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Fine localization of a new cataract locus, *Kec*, on mouse chromosome 14 and exclusion of candidate genes as the gene that causes cataract in the *Kec* mouse

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A mouse with cataract, *Kec*, was generated from N-ethyl-N-nitrosourea (ENU) mutagenesis. Cataract in the *Kec* mouse was observable at about 5 weeks after birth and this gradually progressed to become completely opaque by 12 weeks. Dissection microscopy revealed that vacuoles with a radial or irregular shape were located primarily in the cortex of the posterior and equatorial regions of the lens. At the late stage, the lens structure was distorted, but not ruptured. This cataract phenotype was inherited in an autosomal recessive manner. We performed a genetic linkage analysis using 133 mutant and 67 normal mice produced by mating *Kec* mutant (BALB/c) and F1 (C57BL/6 x *Kec*) mice. The *Kec* locus was mapped to the 3 cM region encompassed by *D14Mit34* and *D14Mit69*. In addition we excluded coding sequences of 9 genes including *Rcbtb2*, *P2ry5*, *Itm2b*, *Med4*, *Nudt15*, *Esd*, *Lcp1*, *Slc25a30*, and *2810032E02Rik* as the candidate gene that causes cataract in the *Kec* mouse. [BMB reports 2008; 41(9): 651-656]

INTRODUCTION

Cataracts exhibit the formation of various levels of opacity in the transparent lens, and this can lead to blindness. In regard to the cataract's location within the lens substance, the most frequent morphological type of congenital cataract is confined to the nucleus, the lens sutures, the perinuclear layers (lamellar or zonular cataracts), the cortex, the anterior or posterior polar regions, or combinations of these (1). A number of inherited causes of cataract are known in both human and animals (2). Most of these cataracts (63% of all cataracts) are inherited in an autosomal dominant manner, 6% are autosomal recessive, 10% are X-linked, 16% are associated with other diseases, and the rest are from undetermined causes (3).

In most cases, the human cataract is known to be an age-related disease. Further more the genetic or early onset cataracts have heterogeneous manifestations with a syndromic or non-syndromic pattern (4). Due to complex nature of cataracts and ethical problems, identifying the causative gene for cataract and performing clinical studies in human are difficult. As an alternative measure, several model systems like fly, fish, mouse, and rat have been used for many years. The mouse lens and eye are remarkably similar to the human lens and eye in structure. In addition, both species have many similar ocular disorders (5, 6). Mouse models for inherited ocular disease allow for rapid genetic analysis, pathological characterization, and assessment of disease processes (6-8). Thus, mouse models provide solution and offer an opportunity to study human cataract formation resulting from genetic defects. Several methods, such as spontaneous breeding, genetic engineering, or random mutagenesis using chemicals and radiation, have been tried by the numerous researchers to acquire and study cataract mutants (9). One of these methods is N-ethyl-N-nitrosourea (ENU) mutagenesis, in which the potent mutagen ENU is used to generate mutants to determine the biological roles of the genes which are disrupted in these mice (10, 11).

A mouse with cataract, *Kec*, was discovered from our recessive trait screening scheme at the ENU mutagenesis program in the Korea Institute of Toxicology (KIT) (12). Among the mice generated from the mating between male BALB/c mice injected with ENU (200 mg/kg) and BALB/c female mice, we identified a cataract mouse which clearly showed eye opacity at about the 35th postnatal day. The cataract mutant was backcrossed and the mutation was maintained by sibling mating. The lens opacity was small and irregular at first, but it spreads to an entire lens in the adult mice. This phenotype was shown to segregate in a simple Mendelian fashion with full penetrance in intercrossing & outcrossing experiments. The homozygote exhibiting a nonsyndromic cataract was viable and

