Hum Genet (2002) 110 : 568–577
DOI 10.1007/s00439-002-0729-z

ORIGINAL INVESTIGATION

**Zhaoxia Ren · Pei-Yu Lin · Gordon K. Klintworth
Fumino Iwata · Francis L. Munier · Daniel F. Schorderet
Leila El Matri · Veena Theendakara · Surendra Basti
Madhukar Reddy · J. Fielding Hejtmancik**

Allelic and locus heterogeneity in autosomal recessive gelatinous drop-like corneal dystrophy

Received: 23 October 2001 / Accepted: 22 March 2002 / Published online: 20 April 2002

Abstract Gelatinous drop-like corneal dystrophy (GDLD) is a rare autosomal recessive disease characterized by the deposition of amyloid beneath the corneal epithelium and by severely impaired visual acuity leading to blindness. Although gelatinous corneal dystrophy has previously been mapped to chromosome 1p and seems to be associated with mutations in the M1S1 gene, molecular genetic studies have been limited to Japanese patients. To investigate the cause of GDLD in patients with diverse ethnic backgrounds, we performed linkage analyses in eight unrelated GDLD families from India, USA, Europe, and Tunisia. In seven of these families, the disease locus mapped to a 16-cM interval on the short arm of chromosome 1 between markers D1S519 and D1S2835, a region including the M1S1 gene. In addition, a 1.2-kb fragment containing the entire coding region of M1S1 gene was sequenced in affected individuals. Seven novel mutations (M1R, 8-bp ins., Q118 E, V194 E, C119 S, 870delC, and 1117delA) were identified in six families and two unrelated individuals. No sequence abnormalities were detected in a single family in which the GDLD locus was also excluded from the M1S1 region by linkage analysis. These findings demonstrate allelic and locus heterogeneity for GDLD.

Electronic database information: URLs for the data in this article are as follows:
Online Mendelian Inheritance in Man (OMIM), http://www3.ncbi.nlm.nih.gov/Omim/ (for GDLD: MIM 204870)
Genethon, http://www.genethon.fr/
The Cooperative Human Linkage Center (CHLC), http://Ipg.nci.nih.gov/CHLC/
National Center for Biotechnology Information (NCBI), http://www.ncbi.nlm.nih.gov/

Z. Ren · P.-Y. Lin · F. Iwata · V. Theendakara · J.F. Hejtmancik
National Eye Institute, National Institutes of Health, Bethesda, Md., USA

J.F. Hejtmancik (✉)
OGVFB/NEI/NIH, Building 10, Room 10B10, 10 CENTER DR MSC 1860, Bethesda, MD 20892-1860, USA
e-mail: f3h@helix.nih.gov,
Tel.: +1-301-4968300, Fax: +1-301-435-1598

P.-Y. Lin
Department of Ophthalmology, National Yang Ming University, Taipei Veterans General Hospital, Taipei, Taiwan, ROC

G.K. Klintworth
Departments of Pathology and Ophthalmology, Duke University Durham, N.C., USA

F.L. Munier · D.F. Schorderet
Department of Ophthalmology and Division of Medical Genetics, University of Lausanne, Lausanne, Switzerland

L. El Matri
Institut Hedi Rais d'Ophtalmologie de Tunis, Tunis, Tunisia

S. Basti · M. Reddy
LV Prasad Eye Institute, LV Prasad Marg, Hyderabad 500034, India

Present address:
S. Basti
Department of Ophthalmology, Northwestern University Medical School, Chicago, Ill., USA

Present address:
M. Reddy
Drishti Eye Center, Hyderabad, India

Introduction

Gelatinous drop-like corneal dystrophy (GDLD; MIM 204870), also called familial subepithelial corneal amyloidosis, is a rare autosomal recessive disease that is characterized by the deposition of amyloid in the subepithelial region of the cornea. Progressive opacification of the cornea severely impairs visual acuity leading to blindness. Nakaizumi first reported this disease in 1914 (Nakaizumi 1914). Clinical symptoms appear in the first decade of life with severe photophobia, an ocular foreign body sensation, and blurred vision. Multiple white nodular deposits of amyloid accumulate beneath the epithelium. Repeated keratoplasties are needed for most patients because of recurrent disease (Lasram et al. 1994). Most cases have been reported in Japanese (Akiya et al. 1990, 1991; Ohnishi et al. 1982; Shimazaki et al. 1995; Takahashi et al. 1985), although GDLD also occurs in Indian (Li et al. 1996) and Tunisian (El Matri et al. 1991; Weber and Ba-

