Fine Localization of Nefl and Nef3 and its Exclusion as Candidate Gene for Lens Rupture 2(lr2)

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Abstract: Cataract causing *lr2* gene is found in the CXSD mouse, which is a recombinant inbred strain of BALB/c and STS mice. For the process of positional cloning of *lr2*, several candidate genes were selected in the middle region of chromosome 14, but most of them were excluded by combination of recombination and homozygosity mapping. Components of neurofilament proteins, neurofilament light polypeptide (Nefl) and neurofilament3 medium (Nef3), were linked to D14Mit87 which was not separated from the *lr2* locus in the homozygosity mapping. When the expression levels of Nefl and Nef3 in eyes were compared in CXSD and BALB/c mice, there were no differences in expression levels. The cDNA sequences of the two genes from CXSD, BALB/c and STS mice were subsequently compared. Several nucleotide differences in cDNA sequences were detected between the mice strains but the majority of the changes were silent mutations that did not alter the amino acids. The sole amino acid difference, E567K in the glutamate rich region of Nfm, between BALB/c and CXSD was found to be a simple genetic polymorphism because the same substitution existed in STS, a non-cataract mouse strain. Therefore we excluded Nefl and Nef3 from the candidate genes for *lr2* based on expression and mutation analyses.

Key words: cataract, exclusion, neurofilament genes, rupture

Introduction

Cataract is an opacity formation in the transparent lens, which may lead to blindness. A number of inherited cataracts are known in human and animals (Hejtmancik *et al.* 1998). So far, several model ani-

mals of inherited cataract have been isolated (Iida *et al.* 1997). A recessive hereditary cataract mouse was discovered in the mouse strain, CXSD, which was one of the recombinant inbred (RI) strains derived from the mating of BALB/cHeA and STS/A mice (Hilgers and Arends 1985; Song *et al.* 1997a). The mutant gene has

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been named lens rupture 2 (*lr2*, Mouse Gene Database accession number: MGD-JNUM-37399) because of the histological observation of posterior protrusion of lens materials accompanied by vacuoles in lens fiber. In CXSD mice, the first clinical symptoms of cataract appear at 5 weeks after birth and it seems to be completely penetrated. The *lr2* locus had been mapped to the middle region near *D14Mit28* from the analysis of backcross mice (Song *et al.* 1997b). Subsequently, the *lr2* locus was finely mapped on the critical region of the 0.38 cM interval in the middle region of Chr14. Several candidate genes were selected based on the previously reported maps (Dietrich *et al.* 1992 and 1996; Doolittle 1996) but were excluded by genetic or homozygosity mapping using the F₂ mouse produced by intersubspecific mating between CXSD and *Mus musculus molossinus* (MSM) mice (Rhee *et al.* submitted).

In this report, we mapped *Nefl* and *Nef3* and found that they are located in the critical region of the *lr2* locus on mouse Chr 14, suggesting a strong possibility that they might be candidate genes for *lr2*. Thus the expression pattern and the sequences of the genes were analyzed to determine whether they are responsible for cataract formation in CXSD mouse.

Materials and Methods

Animals and Production of F₂ Mice: All mice were maintained in a barrier system under normal conditions of lighting (07:00–19:00 h), temperature (24 ± 0.5°C), relative humidity (55 ± 5%) and ventilation (10–12 times per hour). Mice were given filtered tap water *ad libitum* in accordance with *Guide for the Care and Use of Laboratory Animals* (NIH). All animal experiments were carried out in accordance with the *Guidelines for Animal Experimentation* and with the permission of the *Institutional Animal Care and Use Committee* (IACUC of KRICT). CXSD mice, bearing the recessive cataract gene, have maintained for 43 generations by brother and sister mating in Osaka Prefecture University and the Korea Research Institute of Chemical Technology. MSM mouse was kindly provided by Drs. Miyashita and Moriwaki (National Institute of Genetics, Mishima, Japan). For genetic mapping, we generated intersubspecifically intercrossed progeny; that is, cataract CXSD mice were mated to MSM, and the (CXSD × MSM) F₁ progeny were intercrossed [(CXSD ×

MSM)F₂].