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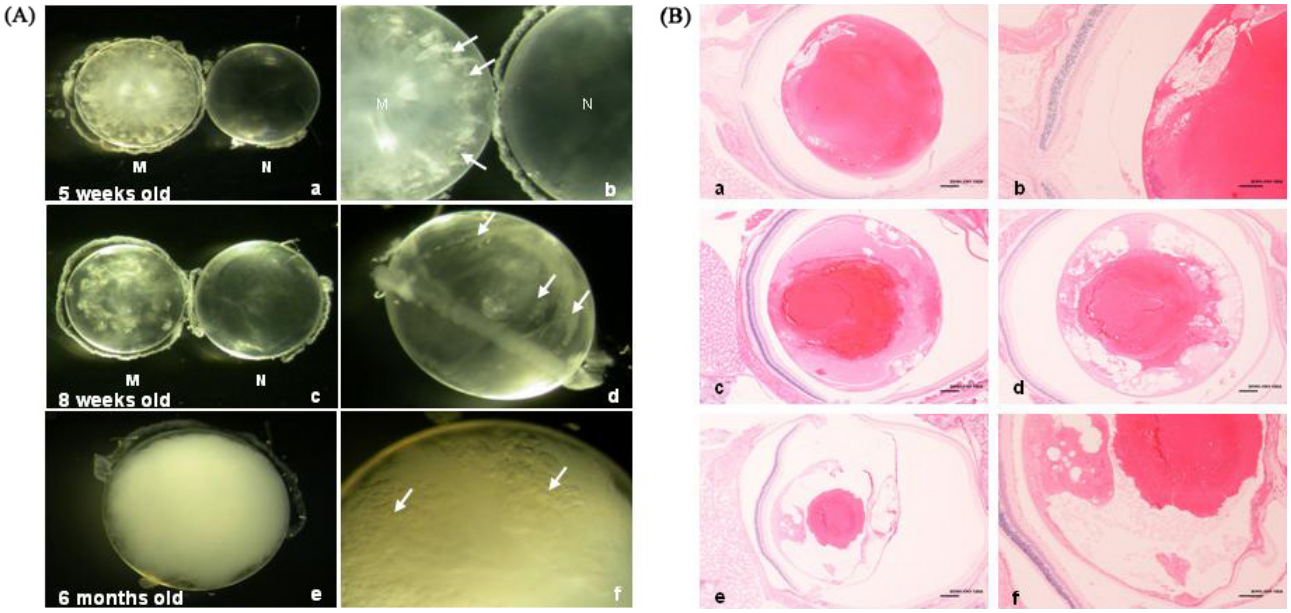

Fig. 1. Photographs of the lens of the *Kec* mouse. (A) The lens vacuoles (arrow) appear primarily in the posterior and the equatorial region (b, d). There was a radial distribution of vacuoles at the early stage (a, c). The whole lens become opaque and many irregularly distributed vacuoles are present at the late stage (e, f). (M: *Kec*, N: normal littermate). (B) Photographs of the *Kec* lens at 1 (a, b), 5 (c, d), and 12 (e, f) postnatal months: degeneration of the lens begins at the posterior cortex and bow region (a, b). Many vacuoles result from degeneration and liquefaction of lens fiber. The vacuoles and degenerated lens fibers increased with age. Degeneration finally causes collapse of the lens structure (e). The scale bar represent (a, c, d, e) 200 μm, (b, f) 100 μm.

there was no difference in the sex ratio of the mutants, indicating that this trait was inherited in an autosomal recessive mode (12). To identify the cataract-causing gene in the *Kec* mutant through the positional cloning approach, we determined the *Kec* locus by genetic linkage analysis and screened the candidate genes located in the critical region for the mutation that co-segregated with the phenotype.

RESULTS

Phenotypic analysis

One of the pheno-deviants identified from screening recessive mutations at the ENU mutagenesis program in the KIT displayed opacity of the eyes without any other specific phenotype. This phenotype persisted in the following generations and this mutant line was established through breeding in the BALB/c background until the 12th generation by mating between homozygotes. The fertility and viability of this mutant mouse were normal and it was named *Kec*.

The lens opacity was small and irregular at first but spread to an entire lens at adulthood. The eye of *Kec* grossly displayed opacity throughout the lens. Microscopically, the *Kec* eye displayed vacuoles in the whole lens and irregularly distributed lens fibers at 5 and 8 weeks after birth. By 6th month, the whole lens was opaque (Fig. 1A). The vacuoles primarily exist in the circumference of the lens from the posterior region to the bow region. The 5-month-old lens showed breakdown of the cortex that caused by the enlarged and increased vacuoles. The 12-month-old lens showed collapse of the lens structure without rupture (Fig. 1B).