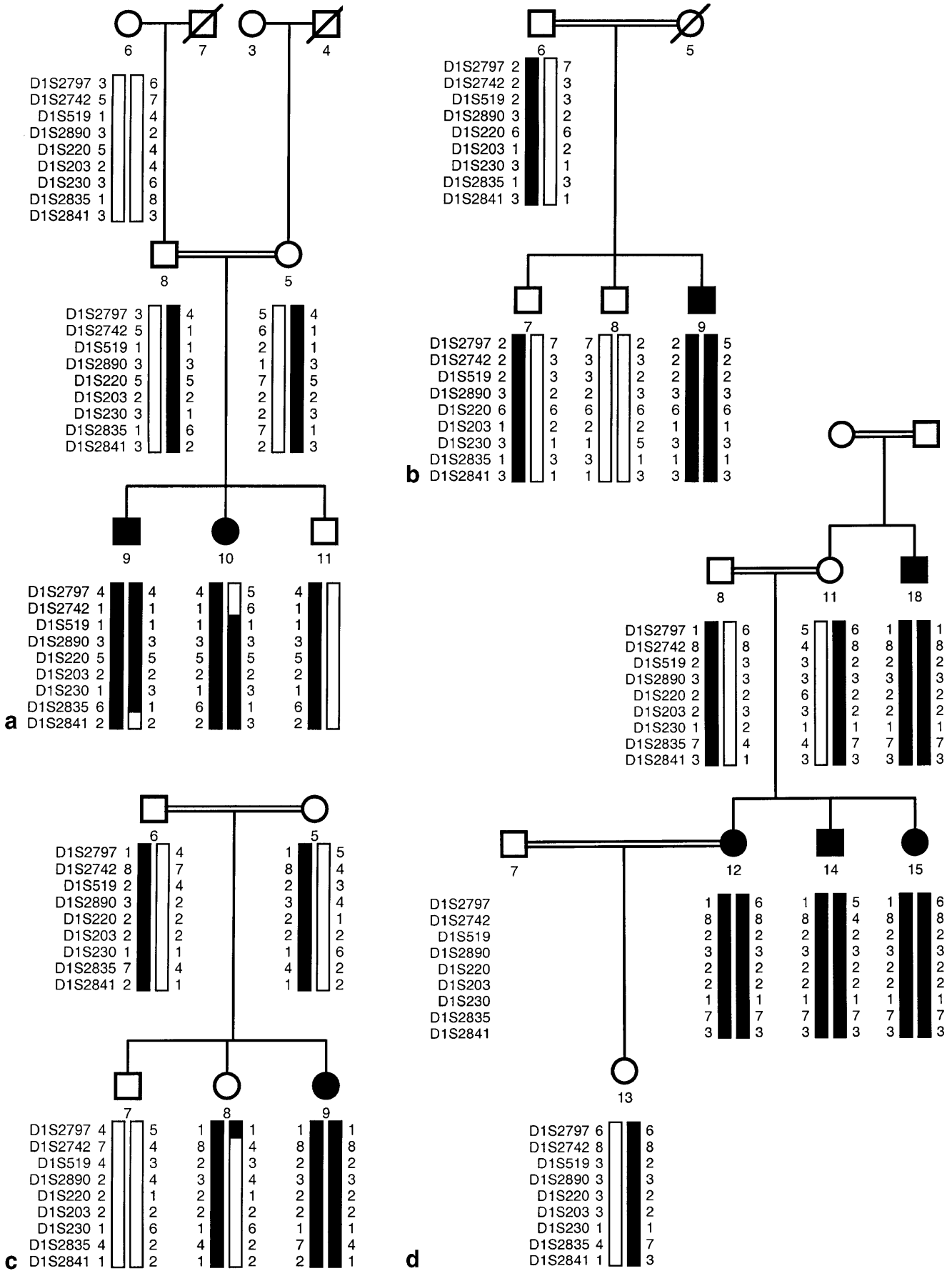
a
b
c
d
6
7
3
4
8
5
9
10
11
D1S2797
D1S2742
D1S519
D1S2890
D1S220
D1S203
D1S230
D1S2835
D1S2841
12
14
15
13
18