Genomic DNA Preparation, Polymerase Chain Reaction (PCR) and Mapping with Single Strand Conformational Polymorphism (SSCP) Analysis: Genomic DNA was isolated from the mouse tail by the procedure described elsewhere (Mori *et al.* 1995). Primers for mapping were derived from 3' UTR of the mouse cDNA sequences as shown in Table 1. The polymerase chain reaction (PCR) was performed on 20 µl reaction mixtures containing 1–10 ng template DNA, 1X Taq Buffer, 1.5 mM MgCl₂, 200 µM dNTPs, 0.2 µM of each primer and 0.75 U of Taq polymerase (Perkin-Elmer, Norwalk, CT, USA). PCR amplification was done with the following conditions using a thermocycler, model T-Gradient (Biometra, Goettingen, Germany): initial denaturation at 94°C for 2 min; 35 cycles at 94°C for 30 s, annealing for 30 s, and 72°C for 1 min 30 s; final extension at 72°C for 5 min. Annealing temperatures are indicated in Table 1. The long PCR products were analyzed with the SSCP technique after digestion with a restriction enzyme, Hae III or Sau3A I. SSCP analysis was done by a slightly modified method of Spinardi *et al.* (1991). The PCR products or its digested products were denatured by boiling in buffer solution containing 95% formamide, 10 mM EDTA, 5 mg/ml bromophenol blue and xylene cyanol. After cooling on ice, DNA fragments were separated by polyacrylamide gel electrophoresis with 4% stacking gel in 120 mM Tris-HCl buffer (pH 7.3) and 8–15% running gel in 760 mM Tris buffer (pH 8.2) with electrophoresis buffer of 12 mM Tris, 96 mM glycine, pH 8.5 at constant temperature of 18°C for 4–5 h, 15 mA. DNA fragments were visualized by staining the gel with ethidium bromide or silver staining (Amersham Pharmacia Biotech, Uppsala, Sweden). The genetic locations of genes were mapped with the previously reported genetic recombination panel (Rhee *et al.*, submitted).

RNA Isolation and cDNA Synthesis: Total RNA was extracted from the eye balls of 5 weeks old animals by guanidine hydrochloride lysis (Sigma, MO) and cDNA synthesis was carried out with a reverse transcription system (Takara, Japan).

Expression of *Nefl* and *Nef3* in the Eyes: The expression levels of *Nefl* and *Nef3* were analyzed with reverse transcription PCR (RT-PCR) methods. The PCR amplification conditions were initial denaturing at 94°C for

Table 1. Primer sequences used in genetic mapping and mutant analysis of *Nefl* and *Nef3*

Name	Forward primer	Reverse primer	Anealing Temp (°C)	Product size (bp)	Source
For Mapping					
<i>Nefl</i>	TCGCAAACATGTTGCCTGGAA	GCCTGTGCTTGTGCGGAGTA	55	820	GB:M55424
<i>Nef3</i>	GGAGTCCGTTGCAAAAGGT	TTGCATGCAGTTCCTCATA	55	140	GB:X05640
For Mutant Analysis					
<i>Nefl</i>					GB:M20480
L1479	GAGGAGGAGAGGAAAGAGGA	TTCGTGTTGTGTGTGTGGTTT	55	540	
L1230	ATTTTCACCAGCGTGGGTAG	CAACTGGTTGGTTGGTGATG	55	500	
L954	CTCCGCTGCTCAAGGCTAA	GGGGCTCATCCTTGGCCTCCT	60	490	
L695	GAAGAAGGTGCACGAGGAAG	AAGCTGCAATCTCGATGTCC	55	500	
L325	ATCGCTTCGCCAGCTTCATC	TCGTACTGAGCGCGGATGTC	55	500	
L20	TGCAGTCACCATGAGTTCGT	AGCGCTTGCTTCTCGTTAGT	55	490	
<i>Nef3</i>					GB:X05640
M2192	GAAGGAGAAGGCAGAGGAGG	TTGCATGCAGTTCCTCATA	55	600	
M1712	TGAGAAGGAAGGCTCCAGTG	ACTCCCTTCCTCCTCACTGC	55	500	
M1313	ATTTTCAGGAAGCATCACCAG	GCATAGGGGATTTTGCTTTC	55	600	
M903	CAGACCAGAACATGCACCAG	GAGTTGGACCTTGAGCTTGG	55	500	
M508	TTACGACCAGGAGATCCGAG	CGATCTCTTCCTTGGCAGAG	55	500	
M55	GGCCTCCAAGATGAGCTACA	TCCTCCAAGTGATCGGAGTC	55	550	

Nefl, Neurofilament light polypeptide; *Nef3*, Neurofilament3 medium.

15 min, followed by 35 cycles consisting of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, with a final extension at 72°C for 5 min. Then the PCR products were analyzed with 1.5% agarose gel electrophoresis. The expression quantity was estimated by comparing the band density with that of housekeeping gene, GAPDH, using a densitometric computer program (ImageMaster Total Lab. Ver 1.0, Amersham Pharmacia Biotech., Uppsala, Sweden).

