Familial Subepithelial Corneal Amyloidosis (Gelatinous Drop-Like Corneal Dystrophy): Exclusion of Linkage to Lactoferrin Gene

Gordon K. Klintworth,1,2 Jeffrey R. Sommer,2 Greg Obrian,3 Lei Han,4 Mohamed N. Ahmed,1 Mazin B. Qumsiyeh,5 Pei-Yu Lin,3 Surrendra Basti,4 Madhukar K. Reddy,4 Atsushi Kanai,7 Yoshihiro Hotta,7 Joel Sugar,8 Govindasamy Kumaramanickavel,9 Francis Munier,10 Daniel F. Schorderet,10 Leila El Matri,5 Fumino Iwata,5 Muriel Kaiser-Kupfer,5 Masao Nagata,1 Kiyoo Nakayasu,7 J. Fielding Hejtmancik,5 and Christina T. Teng1

Purpose: Because corneal tissue with familial subepithelial corneal amyloidosis (FSCA; gelatinous drop-like dystrophy of the cornea) contains lactoferrin the possibility that the FSCA gene was the human lactoferrin (hLF) gene was investigated. Due to contradictory published information we also mapped the hLF gene.

Methods: We mapped the hLF gene using a genomic clone of the entire hLF gene as a probe by fluorescence in situ hybridization (FISH). Utilizing PCR primers that are specific to the hLF gene, we also mapped the hLF via radiation somatic cell hybrid analysis. Linkage of the FSCA gene to the hLF gene was evaluated by genetic linkage analysis using polymorphic markers within and in the vicinity of the hLF gene.

Results: The hLF gene mapped to the short arm of chromosome 3 at 3p21. Linkage analysis using polymorphic markers for hLF and haplotype analysis of the 3p21 loci indicates that the FSCA gene is not linked to the 3p21 locus.

Conclusions: The gene for FSCA is not the hLF gene in these families.

Familial subepithelial corneal amyloidosis (FSCA) (gelatinous droplike dystrophy of the cornea; Mendelian Inheritance in Man (MEM) Number ) is a rare bilateral autosomal recessive corneal disorder that was first described in Japan [1]. Beginning in the first or second decade of life, protuberant nodular deposits of amyloid accumulate beneath the epithelium producing a mulberry shaped mass in the absence of systemic abnormalities. The condition has been reported most often in persons from Japan [2-10], India [11], and Tunisia [12,13], but it has also been detected in other countries [14].

The gene for the multifunctional iron-binding glycoprotein lactoferrin (LF), which is found in numerous tissues, surface mucosa, and biological fluids [15], became a candidate gene for FSCA, when LF was isolated in excessive amounts from corneas with FSCA and the amyloid was found to react immunohistochemically with antibodies to human lactoferrin (hLF) [14].

Based on knowledge about other amyloidoses we would anticipate that a single protein accounts for the amyloid in FSCA. Single amino acid substitutions have been detected in the inherited amyloidoses in which the amyloid protein is derived from gelsolin, transthyretin, or apolipoprotein A-1 [16-22].

Amyloid accumulates within the cornea not only in FSCA, but also in apparently unrelated chronic nonspecific disorders and in the inherited lattice corneal dystrophy (LCD) types I (Biber-Haab-Dimmer), II (Meretoja), III (Hida), and IIIA (Stock) [22]. Mutations in specific genes cause amyloid deposition in some of these inherited disorders: BIGH3 on human chromosome 5 (5q31) (LCD type I, combined GCD-LCD and LCD type IIIA) [23,24] and the gelsolin gene on chromosome 9 (9q32-34) (LCD type II) [18].

The literature concerning the chromosomal location of the hLF gene, a member of the transferrin gene family [25], is confusing. The gene was initially assigned to the long arm of chromosome 3 at 3q21-qter by somatic cell hybrid mapping [26] and to 3q31-23 by radioactive in situ hybridization [27] without the use of markers to identify parts of specific chromosomes. This localization was later corrected to 3p21 [28] and Naylor et al. [29] included hLF at the short arm in the mapping of 638 sequence tag sites (STSs) on chromosome 3 by somatic cell hybrid analysis, but without comment on the earlier discrepancy. The National Center for Biotechnology Information (NCBI) database (UniGene) lists hLF (Hs.347) as mapped to both chromosome 3 (3q21-q23 and SHGC-11211) and chromosome 5. Earlier human mapping studies were carried out with mouse LF cDNA as the probe with conflicting results [26-29]. Because the entire human LF gene is now available, we reexamined its chromosomal location.

