



second report of a mutation in the *LCA5* gene and the fifth mutation reported.

## METHODS

**Clinical examination:** The research protocol was approved by the ethics review board of the Vision Research Foundation, Sankara Nethralaya. The study procedures were performed in accordance with institutional guidelines and the Declaration of Helsinki, and informed consent was obtained from each participant. The two affected sisters, their unaffected parents aged 30 (mother) and 36 years (father) and 50 ethnically matched controls, which included 28 males and 22 females with an average age of 62.32 (ranging between 38 and 79 years), underwent a detailed ophthalmic examination including recording of best corrected visual acuity, refraction, slit lamp examination, and post dilatation binocular indirect ophthalmoscopy. Additionally electroretinography was done on patients.

**DNA extraction:** We drew 10 ml of heparinized blood from both affected patients and their parents after obtaining informed consent from both parents. 10 ml of blood was also drawn from 50 ethnically matched controls after obtaining informed consent. Genomic DNA was extracted immediately using QIAamp Blood DNA maxi kit (Qiagen, GmbH, Germany) according to the manufacturer's instructions. Also consent was obtained from the parents on behalf of the patients because of their underage.

**Genotyping:** Single nucleotide polymorphism (SNP) genotyping was performed with the GeneChip Mapping 10K Xba I Array and Assay Kit (Affymetrix, Santa Clara, CA). All incubations were done using a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA). Internal positive and negative GeneChip controls were performed in parallel using the supplied genomic DNA. Xba I (New England Biolabs, Ipswich, MA) was used to digest 250 ng genomic DNA for 2 h at 37 °C followed by heat inactivation for 20 min at 70 °C. Digested DNA was then incubated with a 0.25 M Xba I adaptor (Affymetrix) and DNA ligase (New England Biolabs) in standard ligation buffer for 2 h at 16 °C followed by heat inactivation for 20 min at 70 °C. Ligated products were amplified in quadruplicate using 10 µM generic primer in PCR buffer II (Applied Biosystems) with 2.5 mM MgCl<sub>2</sub>/2.5 mM deoxyribose nucleotide triphosphates (dNTPs)/10 units of AmpliTaq Gold polymerase (Applied Biosystems) under the following PCR conditions: 95 °C for 5 min, followed by 35 cycles (95 °C for 20 s, 59 °C for 15 s, and 72 °C for 15 s) and a final extension at 72 °C for 7 min. Fragments ranging in size from 250 to 1,000 bp were preferentially amplified under the conditions [6]. PCR products were purified with Qiagen MinElute 96 UF PCR Purification Kit and concentrated with a Qiagen PCR purification column (Qiagen,) according to the manufacturer's recommendations. A 10K genotyping assay kit fragmentation reagent (Affymetrix) was used to digest 20 µg of DNA, which was then labeled with 30 U/µL terminal

deoxynucleotidyl transferase and 5 mM DNA labeling reagent (Affymetrix 10K genotyping assay kit). After undergoing heat inactivation at 95 °C for 10 min, samples were injected into microarray cartridges and hybridized overnight. Microarrays were washed in a fluidics station 450 (Affymetrix), followed by staining with streptavidin Avidin Phycoerythrin (Molecular Probes, Eugene, OR), and biotinylated antistreptavidin (Vector Lab, Burlingame, CA), followed by a final wash with SSPE buffer. Microarrays were scanned according to manufacturer's directions (Affymetrix). The data was analysed using Exclude AR program (ExcludeAR sheet; Excel, Microsoft, Redmond, WA) [7].

**Sequencing of the *LCA5* gene:** The seven coding exons of the *LCA5* gene were amplified using 11 sets of primers with exon 7 amplified using five sets of overlapping primers (same primers used by den Hollander et al. [5]). A 20 ml reaction was set up containing 10 mM Tris (pH 9.0), 50 mM KCl, 1.5mM MgCl<sub>2</sub> and 0.01% gelatin, 1 mM dNTP each (GeNei, Bangalore, India), 10 mM of each forward and reverse primer, 1U of Taq DNA polymerase (GeNei, Bangalore, India) and 5 mM betaine (Sigma Aldrich, St. Louis, MO). 100 ng of genomic DNA was amplified with initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 20 s, annealing at (56 °C for exons 1, 2a, 2b, 2c, 5, 6, 7e, 57 °C for exon 7a, 7b, 7c, 60 °C for 7d, and at 65-58/58 touchdown for exon 3 and 4) for 20 s and extension at 72 °C for 45 s and final extension at 72 °C for 7 min. PCR products were digested with exonuclease I, *E. coli*, and shrimp alkaline phosphatase (Fermentas Life Sciences, Glen Burnie, MD) sequenced unidirectionally using BigDye Terminator v.3.1 kit (Applied Biosystems) with specific primers in ABI3100 Avant, (Applied Biosystems). The sequences were analyzed in Sequence Analysis software v 3.1.1. (Applied Biosystems, Foster City, CA). Any DNA sequence variations were confirmed in the reverse direction. Fifty ethnically matched normal controls were also amplified and sequenced to confirm the mutation.

