci responsible for differences in CCT among non-glaucomatous strains. To accomplish this, three strains of inbred mice with differing CCT were utilized in genetic association studies: KS, SJL/J, and NZB/BINJ (NZB). With the finding that CCT manifests as a continuous trait among (KS × SJL/J) F2 progeny, a genomewide quantitative trait locus (QTL) analysis was utilized to identify the Cctg1 locus on chromosome 7 (Chr 7). Support for the importance of this locus was subsequently confirmed in independent studies with (KS × NZB) F2 mice and with low-generation congenic mice. These data demonstrate that although CCT is a complex trait regulated by the combined action of multiple genes, these loci are tractable and should be amenable to continued genetic dissection.

**METHODS**

Mouse husbandry. C57BLKS/J (abbreviated throughout as KS), SJL/J (abbreviated throughout as SJL), and NZB/BINJ (abbreviated throughout as NZB) mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were subsequently housed and bred at the University of Iowa Research Animal Facility. Mice were maintained on a 4% fat NIH 31 diet provided ad libitum and housed in cages...
containing dry bedding (SoftZorb Enrichment Blend; Northeastern Products, Warrensburg, NY). The environment was kept at 21 °C with a 12:12-h light:dark cycle. All animals were treated in accordance with the American Psychological Society’s “Guiding Principles in the Care and Use of Animals.” All experimental protocols were approved by the Animal Care and Use Committee of the University of Iowa.

Phenotyping. An ultrasound pachymeter (Corneo-Gage Plus; Sonogage, Cleveland, OH) was used to measure murine CCT (33). Probe movements of the ultrasound pachymeter were controlled with a micromanipulator under the guidance of a dissecting microscope to achieve perpendicular alignments between the probe and central cornea. Mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg). Balanced Salt Solution (Alcon Laboratories, Fort Worth, TX) was applied to the eye to maintain a consistent tear film.

A stringent set of criteria for data inclusion was established before collection of genotypes. All measurements were recorded from mice 100–120 days of age with clear corneas free from overt corneal disease. Both eyes of all mice were measured in three separate sessions within this 20-day period, yielding six measurements per mouse. With the four measurements closest to the median, the standard deviation (SD) of these four measurements was used to judge the reliability of the data. On the basis of empirical experience with measuring murine CCT (including repeat measurements of individual mice and surveying interstrain variability (33)), a SD of 1–2 μm is an estimate of the inherent biological and technical variability. Therefore, it was decided a priori that only F2 mice with a SD close to this limit (<2.5 μm) among these four measurements would be used for genetic analysis. Because CCT shows only trace biological variability in adults over a time course of days to weeks (14, 18), this inclusion criterion simply served to identify mice whose CCT measurements were as accurate as possible. According to these criteria, measurements from 92 of 215 (KS × SJL) F2 mice and 84 of 121 (KS × NZB) F2 mice were judged highly reliable. From mice meeting these criteria, the mean of the four most precise measurements was utilized for QTL analysis.

Genotyping. Genomic DNA was extracted from the spleen of each mouse. In the genomewide QTL analysis of (KS × SJL) F2 mice, mice from the 25% extremes (22 thinnest and 22 thickest CCT measurements) were genotyped by the Genomics Resource Center at Rockefeller University (New York, NY) with a commercial single nucleotide polymorphism (SNP) panel (Mouse Medium Density SNP Panel, Illumina, San Diego, CA). The panel contained 1,449 loci, of which 705 were informative for (KS × SJL) F2 progeny. Thus informative SNPs were spaced on average every 3.77 Mb over the genome (Fig. 1). In the subsequent analyses of Chr 7, the 92 (KS × SJL) F2 mice and 84 (KS × NZB) F2 mice were genotyped with 6 simple sequence length polymorphism (SSLP) markers within the region of interest (D7Mit318, D7Mit31, D7Mit321, D7Mit220, D7Mit238, D7Mit71).