To understand the organization of the hLF gene and to verify its chromosomal assignment, we isolated a full-length human genomic clone from a cosmid library. This 40 kbp
genomic DNA was used to construct the physical map and to locate the chromosomal position of the hLF gene. Once the position and structure of the hLF gene were clarified, it was possible to investigate whether FSCA links to the hLF gene using polymorphic markers within and in the vicinity of the hLF gene.

METHODS

Genomic clone isolation: The human genomic DNA was purchased from ClonTech Co. (Catalog No. 6552-1; Palo Alto, CA). The cosmid clone CT6-1 (approximately 40 kbp) was obtained by screening a human male placenta genomic library (Catalog No. 951202, Stratagene, La Jolla, CA; the insert was cloned in the BamHI site of the cosmid vector pWE15) with the 2.1 kbp insert of the hLF cDNA, p1212 [30]. The full length hLF gene was confirmed by hybridization to probes specific to exons 1 and 17.

Mapping of hLF gene by fluorescence in situ hybridization: The cosmid vector (CT6-1) containing genomic hLF was purified using a Qiagen column (Qiagen, Valencia, CA) and 1 µg of DNA was labeled by nick translation using a biotin-16-dUTP kit (Boehringer-Manheim Inc., Indianapolis, IN). A telomeric probe of 3p (Oncor Inc, Gaithersburg, MD) was used as a control marker of the short arm of chromosome 3. This probe consisted of a subclone from a Y AC including D3S1792 mapped to p3 terminal by Brown [31]. We confirmed its 3p localization using banded preparations. The biotinylated probe and the telomeric 3p control probe were allowed to hybridize with normal human lymphocyte chromosomes. The hLF probe was detected with avidin-fluorescein isothiocyanate (FITC; green) and the control probe with rhodamine (red). The preparation was counterstained with 4,6-diamidino-2-phenylindole-dihydrochloride (DAPI; blue). For the FISH experiments, at least 50 cells were examined.

Radiation hybrid analysis: PCR primers were designed specifically to amplify exon 14 of the hLF gene. The primers were situated in the introns flanking exon 14 and had the sequence (5'-3') LF14F = CCTGGCTGAGAA TGCTGGA; LF14R = TCCCAAAGGCAAATCTGAA CA. PCR was performed using Qiagen Taq DNA polymerase and the Qiagen buffer system and in a DNA Thermal Cycler 480 (Perkin Elmer Applied Biosystems, Foster City, CA), 94 °C, 1 sec; 55 °C, 1 sec; and final extension of 10 min at 72 °C. This produced a band of 150 bp which was sequenced for verification. PCR was run with this primer set on human and hamster genomic DNA and the results from the amplification were unique. Radiation hybrid analysis was performed at the Genome Core Laboratory at Duke University using the hLF gene exon 14 primer set and the results were submitted to the Stanford Human Genome Center for analysis.

Linkage and haplotype mapping: Genomic DNA was isolated from blood samples of FSCA family members using the QIAamp Blood Maxi Kit (Qiagen). The following PCR primer pairs were used: LF2F/R (TCTGGAGAGTTAGACTTTGT T, CCGCACCCTTGGAACTCC), D3S1298 F/R (AGCTCTC AGTGCCACCC, GAAAAATCCCTGGTAAGCG), D3S1260 F/R (AGCTACAGGGAAGCACTGT, CTATGCAATCACTGCCCATT) and D3S2968 F/R (GAAGCCTTTGTCAGAAGGAG, AATGGATTAGGA ACGAGGCC). PCR was performed using Taq DNA polymerase and buffers obtained from Qiagen and the following cycling conditions: 94 °C 4 min, 40X (94 °C 1 sec, 55 °C 1 sec, and 72 °C 1 sec), 72 °C 5 min.