**In silico splice site prediction:** The effect of the single base substitution identified in the last base position of exon 6 was evaluated using a splice site prediction algorithm (<http://violin.genet.sickkids.on.ca/~ali/splicesitefinder.html>) [8,9].

**RNA isolation and cDNA sequencing:** RNA was isolated from the lymphocytes separated from 10-ml heparinized blood samples of the affected (proband), unaffected parents, and one unrelated normal control by using Trizol reagent (Sigma-Aldrich, St. Louis, MO), according to the manufacturer's instructions, and dissolved in diethyl pyrocarbonate (DEPC)-treated water. Total RNA was used to generate a cDNA pool by RT-PCR using a Qiagen Sensiscript reverse transcriptase kit (Qiagen, GmbH, Germany) according to the manufacturer's instructions (Qiagen). PCR primers [10] for the *GAPDH* housekeeping gene were used as the internal control. For the amplification of the *LCA5* gene, exonic





frameshift mutations, and one promoter mutation in consanguineous LCA families [5]. Here we used homozygosity mapping in a consanguineous Indian pedigree to identify a novel *LCA5* mutation, c.955G>A, that disrupts the correct exon 6 splice donor site and leads to splicing at a cryptic donor consensus sequence 5 bp into the adjacent intron. This finding further underlines the importance of homozygosity mapping as a tool for identifying genes and mutations involved in recessively inherited diseases, and of nonsense mutations in the *LCA5* gene as a cause of LCA. The mutation identified is interesting as it serves to further emphasize that defects in splicing, as well as direct alterations of the protein code, can cause human inherited diseases. A similar change in the third base of a codon could easily be overlooked in such analyses since at first glance it is a silent change in terms of its effect on the mRNA code.

The phenotype of the family described herein is consistent with a diagnosis of LCA and is similar to the phenotype described previously in *LCA5* patients of the same age [4]. Despite the fact that LCA is a congenital abnormality, the previous report suggested some progression of phenotype with age, with macular staphyloma as a complication of disease in adulthood. The two patients observed in this report were both below ten years of age on examination and had no staphylomatous changes. The relatively consistent *LCA5* phenotype, the recessive mode of inheritance, and the growing list of null mutations all point to the *LCA5* phenotype being the result of a lack of functional lebercilin protein rather than the presence of a defective protein. It is not yet known whether truncated proteins are produced in patients or whether the mutated mRNAs are degraded by nonsense mediated decay [11]. However the mutated cDNA was readily amplified from lymphocyte RNA, suggesting that the mutated mRNA is still present at a significant level.

It may be significant that the second affected sibling died of respiratory failure. *LCA5* is known to be a ciliopathy, a disease resulting from a defect in formation or function of cilia. Cilia proteins are essential in the retina because the outer

segments of rod and cone photoreceptors are highly adapted cilia. However, most human cells are ciliated and therefore the majority of these proteins would be expected to serve similar functions elsewhere in the body. To date all reported cases of LCA associated with mutations in or linkage to the *LCA5* gene lack other syndromic features. This is surprising as other ciliopathies affect the kidney and other organs, and the *LCA5* gene is known to be expressed in nasopharynx, trachea, and lungs and was originally identified in the proteome of bronchial epithelium ciliary axonemes [5]. If other *LCA5* cases were found to have respiratory defects this might imply a defect of motor as well as sensory cilia in these patients.

In summary, this is the second report of *LCA5* mutations in LCA patients, further emphasizing the significance of mutations in this gene as a cause of LCA. The mutation identified is novel and causes disease by disrupting an existing splice donor site so that a cryptic donor site in the adjacent intron is favored, leading to a frameshift in the resultant mRNA.

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Normal sequence: 5'-TGCATTAAGAAAAAAT**G**gtatg|gtaaataat -3'

Mutant sequence: 5'-TGCATTAAGAAAAAAT**A**gtatg|gtaaataat -3'

Figure 4. Schematic representation of the 3' end of the exon 6 of the *LCA5* gene. The figure represents 3' portion of the normal and mutant sequence of the exon 6 of the *LCA5* gene. Nucleotides in uppercase represent exonic sequence and that in small lower case represent intronic sequences. The nucleotide in uppercase and in bold represent the last base of the exon and the site of mutation. The first vertical bar in the normal sequence represent the real splice donor site and the second vertical bar represent additional/alternative splice donor site, which is activated in the event of absence of the real splice donor site.

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