Fig. 1a–d

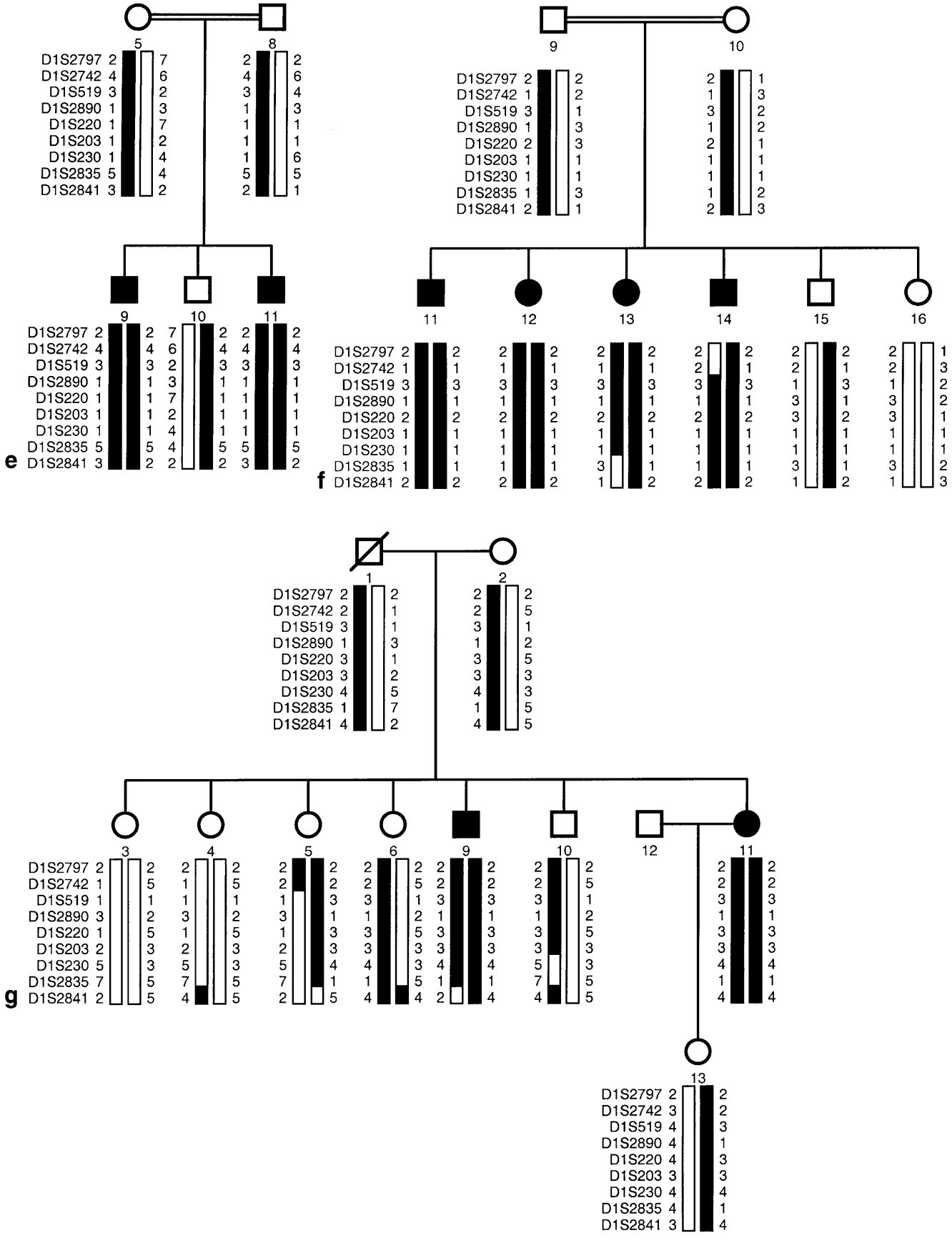
e
f
g
D1S2797
D1S2742
D1S519
D1S2890
D1S220
D1S203
D1S230
D1S2835
D1S2841