Families with familial subepithelial corneal amyloidosis: DNA was obtained from 21 affected and 36 unaffected members of 11 families with FSCA using standard procedures [32]. The families came from the USA (Families 1 and 10), Tunisia (Family 2), India (Families 3, 4, 5, 8 and 9) and Japan (Families 6, 7 and 11). The diagnosis in all affected persons was established by the typical clinical appearance and it was usually also confirmed histopathologically. Data on non-linkage aspects of some families have been previously reported [11,12,14,33-35].

Determination of whether FSCA maps to lactoferrin gene and human chromosome 3: Having mapped the hLF gene to the 3p21 locus on human chromosome 3 we determined if FSCA was linked to this locus. This was done by establishing haplotypes for affected and unaffected individuals in families with FSCA using polymorphic STS markers (D3S1298, D3S1260 and D3S2968) whose order had been established relative to each other in the region of 3p21 of human chromosome 3 [36]. We also used 3 polymorphisms found within exon 2 of the hLF gene.

DNA analysis of members of families with FSCA: Haplotype analysis. Haplotype analysis was accomplished in Family 1 by PCR analysis using the 3p21 linked polymorphic STS markers D3S1298, D3S1260 and D3S2968. 5 µl of amplified product was added to the sequencing loading dye (90% formamide, 10% 10X TBE (tris-borate EDTA) (1X TBE = 89 mM Tris base, 89 mM boric acid and 2 mM EDTA) with bromophenol blue and 0.25% xylene cyanol). The sample was denatured for 2 min at 95 °C and cooled on ice and loaded on a 5% denaturing polyacrylamide gel with 7 mM urea and 1X TBE (89 mM Tris base, 89 mM boric acid and 2 mM EDTA). The samples were electrophoresed at 55 °C and 80 W. The gel was stained with SYBR Green II (FMC BioProducts, Rockland, ME) and photographed with 254 nm epi-illumination. The results of each individual’s banding profile for every marker was compared with their hLF exon 2 banding profile.

Single-stranded conformational polymorphism (SSCP) analysis of hLF exon 2.: SSCP analysis of hLF exon 2 was performed on eleven families with FSCA by adding 5µl of the PCR products to 10µl SSCP loading dye (95% formamide, 10 mM NaOH and 0.25% bromophenol blue), heat denaturing at 94 °C for 2 min and chilled on ice. The samples were then loaded on a 0.5X mutation detection enhancement (MDE) gel (FMC BioProducts, Rockland, ME) prepared with 0.6X TBE (1X TBE = 89 mM Tris base, 89 mM boric acid and 2 mM EDTA) and electrophoresed at 400 V and 8 W for 2 h at 4 °C. The DNA was visualized as described for the haplotype analysis. Heterogeneity in the hLF gene was used as a marker to genotype the affected and non-affected family members.

DNA sequencing: For sequence analysis polymorphic bands were excised from the MDE gel and placed in a 1.5 ml
centrifuge tube with 50 µl H2O and left at 25 °C over night. 1 µl of the solution containing the excised polymorphic band was re-amplified with the appropriate primers. When polymorphism was not present with SSCP analysis, the PCR product from genomic DNA amplification was used for sequencing.

**Chromosome 3 linkage analysis methods:** DNA isolation for linkage was performed on 8 families as described by Smith et al. [32]. DNA was isolated from blood directly or from transformed lymphoblastoid cell lines as described previously [32]. Microsatellite markers from the Applied Biosystems Panel 1 (Perkin Elmer Applied Biosystems, Foster City, CA) were multiplexed for analysis. Each reaction contained 40 ng genomic DNA, 1.65 pm each of 1-4 fluorescent dye-labeled primer pairs, 250 µM of each dNTP, 0.2 units Taq Gold DNA polymerase (Perkin Elmer Applied Biosystems), in 10 mM Tris-HCl pH 8.3, 50 mM KCl and 2.5 mM MgCl2, in 5 µl. The samples were cycled with an initial 12 min denaturation at 95 °C followed by 10 cycles of 15 sec denaturation at 95 °C, 15 sec annealing at 55 °C, and 30 sec elongation at 72 °C.