Sequence Analysis: The primers for obtaining cDNA sequences were derived from the reported sequences (GenBank: accession numbers listed in Table 1) and purchased from Bioneer Corp. (Cheongju, Korea). RT-PCR products were cloned into pCR2.1 vector with a TA cloning kit from Invitrogen (Leek, the Netherlands) and both strands were sequenced using an ABI Prism System (Perkin-Elmer Corp. Calif. USA).

Results

We previously obtained the mapping panel from 10 mice bearing recombinations between *D14Mit33* and *D14Mit67*. *D14Mit28* and *D14Mit87* were found to be located in the critical region with homozygosity map-

ping (Rhee *et al.* submitted). The locations of several genes including *Nefl* and *Nef3* that were previously mapped in the vicinity of the middle region of Chr. 14 were determined with SSCP and SSCP analysis. We were able to exclude most of these genes from the candidate genes. But *Nefl* and *Nef3* were found to be closely linked with *D14Mit87* that was not separated from the cataract phenotype in the homozygosity mapping (Fig. 1). In addition, *Nefl* and *Nef3* were located in the same YAC clones in the physical map of the critical region (Rhee *et al.* submitted), thus suggesting these genes are strong candidates for *lr2*.

In order to investigate whether one of these genes is responsible for cataract in CXSD, their mRNA expression levels in CXSD and BALB/c mice were compared. After total RNAs from the eyes of 5 weeks old mice of BALB/c and CXSD mice were obtained, the RT-PCR was used for analyzing whether mRNA expression levels of *Nefl* or *Nef3* were altered or not. The *Nefl* and *Nef3* expression levels in eye of the CXSD mouse, however, were not different from that of the BALB/C mouse as shown in Fig. 2.

In order to analyze the sequences of *Nefl* and *Nef3*, a series of cDNA fragments of all coding regions were

Fig. 1. Genetic mapping of *Nefl* and *Nef3* by SSCP analysis. The mapping panel was composed from 10 recombinant F₂ mice produced by mating between CXSD and MSM mice. SSCP analysis was done by the method described in Materials and Methods. A Microsatellite markers and their map position; B distance between markers in centimorgan (cM); C *lr2* locus base on homozygosity mapping and mating experiment.

Fig. 2. mRNA expression patterns of *Nefl* and *Nef3* in the eyes of BALB/c and CXSD mice analyzed by RT-PCT. Lane 1, β -action; lane2, *Nefl*; lane3, *Nef3*.

obtained from strains of CXSD, BALB/c, and STS mice by RT-PCR, using primer sets derived from the previously reported sequences (GenBank accession numbers; M20480 for *Nefl* and X05640 for *Nef3*). All of the sequences were primarily compared with those cited in Genbank and with each other by BLAST. *Nefl* of the CXSD mouse has several nucleotide changes when compared to that cited in GenBank, but all nucleotide changes are silent and do not result in changes to the amino acid sequence. In the case of *Nef3*, nucleotide changes were also observed at several positions of the sequence of GB dB: X05640, including a G to A transition at the nt4438 position of the cited sequence

Fig. 3. Result of mutation analysis of *Nef3* compared with the cited sequence of GenBank for CXSD, BALB/c and STS mice. * indicates the position of nucleotide exchange in GenBank that resulted in an amino acid change, E567K.

resulting in a Glu567Lys amino acid change. The cDNA sequences from STS and BALB/c mice were subsequently analyzed to confirm the mutation. Only the G4438A change in the glutamate rich region of the carboxyl terminus of *Nef3* was a real nucleotide change between CXSD and BALB/c (Fig. 3), but the nucleotide sequence of the CXSD mouse was the same as that of STS mouse which does not show the cataract phenotype. Thus, these data indicate that the G4438A mutation does not change the protein structure of *Nef3* functionally, but is a simple genetic polymorphism between the mouse strains.

Discussion

Previously, we localized *lr2* gene to *D14Mit28* on Chr. 14 using backcross progeny from the mating of STS and cataract CXSD mice, and by typing 5 polymorphic markers between *D14Mit155* and *D14Mit34* (about 7 cM) (Song *et al.* 1997b). The *lr2* locus has been localized to a 0.38 cM region using a combination of homozygosity mapping and additive mating with classical genetic mapping using intersubspecific mating between CXSD and MSM mice (Rhee *et al.* submitted). In addition to fine mapping of *lr2*, we determined the position of several candidate genes of the cataract phenotype using genetic mapping as well as physical mapping. These genes include B lymphoid tyrosine kinase (*Blk*), cathepsin B (*Ctsb*), A disintegrin and metalloprotease domain 7 (*Adam7*), clustrerin (*Clu*),

bone morphogenetic protein 1 (*Bmp1*), a disintegrin and metalloprotease domain 2 (*Adam2*), protein tyrosine kinase 2b (*Ptk2b*), *Nefl*, *Nef3*, and gap junction protein 3 (*Gja3*). *Blk*, *Ctsb*, *Clu*, *Bmp1*, *Adam2* and *Ptk2b* were not present in the candidate region. *Nefl*, *Nef3*, *Adam7* and *Gnrh*, which were not mapped in genetic mapping because of their non-polymorphism between MSM and CXSD mice, were found to be present in the YAC-STS physical map. Therefore, they have been subjected to further investigation.