Fig. 1 e–g

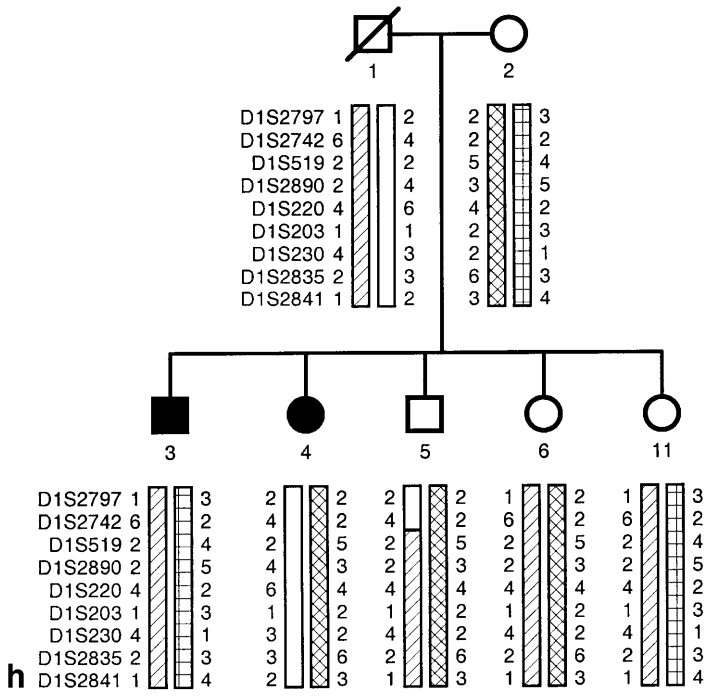

Fig. 1 Haplotypes for pedigrees of multiplex families showing alleles of microsatellite markers in the M1S1 region. The M1S1 gene lies between D1S1835 and D1S2742. (**a**) family 37001, (**b**) family 37002, (**c**) family 37003, (**d**) family 37004, (**e**) family 37005, (**f**) family 37009, (**g**) family 37010, (**h**) family 37011

bel 1980) patients. Some cases have also been reported in Americans and Europeans (Kirk et al. 1973; Klintworth et al. 1997; Stock and Kielar 1976).

Three genes, viz., TGFBI (also known as BIGH3), GSN, and M1S1, have been implicated in the deposition of amyloid in the corneal dystrophies (Hiltunen et al. 1991; Klintworth 1999; Munier et al. 1997; Tsujikawa et al. 1999; Yamamoto et al. 1998). The M1S1 gene, which encodes a gastrointestinal tumor-associated antigen, has been shown to cause GDLD (Tsujikawa et al. 1999), whereas the TGFBI and GSN genes are responsible for granular corneal dystrophy types I, II, and III and lattice corneal dystrophies types I, II, and IIIA (Klintworth 1999), all of which are autosomal dominant diseases. M1S1 is located on chromosome 1p (Tsujikawa et al. 1999), whereas the TGFBI and GSN genes have been mapped to chromosomes 5q31 and 9q34, respectively (Klintworth 1999; Kwiatkowski et al. 1988; Skonier et al. 1992).

Genetic heterogeneity occurs commonly in inherited ocular diseases and has been found in many corneal dystrophies (Klintworth 1999). However, heterogeneity of GDLD has not previously been reported. In the current study, we have mapped GDLD to the M1S1 regions on chromosome 1p in seven families, in agreement with the results of Tsujikawa et al. (1998, 1999) and identified seven novel mutations in M1S1 in the affected members of seven unrelated non-Japanese families and two isolated individuals from Tunisia. The GDLD locus in a single family has been excluded from this region by linkage analysis, and no sequence abnormalities have been identified in the M1S1 gene in this family, suggesting the existence of second locus for GDLD.

Materials and methods

Patient samples and pedigree

Detailed ocular, medical, and family histories were obtained from each available family member. Genomic DNA was isolated from members of ten families (eight multiplex families and two with a single affected individual, one of Tunisian and one of European descent) including five Indian (families 37001–37005), one Tunisian (family 37009), and two Caucasian (families 37010 and 37011) multiplex families containing individuals affected with GDLD (Fig. 1). This study conformed to the Helsinki Accord and was approved by the National Eye Institute IRB. The patients admitted to the study gave their informed consent.