Statistical analysis. To compare CCT between parental strains and F1 mice, P values were calculated with an unpaired two-tailed Student’s t-test. QTL analysis was performed with R/qtl (6, 7), using the logarithm of odds (LOD) score at 2.5-cM steps computed with 705 SNPs distributed over the entire genome with standard interval mapping by the EM algorithm (9, 28). For the genomewide scan, a significance threshold was set at a LOD score of 3.87 with stratified permutation testing (34). For the region-specific analysis of Chr 7, significance thresholds were computed by traditional permutation analysis (1,000 permutations, α values of 0.05 and 0.10 for significant and suggestive, respectively). Confidence intervals were computed by finding the area under the 100% LOD curve that contains 95% of the total area (Bayesian credible interval). Heritability was calculated by a R/qtl suggestion [1–10^(-1.96 LOD/n)], where n = sample size; Ref. 7]. The effect of sex was analyzed by including sex as an additive covariate and by including sex as an interactive covariate (6). Gene-gene interactions were determined by performing a two-dimensional genome scan with a two-QTL model versus none (7). For the genomewide gene-gene interaction analysis, significant and suggestive thresholds were set at LOD scores of 9.62 and 9.17, respectively, with stratified permutation testing (34). To compare differences of allelic effects at a single SSLP marker, a one-way ANOVA was used, with a P value < 0.05 considered significant. Unless otherwise stated, all CCT values are stated as means ± SD.

Generation and analysis of low-generation KS.SJL-Cctq1SJL congenic mice. Low-generation KS.SJL-Cctq1SJL mice were generated by reiterative backcrossing to KS mice for 4 generations, breeding mice carrying the SJL-derived allele of D7Mit321 in each successive generation. CCT was measured in N0F2 offspring by ultrasound pachymetry at 100–120 days of age.

RESULTS

Initial KS × SJL intercross. Because KS and SJL mice are from opposite ends of the known CCT spectrum and free from overt corneal disease (33), they represent an ideal resource for studying CCT genetics. In reciprocal matings, (KS × SJL) F1 progeny exhibited CCT values intermediate to the two parental strains (114.1 ± 6.2 μm; n = 16 eyes). When intercrossed, CCT of (KS × SJL) F2 progeny had a continuous distribution ranging from 94.7 to 123.1 μm (Fig. 2), suggesting multigenic inheritance. CCT values of male and female F2 mice were not statistically different (110.3 μm and 111.9 μm, respectively; P > 0.05). Interestingly, mean CCT values of the (KS × SJL) F1 and (KS × SJL) F2 progeny were not precisely midway between the parental values but were slightly skewed toward thicker CCT values (106.5 μm expected; 114.1 μm observed, 110.5 μm F2 observed). Combined, these results indicated that CCT was likely under multigenic influence and that a QTL method of analysis would be required to identify loci responsible for differences in CCT.

Identification of QTL for CCT. A genomewide scan was conducted with 92 (KS × SJL) F2 mice; 44 of these mice were genotyped across 705 SNP markers and represented the 25% phenotypic extremes, while 48 of the mice were initially not
genotyped. A region of interest was identified on Chr 7 (Fig. 3A; max LOD score 2.87 at 115 Mb) that exhibited a maximum heritability of 0.13 and a Bayesian credible interval extending from 65 to 139 Mb. Including sex as an additive covariate or an interactive covariate had no effect on the results of the genome-wide scan. Significance of the Chr 7 region was further tested by genotyping all 92 (KS/SJL) F2 mice with 6 SSLP markers flanking the position of max LOD score (Fig. 3B). This analysis confirmed existence of a significant QTL, Cctq1 (for “central corneal thickness QTL 1”). Cctq1 was characterized by a max LOD score of 3.28 at 105 Mb (Fig. 3C), well above the significant LOD threshold of 1.99 established by traditional permutation testing. The allelic effects of Cctq1 demonstrated a pattern of overdominance (Fig. 3D). To our knowledge, Cctq1 is the first known locus influencing CCT in otherwise healthy corneas that has been identified in mice or humans.

Genomewide gene-gene interactions were assessed by using the scantwo function of R/qtl (Fig. 4). The genomewide analysis of 92 (KS × SJL) F2 mice identified one suggestive interaction between loci on Chr 11 (Cctq2, 21.3 Mb) and Chr 17 (Cctq3, 92.7 Mb). This pairing resulted in a full LOD (LODf, i.e., 2 QTL vs. none) score of 9.16 and an interactive LOD (LODi, i.e., evidence for an interaction between QTL) score of 7.18. The thresholds for suggestive significance were 9.17 (LODf) and 6.20 (LODi), as determined by stratified permutation testing (6, 34).