This was followed by 20 cycles with the denaturation step at 89 °C, and a final extension period of 20 min at 72 °C. Samples were electrophoresed on a 377 Prism automated DNA sequencer (Perkin Elmer, ABI) as recommended by the manufacturer. Data were collected with Genescan Analysis Software 2.1 converted to allele size with Genotyper (Perkin Elmer, ABI). All data were independently interpreted by 2 individuals without reference to pedigree or disease status, and disagreements resolved by a third independent observer. If conflicting readings were not resolved the marker was retested. Family relationships were confirmed by observation of Mendelian Inheritance in a total of 61 microsatellite markers in Panels 1-4 of the ABI Prism marker set. Linkage analysis was carried out with the FASTLINK program [37]. FSCA was analyzed as a fully penetrant autosomal recessive trait with a gene frequency of 0.00563 [38], although varying this value by an order of magnitude had minimal effects on the results. Marker alleles were arbitrarily set equal frequencies.

**RESULTS**

**Chromosomal localization of human lactoferrin gene:** Using FISH we found the hLF gene to be located on the short arm of chromosome 3 at band level 3p21.2-3 (Figure 1). Of the fifty cells examined twenty had one specific signal and 30 had two signals. The Stanford Human Genome Center Radiation Hybridization Screen linked the hLF exon 14 amplimer most closely to the marker SHGC-11211 with a LOD score of 10.7.
Detection of polymorphism in lactoferrin gene: Exon 2 of the hLF gene was found to contain 3 different banding patterns when using SSCP analysis (Figure 2). These polymorphisms were characterized by nucleotide sequencing and correlated with base pair alterations in codons 15, 30, and 48. The exon polymorphisms were as follows:

1. Polymorphic band a = Codon 15 (GGG) Gly
   Codon 30 (ACC) Thr
   Codon 48 (AGA) Arg
2. Polymorphic band b = Codon 15 (GGA) Gly
   Codon 30 (ACC) Thr
   Codon 48 (AGA) Arg
3. Polymorphic band c = Codon 15 (GGA) Gly
   Codon 30 (GCC) Ala
   Codon 48 (AAA) Lys

The codon 15 mutation does not change the amino acid sequence of the translated protein. The codon 30 (GCC:Ala and ACC:Thr) and codon 48 differences (AAA:Lys and AGA:Arg) have also been documented in with Genbank Accession Numbers U07643 and M93150, respectively.

Analysis of lactoferrin gene haplotype: A screening of Family 1 using SSCP of hLF exon 2 disclosed three polymorphisms (Figure 2A). The seven siblings contained three different SSCP banding patterns. The two affected individuals had different SSCP profiles, indicating that they did not share the same chromosome 3 pairs. Moreover each affected person had SSCP profiles resembling that of unaffected siblings. Thus the SSCP findings in Family 1 suggested that the hLF gene was not the FSCA gene.

Haplotype analysis of Family 1 using polymorphic STS markers (D3S1298, D3S1260 and D3S2968) that link to 3p21.2-3 revealed that the hLF exon 2 polymorphisms cosegregated with these markers providing further evidence of the linkage of the hLF gene to 3p21.2-3 (Figure 2). A comparison of the haplotype profiles for each sibling ruled out this locus’ involvement as the candidate gene for FSCA in this family. Affected siblings did not share the same 3p21 profile as each other and unaffected siblings had the same haplotype as persons with FSCA.

Using only SSCP polymorphisms in hLF exon 2, the hLF gene could be ruled out as the FSCA candidate gene in two more of the investigated families (Families 2 and 3; Figure 3). In both of these instances affected siblings with unaffected parents, had different banding patterns of hLF exon 2. In Family 2 (Figure 3A) with four affected progeny from a consanguineous union the presence of two different hLF genotypes ruled out the hLF gene as the FSCA gene in this family. Family 3 (Figure 3B), which contained two separate consanguineous unions of Family 1 using SSCP of hLF exon 2 disclosed three polymorphisms (Figure 2A). The seven siblings contained three different SSCP banding patterns. The two affected individuals had different SSCP profiles, indicating that they did not share the same chromosome 3 pairs. Moreover each affected person had SSCP profiles resembling that of unaffected siblings. Thus the SSCP findings in Family 1 suggested that the hLF gene was not the FSCA gene.