Linkage analysis

Determination of the *Kec* locus was achieved by two steps. The first step was to identify the chromosome linked to the *Kec* locus. Twenty one cataract mice (N2) generated from the backcross of F1 (*Kec* X C57BL/6) to *Kec* mice were analyzed by simple sequence length polymorphism (SSLP). A total of 49 microsatellite markers which show polymorphism between the BALB/c and the C57BL/6 were selected, and the genotyping was carried out by polyacrylamide gel electrophoresis (PAGE) analysis following polymerase chain reaction (PCR).

Since recombination was not observed with the markers of *D14Mit113, D14Mit203*, and *D14Mit193*, this initial segregation analysis identified the *Kec* locus to the 24.8 cM region between *D14Mit259* and *D14Mit195* on chromosome 14 (Table 1).

For fine mapping of the *Kec* locus, the second step genotyping was performed with the markers in between *D14Mit259* and *D14Mit195; D14Mit28, D14Mit114, hairless, D14Mit193, D14Mit34*, and *D14Mit69* makers on 112 mutant mice and 67 normal mice that were generated from the same backcross used for the initial mapping. The haplotypes of 179 N2 mice were summarized in Fig. 2. There was no recombination with

Table 1. Chromosome 14 markers used for initial mapping

Markers	Position	Affected		CC ratio (CC/total)	x^2 value
		CC[a]	BC[b]		
D14Mit141	15 cM	20	1	0.95	17.190
D14Mit214	19 cM	20	1	0.95	17.190
D14Mit259	19.5 cM	20	1	0.95	17.190
D14Mit113	25 cM	21	0	1.00	21.000
D14Mit203	28.3 cM	21	0	1.00	21.000
D14Mit193	40 cM	21	0	1.00	21.000
D14Mit195	44.3 cM	20	1	0.95	17.190
D14Mit107	63.0 cM	12	9	0.57	0.429

a; Mutant allele, b; BALB/c, C57BL/6 heterozygous.

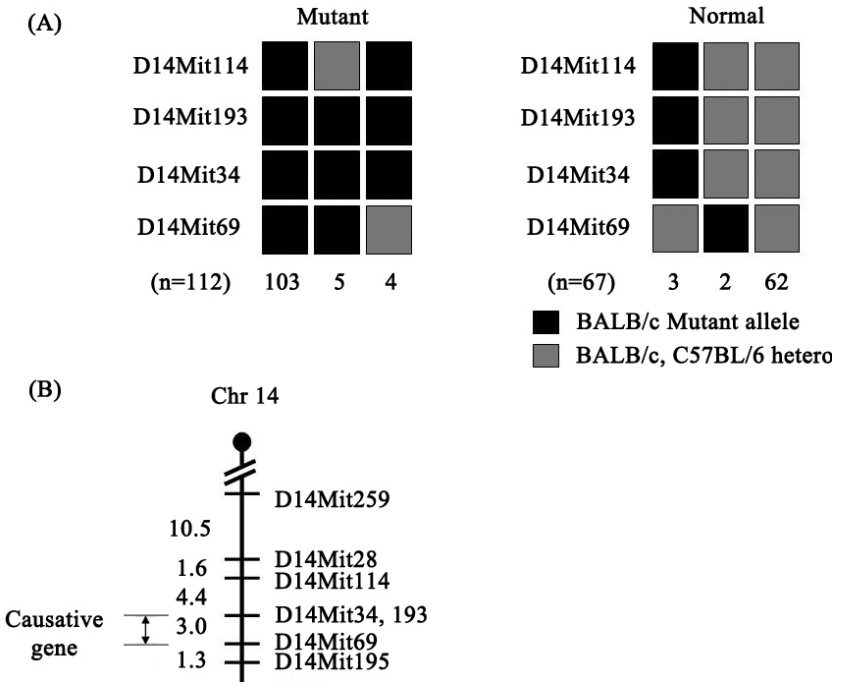

Fig. 2. Genotype summary and genetic map. (A) Genotype summary of 112 mutant and 67 normal offspring resulted from the *Kec* X F1 (*Kec* X C57BL/6) backcross. The black squares represent mutant alleles and the gray squares represent BALB/c and C57BL/6 heterozygous alleles. (B) Genetic map of the causative gene region on mouse chromosome 14. The position of each marker, causative gene, and genetic distances are shown.