Genotype analysis

Genotyping was carried out with microsatellite markers (ABI Prism Linkage Mapping Set MD-10) and the products were subsequently separated on a 5% denaturing polyacrylamide gel in an Applied Biosystems 377 sequencer as described (Jiao et al. 2000). Briefly, the polymerase chain reactions (PCRs) were prepared following the manufacture's protocols. Multiplex PCR amplifications were performed in an ABI Prism 8700 thermocycler workstation. The GENESCAN and GENOTYPER software packages (Applied Biosystems/Perkin-Elmer) were used to analyze the alleles. Two independent masked individuals interpreted all gels, with conflicts being resolved by a third independent reader. Data producing conflicts that could not be unambiguously resolved were discarded or, in an area of interest, repeated. Family relationships were confirmed by observation of Mendelian inheritance of alleles of 31 microsatellite markers from panels 1 and 2 of the ABI Linkage Mapping Set MD-10. Genotyping of markers for family 37011 was performed twice on independent DNA samples with identical results.

Linkage analysis

Linkage analysis was performed on 52 individuals from eight families by using the FASTLINK implementation of the LINKAGE

program package, version 5.1 (Cottingham et al. 1993; Schaffer et al. 1994) . Admixture heterogeneity tests were performed by using the HOMOG program (Ott 1983). Two-point linkage analysis was performed for all markers by means of the MLINK program, and maximum lod scores were calculated by using ILINK. LINKMAP was employed for multipoint analysis carried out by using overlapping sets of three markers against the map cetromere–D1S2797–0.08–D1S2742–0.01–D1S519–0.02–D1S2890–0.03–D1S220–0.04–D1S203–0.04–D1S230–0.01–D1S2835–0.13–D1S2841. Distances between markers are taken from the Centre d'Étude du Polymorphisme Humain and Genethon maps. GDLD was analyzed as a fully penetrant autosomal recessive trait with the frequency of the GDLD allele set at 0.005. Population-specific marker-allele frequencies were estimated from 29 southern Indian (Hindu), 8 Tunisian, and 13 Caucasian individuals (100 total chromosomes). These same individuals were used for mutation screening in controls.

PCR amplification of the M1S1 gene

A pair of primers flanking the M1S1 gene (human M1S1 gene, Genbank accession no. J04152) was used to amplify a 1.2-kb DNA fragment including the entire coding sequence. The sequences of the primers were: F1 (5′AATACCAGTGGGGACGGTCG3′) and R1 (5′GTGTGTGCGCAAAAGGGAGG3′). Amplification was carried out in a Perkin-Elmer 9700 thermocycler with 100 ng genomic DNA mixed with reaction buffer from the FailSafe kit (Epicentre, Madison, Wis.). A touchdown amplification protocol beginning with two cycles consisting of a denaturation step at 94°C for 30 s, an annealing step at 55°C for 30 s and an extension step at 72°C for 60 s was used. In succeeding cycles, the annealing temperature was decreased by 1°C every two cycles to 50°C after 10 cycles. A further 25 cycles were then carried out with an annealing temperature of 50°C followed by a final extension step at 72°C for 5 min.