Haplotype analysis of Family 1 using polymorphic STS markers (D3S1298, D3S1260 and D3S2968) that link to 3p21.2-3 revealed that the hLF exon 2 polymorphisms cosegregated with these markers providing further evidence of the linkage of the hLF gene to 3p21.2-3 (Figure 2). A comparison of the haplotype profiles for each sibling ruled out this locus’ involvement as the candidate gene for FSCA in this family. Affected siblings did not share the same 3p21 profile as each other and unaffected siblings had the same haplotype as persons with FSCA.

Using only SSCP polymorphisms in hLF exon 2, the hLF gene could be ruled out as the FSCA candidate gene in two more of the investigated families (Families 2 and 3; Figure 3). In both of these instances affected siblings with unaffected parents, had different banding patterns of hLF exon 2. In Family 2 (Figure 3A) with four affected progeny from a consanguineous union the presence of two different hLF genotypes ruled out the hLF gene as the FSCA gene in this family. Family 3 (Figure 3B), which contained two separate consanguineous unions of Family 1 using SSCP of hLF exon 2 disclosed three polymorphisms (Figure 2A). The seven siblings contained three different SSCP banding patterns. The two affected individuals had different SSCP profiles, indicating that they did not share the same chromosome 3 pairs. Moreover each affected person had SSCP profiles resembling that of unaffected siblings. Thus the SSCP findings in Family 1 suggested that the hLF gene was not the FSCA gene.
ous unions, resulted in four affected progeny with two different hLF genotypes (two homozygous and two heterozygous). Since the parents of affected individuals (3, 5 and 6) were not affected the presence of two different hLF genotypes in their affected progeny ruled out the hLF gene as the FSCA gene in this family.

In several of the FSCA families screened by hLF exon 2 SSCP analysis the progeny were of consanguineous unions (either from first cousin or uncle-niece relationships). In such cases one would expect homogeneity at the loci involved in this autosomal recessive disease. Investigation of Families 4, 5, 6 and 7 in which the affected individuals were from consanguineous unions revealed two unique polymorphic bands with SSCP analysis of hLF exon 2 (Figure 4). Since one would expect homogeneity in affected offspring of consanguineous unions, these cases provide strong evidence that the hLF gene is not associated with FSCA. In Family 8, the affected individuals are homozygous in regards to their hLF exon 2 SSCP banding profile, but the analysis of chromosomal segregation indicates that one of the hLF gene copies was not inherited from direct lineage (Figure 5).

In three families with FSCA (Family 9, 10, 11) a lack of hLF exon 2 polymorphisms or an insufficient sample size made it impossible to draw any conclusions about involvement of the hLF gene in FSCA (Figure 6).

Chromosome 3 linkage analysis results: Linkage analysis with markers on human chromosome 3 excludes the FSCA locus from the region of the hLF gene (3p21) (Table 1).

**DISCUSSION**

Because of confusion about the location of the hLF locus we initially verified the site of this gene and found that it mapped to the short arm of chromosome 3 (3p21) as documented in some reports [28,29] and not to the long arm (3q21-3qter) as previously described [26,27]. While hLF maps to this part of chromosome 3, other members of the transferrin gene family, such as transferrin (3q21) [39], transferrin receptor (3q26.2-qter) [40] and melanoma antigen p97 (3q24-qter) [41] have been assigned to the long arm of this chromosome. All members of this gene family are a product of an ancient intragenic duplication that occurred several hundred million years ago [42,43]. Hence the separation of the hLF gene from the other family members on chromosome 3 must have occurred during gene rearrangements on this chromosome during the primate evolution which separates man and orangutan [44]. Thus far, *Homo sapiens* is the only species known to separate LF from other members of the transferrin gene family.