hairless, D14Mit193 and *D14Mit34* in the mutant mice, thus *Kec* locus was mapped to the 8.76 cM region between *D14Mit114* and *D14Mit69*. To exclude the region not linked to the locus, 67 normal N2 mice were genotyped. The proximal and distal boundary of the *Kec* locus was determined by three and two wildtype mice with the BALB/c homozygous genotype for *D14Mit34* and *D14Mit69*, respectively. This exclusion mapping placed the *Kec* locus to the 3 cM region between *D14Mit34* and *D14Mit69* (Fig. 2B).

Mutational analysis

To identify the genes located in the 3 cM region between *D14Mit34* (40.0 cM) and *D14Mit69* (43.0 cM), we searched the database (http://www.ncbi.nlm.nih.gov/mapview). Among the transcripts mapped in this region, we excluded pseudogenes and the genes with no expression in eye for the obvious reason. We selected nine candidate genes based on their positive expressions in eye as well as their functional relatedness. Those candidate genes included regulator of chromosome condensation (RCC1) and BTB (POZ) domain containing (*Rcbtb2*), purinergic receptor P2Y, G-protein coupled, 5 (*P2ry5*), integral membrane protein 2B (*Itm2b*), mediator of RNA polymerase II transcription, subunit 4 homolog (yeast) (*Med4*), nudix (nucleoside diphosphate linked moiety X)-type motif 15 (*Nudt15*), esterase D/formylglutathione hydrolase (*Esd*), lymphocyte cytosolic protein 1 (*Lcp1*), solute carrier family 25, member 30 (*Slc25a30*), and the RIKEN cDNA

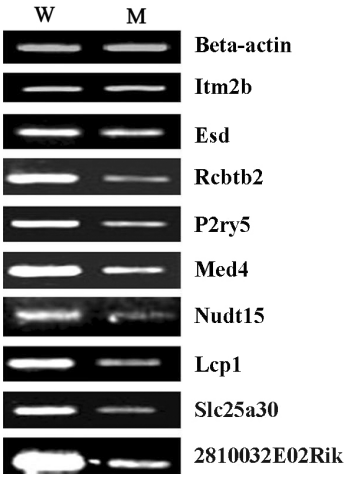

Fig. 3. RT-PCR analysis of mRNA expression of the candidate genes in the eyes of BALB/c and *Kec* mice. W denotes 8-week-old BALB/c, and M denotes 8-week-old *Kec*.

2810032E02 gene (*2810032E02Rik*). First, we determined the expressions of the candidate genes in the *Kec* mouse by reverse transcription-PCR (RT-PCR) and then compared them to those in the wildtype BALB/c mouse to confirm their expression in eye and to investigate the possibility of a mutation in the regulatory region. All the genes we tested were expressed in eyes of *Kec* as well as in the wildtype mice. Although they displayed various expression levels, there was no obvious mutation causing the absence of expression, as shown in Fig. 3.

To search for the mutation in the candidate genes that causes cataract, the sequences of all the exons with flanking introns were analyzed by direct sequencing following PCR. For a few genes, a series of cDNA fragments were amplified by RT-PCR and sequenced; thus, they were screened for the coding regions. The gene specific primers were derived from the reference sequences in Supplemental Table 1. The sequences of the genes in the mutant were determined and they were primarily compared with those in the database using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Any discrepancy in the sequences was evaluated by sequence comparison with that of the wildtype mouse. Despite an assiduous effort, there was no mutation present that would cause either amino acid changes or aberrant splicing in these genes we investigated. Thus, this excluded these nine genes as the candidate genes for the cataract phenotype in the *Kec* mouse.