Nefl and *Nef3* are the members of the intermediate filament (IF) protein composed of *Nefl* (61 kDa), *Nef3* (90 kDa) and *Nefh* (110 kDa) (Julien 1997), and form the neurofilament by copolymerization. Neurofilaments are present in most populations of neurons in the nervous system to provide mechanical support to the neuron and play a role in modulating the caliber of large myelinated axons. Neurofilament deposit in degenerating motor neurons is related to diseases such as amyotrophic lateral sclerosis (ALS), and dramatic hypotrophy of axon was observed in the quivering quail mutant which is deficient in *Nefl* protein (Yamasaki *et al.* 1992), and in the *Nefl* knockout mouse (Julien 1999). The overexpression of *Nefl* by murine sarcoma virus long terminal repeat promoter in the mouse led to cataract formation (Monteiro *et al.* 1990).

When *Nefl* and *Nef3* were mapped with SSCP using the primers newly obtained from the 3' UTR of cDNA sequences of the genes, they were linked to *D14Mit87* which was not separated from *lr2* in the homozygosity mapping. Previous reports, that overexpression of *Nefl* is a cause of cataract (Monteiro *et al.* 1990), and that *Nefl* and *Nef3* are candidate genes of rupture of lens cataract (*rlc*) which has similar phenotypes to *lr2* mutant (Matsushima *et al.* 1996), led us to suppose that these two genes were strong candidates for *lr2*. Therefore, the expression level in the eyes and the cDNA sequence of *Nefl* and *Nef3* in several strains of mice were analyzed for mutations. The expression of *Nefl* and *Nef3* in the eye was not different between BALB/c and CXSD mice. Several nucleotide changes were detected but most of them were silent mutations, which did not change the amino acid composition. Amino acid substitution was found at E567K in the sequence of *Nef3*, in which the acidic amino acid was changed to a basic amino acid, but this difference may not be a cataract causing mutation because glutamate (E) was

found in the amino acid sequence in the BALB/c mouse and lysine (K) in STS and CXSD mice. It may be a simple genetic polymorphism between the mouse strains for two reasons: 1) STS mouse that has lysine at position 567 does not have cataract; 2) the segregation ratio of phenotypes and genotypes in Mendelian analysis of the cataract gene was not different between backcross matings of (CXSD $\times$ BALB/c) F_1 $\times$ CXSD and (CXSD $\times$ STS) F_1 $\times$ CXSD in a previous report (Song *et al.* 1997), which showed that the genetic background of the STS mouse did not influence the cataract formation. The single amino acid change from acidic amino acid to basic amino acid buried in the long glutamate rich domain may not induce sufficient conformational change in the structure of *Nef3* to induce cataract formation in the STS mouse. Thus we excluded the two neurofilament genes from the candidate genes for *lr2*.

To date, there have been many genes reported to cause cataract. The several crystallins responsible for the maintenance of lens transparency (Stephan *et al.* 1999; Pras *et al.* 2000; Smith *et al.* 2000), transcription factors having important roles in the developmental process of the eye (Francis 1999), such as *SOX1* (Nishiguchi *et al.* 1998), *PITX3* (Semina *et al.* 1998), *SIX5* (Sarkar *et al.* 2000) and *PAX6* (Azuma *et al.* 1999), and the various proteins maintaining the intracellular environment including the gap-junction proteins such as *Gja8* (Shiels *et al.* 1998; Steel *et al.* 1998) and *Gja3* (Gong *et al.* 1997; Mackay *et al.* 1999) and L-ferritin gene (Beaumont *et al.* 1995) have been related to cataract formation. In addition, a mutation in the cytoskeletal protein was also found as a minor mutation of cataract (Jakobs *et al.* 2000). Most of the genes reported so far are responsible for the cataract with autosomal dominant inheritance. The phenotype of *lr2* mice is distinct from others in that the lenses of mice affected appear clear at birth, but with time focal opacities develop, and the lenses eventually rupture. The progressive nature of the cataract with rupture may be suggestive of a gene developmentally regulated and involved in the cellular structure, but with a mechanism distinctive from other cataract mutants. Certain mutations in *Nefl* and *Nef3* might be linked to the lens rupture because they maintain the cellular structure, but the critical mutations were not detected in this study, which suggests that *lr2* might be a novel gene.

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