Confirmation of QTL for CCT. As an independent test for the biological significance of the Chr 7 locus, a genetic association study was performed with F2 mice from related, but distinct, genetic contexts. Because of the particular pathological importance of decreased CCT and because KS mice have uniquely thin CCT, the KS strain was reutilized in an intercross with NZB mice (Fig. 5). NZB mice have a relatively thick cornea.
F2 progeny were not precisely midway between the parental \( \text{H9262} \) values (Fig. 5; 106.5 measurements but were slightly skewed toward thicker \( \text{KS} \)/\( \text{H11003} \) mice, mean CCT values of the (KS \( \times \) NZB) F2 progeny were intercrossed at generation N4 to assess whether the \( * \)Significance established by 1-way ANOVA, \( o \)vert corneal disease (33). In reciprocal matings, (KS \( \times \) NZB) F1 progeny had CCT values intermediate to the two parental strains (116.8 \( \pm \) 3.6 \( \mu \text{m} \); \( n = 14 \)). When intercrossed, CCT of (KS \( \times \) NZB) F2 progeny had a continuous distribution ranging from 97.4 to 131.7 \( \mu \text{m} \). As observed in the intercross of KS \( \times \) SJL mice, mean CCT values of the (KS \( \times \) NZB) F1 and (KS \( \times \) NZB) F2 progeny were not precisely midway between the parental measurements but were slightly skewed toward thicker CCT values (Fig. 5A; 106.5 \( \mu \text{m} \) expected; 116.8 \( \mu \text{m} \) F1 observed, 112.2 \( \mu \text{m} \) F2 observed). Potential significance of the Chr 7 region of interest was tested by genotyping all 84 (KS \( \times \) NZB) F2 mice with the same 6 SSLP markers utilized to assess the KS \( \times \) SJL intercross (Fig. 5B). A significant QTL was found at 131 Mb (max LOD score = 3.13; significant LOD threshold established by traditional permutation testing = 2.14). In this context, the NZB allele demonstrated a recessive influence (Fig. 5C). Given that the Bayesian credible interval for \( \text{Cctq1} \) extended from 65 to 139 Mb, it is likely that the peaks identified at 105 and 131 Mb from the two independent crosses represent the same locus.

To further test the existence of \( \text{Cctq1} \), we initiated creation of a congenic strain transferring the SJL-derived \( \text{Cctq1} \) allele to the KS genetic background (KS.\( \text{Cctq1}^{\text{SJL}} \)). A group of mice were intercrossed at generation N4 to assess whether the \( \text{Cctq1} \) locus conferred a biological influence on CCT in this more defined genetic context. Mirroring the overdominance observed with (KS \( \times \) SJL) F2 progeny, mice heterozygous for SJL alleles in the congenic interval had a CCT of 100.5 \( \pm \) 4.2 \( \mu \text{m} \) (\( n = 17 \) mice), which is significantly greater than the CCT of inbred KS mice (1-way ANOVA; \( P < 0.01 \)). Mice homozygous for SJL alleles in the congenic interval had a CCT of 94.7 \( \pm \) 3.8 \( \mu \text{m} \) (\( n = 12 \)), which was not statistically different from the CCT of inbred KS mice. Thus both the crosses with NZB mice and the analysis of low-generation congenic mice supported the existence of a significant QTL influencing CCT on Chr 7.

**DISCUSSION**

Decreased CCT has recently become recognized as an important glaucoma risk factor. An understanding of the biological reasons for this association is currently lacking, but could lead to identification of pathways important to glaucoma pathogenesis and treatment. Although CCT appears to be highly heritable, no genes influencing CCT in the absence of a concurrent ocular disease have yet been described. Here we initiated steps to find CCT-regulating genes by performing a QTL analysis from two intercrosses of inbred mouse strains with strain-specific differences in CCT. In both experiments, a pattern of multigenic inheritance was observed. A locus on Chr 7 was found, as well as interacting loci on Chr 11 and Chr 17.

The realization that decreased CCT is a significant glaucoma risk factor has influenced clinical practices and led to a reevaluation of many hypotheses of glaucoma pathophysiology. Increased intraocular pressure is the glaucoma risk factor that has traditionally received the most attention and is the only glaucoma risk factor currently treatable. Intraocular pressure is typically measured by tonometers in which corneal thickness and rigidity influence measurements. Unless corrected, variation in CCT between individuals will distort intraocular pressure measurements. Thus measurement of CCT has become a clinical standard of care so that intraocular pressure can be more accurately monitored. However, even after correction for the “true intraocular pressure,” decreased CCT remains associated with worse glaucoma outcomes (20). Therefore, there appears to be more to the association of decreased CCT and glaucoma risk than a simple indirect influence mediated by inaccurate intraocular pressure recordings.