DISCUSSION

There are roughly 4 types of cataract, including cortical cataract, nuclear cataract, lens extrusion cataract and diffuse cataract, according to the lens morphology (13). In the mouse, diffuse cataract means that the lesion simultaneously appeared in various sites of the lens like the capsule, cortex and epithelium (13). Nuclear cataract involves the lens nucleus. Because it is the oldest part of the lens, the existence of a nuclear change tells us that the causative gene works during early ocular development (14). The cortical type exhibits degeneration or other abnormal changes in the lens cortex. It includes a breakdown of cortex components, shift of the lens nuclei to the posterior area and production of abnormal lens fiber (15). The epithelial cataract is caused by abnormal lens epithelium. It may generate focal or diffuse overproduction of lens capsular material. In some cases, the rupture of the posterior lens capsule often occurred (16). Classifying human cataract is difficult due to the wide variations in lens morphologies. Thus categorization by the location of the opacity rather than its appearance accommodates developmental considerations and reflects the underlying genotype.

The cataract in the *Kec* mice shows characteristics of vacuolation of the whole lens and distortion of lens structure occurring later. The degeneration of the lens started in the cortex of the posterior and polar regions at the early stage of cataract development. The cataract lens could be grossly distinguishable from the normal lens at 5 weeks after birth. Small vacuoles and degenerative lens fibers were mainly arranged to the direction of proliferation of the secondary lens fiber. We also observed vacuoles located in the anterior suture and other areas, but this was rare. At the late stage, the vacuoles increased in size and number and they appeared in the whole lens, including the nucleus, cortex and the anterior and polar areas, yet no disruption of the lens capsule or lens rupture was observed. Thus, morphologically, the cataract in the *Kec* mouse is defined to be the cortical type.

The cataract phenotype in *Kec* mice is similar to zonular pulverulent cataracts, which display a powdery, snowflake appearance for the opacification (17). Autosomal dominant zonular pulverulent cataracts have been described in association with mutations in the gap-junction protein genes alpha 3 (*GJA3*) (3, 18) and alpha 8 (*GJA8*) (19-21) and the crystalline gene, gamma C (*CRYGC*) (18). Since *GJA3* is located on mouse chromosome 14, we screened for a mutation in *GJA3* that was responsible for the cataract in the *Kec* mouse but failed to find it (data not shown). Thus, there must be other defect responsible for cataract in the *Kec* mouse.

Although mutations in many genes with variety of functions have been reported to cause cataract to date, the cataract causing mutations are not randomly distributed. Rather, these genes play essential roles in eye and lens development and for maintaining the transparency and integrity of the eye. These include the crystalline genes, transcription factors and lens membrane genes (22). In the *Kec* locus encompassed by *D14Mit34* and *D14Mit69*, there was no gene whose function obviously fits to the categories mentioned above. Thus, we selected the candidate genes in this region mainly on the basis of their positive expression in eye and we confirmed their expressions by RT-PCR. All nine genes were expressed in both the wildtype and mutant eyes. There was no mutation causing

an absence of expression of the genes, although some of them showed a reduced level of expression.

The sequencing analysis indicated that none of the nine candidate genes contain any mutation in their coding regions. At this point, we can not completely rule out these genes from the cataract-causing candidate genes. Further study is required to discern the possibility that there is a mutation causing cataract in the regulatory region of one of these genes and its reduced expression may result in cataract. Alternatively, other gene in this critical region could be responsible for the cataract phenotype in the *Kec* mouse. Identification of this gene will facilitate clarifying the mechanism of cataract in the *Kec* mouse.

MATERIALS AND METHODS

Mice

The mutant breeding stock was developed and maintained at the KIT of the Korea Research Institute of Chemical Technology. An ENU derived cataract mutant was designated *Kec*. The mutant mice were maintained under specific pathogen-free conditions with regulated light (07:00-19:00), temperature (23 $\pm$ 1^{o}C), humidity (50 $\pm$ 5%), and ventilation (10-12 times per h). The care and treatment of the animals were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All animal experiments conformed to the Guidelines for Animal Experimentation (Decision of the University Council, 1989). The ages of mice used in this study ranged from newborn to 12 months. The control animals were age-matched littermates or wild type BALB/c.