The SHGC-11211 amplicon by which the hLF gene was linked most closely was developed from the 3’ end of the hLF gene and amplifies from nucleotide 2179 to nucleotide 2273 of the mRNA for hLF (Genbank Accession Number M93150). When the cytogenetic location of this marker is searched confusing results are obtained. The Genome DataBank (GDB) has SHGC-11211 mapped to chromosome 3, 3pter and 3qter. The Database UniGene has the cytogenetic location of this marker (referenced as Hs.347) on both chromosomes 3 and 5.

**Figure 5.** Family 8: Pedigrees and SSCP analyses of polymorphisms in exon 2 of the hLF gene used to investigate the relationship between the hLF genotype and FSCA in Families 2-11. Individual #1 is homozygous for the X allele of hLF and individual #2 is homozygous for the Y allele of hLF. Therefore one of the X alleles in affected individuals 4 and 5 had to come from the non-family member #8. Although #4 and #5 are homozygous for the X allele of hLF, one of the X alleles is from the non-family member #8. In this family it is hence highly unlikely that the autosomal recessive phenotype is a result of the lactoferrin gene. Because of the inbreeding, homogeneity of recessive alleles would be expected to cause FSCA.

**Figure 6.** Families 9, 10, and 11: Pedigrees and SSCP analyses of polymorphisms in exon 2 of the hLF gene used to investigate the relationship between the hLF genotype and FSCA in Families 2-11. Family 9 (A). Due to a lack of polymorphisms in exon 2 of the hLF genes of this family, it is not possible to either include or exclude the involvement of hLF. Families 10 (B) and 11 (C). The affected individuals are heterogeneous for the hLF allele, but there is no evidence of inbreeding.
An analysis of our findings in most families in the present study (Families 1-8; Figures 2-5) excludes the hLF gene on 3p21 as the FSCA gene. The elimination of this candidate gene is based on the chromosome 3 linkage data and the haplotype analyses in informative families with FSCA. An examination of the haplotypes in Family 1 indicates that the disease gene in this autosomal recessive disease cannot be linked to the hLF locus. Affected individuals in Family 1, that was part of the original investigation suggesting the hLF gene as a candidate for the FSCA gene, do not share the same haplotype (chromosomal pairing) with each other. Taken together the seven siblings in this family represent 3 of 4 possible chromosomal combinations from their parents. The hLF gene is excluded as a candidate in the majority of families tested and is unlikely to be the cause in the additional families (especially considering the recent mapping of the FSCA gene to the short arm of human chromosome 1 (1p)[46]). The possibility of genetic heterogeneity in FSCA remains an open question.

A failure to map FSCA to the hLF gene is remarkable in view of the prominent accumulation of lactoferrin within the amyloid deposits of affected corneas. The observation also negates the hypothesis that the amyloid accumulation in FSCA results from a mutation in the hLF gene. The alternative hypothesis that hLF binds to other constituents of a yet to be identified amyloid protein gains credence. In this regard it is noteworthy lactoferrin has been identified on immunohistochemical evidence in amyloid associated with Alzheimer disease [47,48] and seminal vesicles in elderly men [49,50].

The identification of hLF in the amyloid of FSCA suggested that the relevant mutant gene may be at the hLF gene locus on human chromosome 3 (3q21-q23). This study indicates that this is not the case. Because the hLF gene can be excluded as the FSCA gene in the majority of families investigated, one would suspect that hLF within corneas with FSCA is normal, but hLF may be upregulated by another gene. It is also possible that FSCA results from a mutation in a modifier of hLF or changes in the permeability of the cornea to hLF. Because FSCA is only expressed clinically in the cornea and since the LF gene is abnormally regulated in specific tissues [51] an upregulation of hLF may involve specific tissues where it is produced, such as the lacrimal gland.

**ACKNOWLEDGEMENTS**

Supported in part by grants from the National Eye Institute (R01EY 08249, EY 00146 and P30 EY 5722), the Swiss National Science Foundation (32-053750.98) and a grant-in-aid for Scientific Research from the Ministry of Education, Japan (B07457417).

**REFERENCES**

3. Akiya S, Ho K, Matsui M. Gelatinous drop-like dystrophy of the