**Fig. 5. Confirmation of QTL regulating CCT in (KS \( \times \) NZB) F2 mice. A: distribution of CCT in (KS \( \times \) NZB) F2 progeny: mean CCT of both eyes from triplicate measurements of 121 F2 mice recorded on separate days in mice 100–120 days of age. B: interval map based on 6 SSLP markers within the Chr 7 region of interest and assessed in 84 F2 mice. Suggestive (\( P = 0.10 \)) and significant (\( P = 0.05 \)) LOD scores are shown as dotted lines and based on traditional permutation testing. C: allelic effects for \( \text{D7Mit238} \) located at 126 Mb, near the max LOD score at 131 Mb in the analysis of (KS \( \times \) NZB) F2 mice (no. of mice in parentheses). \( ^* \)Significance established by 1-way ANOVA, \( P = 0.008 \).**
We hypothesize that there is a biological correlation between CCT regulation and glaucoma risk. The cornea itself is not involved in any of the known clinical features of glaucoma pathophysiology. However, genes influencing CCT may also act in other tissues of more direct glaucoma relevance such as the trabecular meshwork, retinal nerve fiber layer, or lamina cribrosa (19, 23). In this scenario, CCT would constitute a biomarker for a glaucomatous genotype.

On the basis of corneal anatomy and physiology, there are at least two major candidate pathways influencing CCT, extracellular matrix and electroosmosis regulators. Of the three main cellular layers of the cornea, the corneal stroma constitutes the majority of corneal thickness. The stroma contributes 90% of total CCT in humans (17, 32) and 66% of total CCT in mice (46). The stroma itself largely consists of a highly structured extracellular matrix with collagen fibrils arranged in lamellar sheets. With respect to glaucoma, the trabecular meshwork and lamina cribrosa also contain key extracellular matrix proteins. Therefore, genes influencing the extracellular matrix of the corneal stroma might influence one of these other tissues, thus simultaneously influencing CCT and glaucoma. Several candidates within the Cctq1 region share links to regulation of the extracellular matrix, including Adams13 and Adams17, predicted to encode regulatory components of the extracellular matrix (40).

Another pathway for CCT regulation pertains to electroosmosis. Fluid transport across the corneal endothelium and epithelium is a key component in regulating constant corneal thickness. Both layers continuously move water out of the cornea, prompting a relatively dehydrated stromal collagen thickness. Fluid transport across the corneal endothelium and matrix (40).

Among genes predicted to encode regulatory components of the extracellular matrix, Adamts13 and Adams17 have been identified in mice (47). Reduced expression or acting in corneal development.

Neither the loci identified here nor the regions of conserved synteny in human (max LOD score Cctq1, 11q14; max LOD score Cctq2, 2p13; max LOD score Cctq3, within a syntenic breakpoint between 2p16 and 18p11) contain known disease-causing mutations that influence the cornea or glaucoma. However, the Cctq2-Cctq3 interaction is interesting because the loci overlap a region of conserved synteny associated with glaucoma in humans (22). In a previously published study (33), we examined differential gene expression patterns between KS and SJL corneas. Key differentially expressed genes in the Cctq1 region are Spon1 and Adams13, both of which are associated with extracellular matrix tissues. Notable differentially expressed genes in the Cctq2 and Cctq3 region include Otx1 and Nrxn1, respectively. The vast majority of genes previously linked with CCT cause more broad corneal diseases such as keratoconus (13), corneal dystrophies (24), and Axenfeld-Rieger syndrome (3). Although important, these changes may not be typical of more subtle genetic differences that cause CCT to vary widely among individuals with healthy corneas. To date, linkage studies have identified chromosomal locations for 14 glaucoma-related genes in humans (GLC1A through GLC1N; Refs. 25, 48), none of which overlaps Cctq1, 2, or 3.

A limited number of QTL for intraocular pressure in human populations have been identified (12, 43), but none overlaps regions identified here. QTL for intraocular pressure have not yet been reported for mice; it will be interesting in future experiments to determine whether the Cctq1–3 loci might also influence intraocular pressure.

In conclusion, this work has utilized intercrosses of inbred mice and congenic mice to demonstrate that murine CCT is heritable and follows a multigenic pattern of inheritance. With QTL analysis, a significant QTL located on mouse Chr 7 has been identified (Cctq1). Suggestive QTL at two other locations were found, 21.3 Mb on Chr 11 (Cctq2) and 92.8 Mb on Chr 17 (Cctq3). To our knowledge, these findings represent the first identification of loci regulating normal variability of CCT in the absence of overt corneal disease. By extension, the regions of conserved synteny are evoked as candidates for regulating CCT in humans. In our ongoing work, we intend to narrow these QTL intervals to find individual genes regulating CCT and study the mechanisms by which these genes influence CCT and glaucoma susceptibility.

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DISCLOSURES
The authors have no conflicts of interest to disclose.

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