Morphological observations

For phenotypic characterization, all the removed specimens were examined with a Nikon SMZ800 dissecting microscope (Nikon, Tokyo, Japan). The lens specimens obtained from 5-week-old, 8-week-old, and 6-month-old mice were enucleated. The sites examined for the presence of cataracts included the anterior and posterior sutures, and the polar or the bow regions. Photographs were taken using Nikon Coolpix 5400 digital camera system and Photoshop ver. 6.0 software.

Histology

Mice were sacrificed by cervical dislocation at 1, 5, and 12 months of age. The eyes of the mutant mice and their normal littermates were removed surgically and fixed in 10% phosphate buffered formalin. The eyeballs were dehydrated in a series of increasing ethanol concentrations, embedded in paraffin (Leica, Wetzlar, Germany), after and lucidification in xylene. Thin sections (4 μm) were stained with hematoxlyin and eosin following standard methodology. Light microscopic examination utilizing a Nikon E600 instrument was performed for histological analysis.

Genetic mapping

Intra-specific progenies were generated to genetically map the *Kec* cataract locus. *Kec* cataract BALB/c (*Kec*) mice were outcrossed to normal C57BL/6 (B6) mice. The F1 progeny of the *Kec* x B6 mating were backcrossed to the *Kec* mice. Twenty-three cataract progeny were used for initial mapping, with 112 cataract and 67 normal progeny being used for the fine mapping. The markers were selected based on their positions within the genome and the primer sequences were obtained from the database (http://www.informatics.jax.org). The markers for the initial mapping are summarized in Supplemental Table 2. Five additional polymorphic microsatellite markers (*D14Mit28, D14Mit114, D14Mit193, D14Mit34,* and *D14Mit69*) and a single nucleotide polymorphism marker, *hairless*, were used for the fine mapping by a standard method. Briefly, genomic DNAs were isolated from the mouse tail as previously described (Transgenic Animal Web; http://www.med.um-ich.edu/tamc/tDnA.html). PCR was performed in a 20 μl reaction mixture that contained 25-50 ng of template DNA, 1X Taq buffer, 200 μM each dNTP, 1 U of Taq polymerase, and 0.2 μM of each primer. PCR amplification was carried out with an initial denaturation step at 94^{o}C for 2 min and then 35 cycles of denaturation at 94^{o}C for 30 s, annealing for 30 s, and extension at 72^{o}C for 30 s; a final extension step at 72^{o}C was done for 10 min. The annealing temperatures were variable depending on the markers. PCR products were analyzed by PAGE or agarose gel electrophoresis as described previously (23). Genetic linkage was assessed by segregation analysis and the statistical significance of the recombination was determined by χ^2 analysis (24).

Mutational analysis

Mutational analysis was carried out with either cDNAs or genomic DNAs. Total RNA was extracted from the eyeball of 8-week-old mice with TRIZOL reagent (Invitrogen, Carlsbad, CA) as recommended by the manufacturer. The cDNA synthesis was carried out with reverse transcriptase (Invitrogen) at 37^{o}C for 1 h using a mixture of random hexamer and oligo dT nucleotides as primers. The gene specific primers were designed using the primer3 program (http://frodo.wi.mit.edu/cgibin/primer3/primer3_www.cgi) from the cDNA or genomic DNA sequences. All DNA fragments were purified using the GENECLEAN® Turbo kit (Qbiogene, Irvince, CA) and were directly sequenced using an ABI Prism System (Applied Biosystems, Foster City, CA) as described previously (25). The sequences were analyzed using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). Amplification was carried out as described above using a Peltier PTC-100 Thermal Cycler (Bio-Rad, Hercules, CA).

The expression of the candidate genes was analyzed by RT-PCR. PCR amplification conditions were the same as above except the annealing temperature was 60^{o}C The PCR products were separated on 2.5% agarose gels. The primer sequences are summarized in Supplemental Table 3.

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