PCR amplification of the lactoferrin gene

Forward and reverse primers used for sequencing of the lactoferrin gene on chromosome 3 for exons 1–17, respectively, included: F1 (5′GGGGAGTGGGGAGGGAAGG3′), R1 (5′CCAGCCAACCGGCACAAGG3′); F2 (5′CATCAGAGGGTGCGGTGGTC3′), R2 (5′TGAAGCAGAGGAAGTAAGGAGAGC3′); F3 (5′TTGCTGGGTTTGGGTGAGTTTTCT3′), R3 (5′CCTCCACATGTTCCCCCAGTCTTA3′); F4 (5′GCACAGCATTCCCCCTTCC3′), R4 (5′GTGTGGCCTGTGCTTACAACTGG3′); F5 (5′CCTTGCCCACGGAGACCTCA3′), R5 (5′GCTCTATGTGGGGCCAGAGAAAAG3′); F6 (5′CTGCCCTGCAGGGAGTAGAAAC3′), R6 (5′TGTGCCCTGTAGGAATCTTGAAAA3′); F7 (5′GTCAGCCCGTGTGACAGAAGAGTT3′), R7 (5′GCTTTTGGGGCACTACCTTTACCT3′); F8 (5′TCCACGATGACCCCACAGTGTC3′), R8 (5′AACCGAGGCTCCTGCTCTCAGTTA3′); F9 (5′CCGTGGCCTCTTTGACTGTTGA3′), R9 (5′GAAGTGGAGGAGGAGAGGGTATGG3′); F10 (5′CGGCCCCTTTCATTTCTTCTT3′), R10 (5′CTGGCTGCTCAGTTTGAATAGTCC3′); F11 (5′AGGGCTGCAATTCTTTCTGTTTT3′), R11 (5′GAACTGCTGGGACGGTCTCTTT3′); F12 (5′GAGGCCTGCAGGCCACTATCAG3′), R12 (5′AAGGCCAGTTCTTTGAGGGAATCA3′); F13 (5′GATGACCCCCACTCTGCTGTG3′), R13 (5′TTGCTGTCCTGGGAAGTCTAATGA3′); F14 (5′GATGTGTTGTGATGCCAAAGACTC3′), R14 (5′CTGTGTTCAATCTGCCGCTGTA3′); F15 (5′TCCTCACCTAACATGAGCCCACAC3′), R15 (5′GCCACACCCACCCAGAAGAGAC3′); F16 (5′GCCCTCAAACCTTGACCTTCAC3′), R16 (5′TCTTTTCCTTAGCTACTCACTGTCTGC3′); F17 (5′GAAGAGCTGGGGGCAGTGAATG3′), R17 (5′AGGGGAGTGGGAATATGAGTGTGG3′). Amplification was carried out in a Perkin-Elmer 9700 thermocycler with 100 ng genomic DNA mixed with GeneAmp PCR reaction buffer (Applied Biosystems, Foster City, Calif.). A PCR amplification protocol beginning with a denaturation step at 94°C for 9 min, followed by 35 cycles, each consisting of a denaturation step at 94°C for 30 s, an annealing step for 30 s, and an extension step at 72°C for 60 s, followed by a final extension at 72°C for 10 m was used. The annealing temperature for exons 10, 11, 13, 14, 16, and 17 was 55°C, for exon 6 was 56°C, for exons 2, 3, 4, 5, 7, 8, 9, 12, and 15 was 58°C, and for exon 1 was 59°C. Amplification of exon 1 was carried out with the addition of 10% dimethylsulfoxide to the PCR mix.

DNA sequencing

Purification of PCR products and sequencing procedures were carried out as previously described (Ren et al. 2000). Primers used for sequencing in both directions include the two primers described above and four additional internal primers, viz., F2 (5′TGTCTGCTGCTCAAGGCGCGC3′), F3 (5′CCGCCGGTGAAGTGGATATCG3′), R2 (5′CGGTGGCGCAGGTCAATGAGGAT3′), and R3 (5′CTTTGCGCCGAGGAATCAGGA3′), to provide redundant bi-directional coverage of the full length of the coding sequence. The PCR amplification primers described above for the lactoferrin gene were also used for bi-directional sequencing.

Analysis of M1S1 gene mutations

For detection of single base changes at nucleotides T308G and T887 A, PCR products produced by primers F1 and R1 were digested according to the supplier's instructions with restriction enzymes *Nco*I and *Alw*44I, respectively (Boehringer Mannheim, Germany) at 37°C for 1 h, and the reaction products were resolved on a 1% agarose gel.

Detection of the insertion and single base deletions of the M1S1 gene (Genbank accession no. J04152) was carried out by using PCR amplification with primers F4 (5′TGCTGGTGCGTGAACTCGGTG3′) labeled with the fluorescent dye (5′FAM; Gene Probe Technologies, Gaithersburg, Md.) and R4 (5′ATCTGGATGGTGGGCTGCTCG3′). In control DNA, these primers amplified a 236-bp fragment including the site of the 8-base insertion and the single base deletion in the mutant M1S1 genes. After an initial 5-min denaturing step at 94°C, PCR was carried out for 35 cycles each consisting of a 94°C denaturation step for 30 s, a 55°C annealing step for 30 s, and a 72°C extension step for 1 min, followed by a final extension at 72°C for 4 min. Amplified fragments were electrophoresed on a 5% denaturing polyacrylamide gel in an Applied Biosystems 377 sequencer. The GENESCAN and GENOTYPER software packages (Applied Biosystems/Perkin-Elmer) were used to analyze the size and the zygosity of alleles.

Results

Clinical description of patients

Patients were diagnosed with GDLD on the basis of the typical clinical appearance and histopathological confirmation (Klintworth et al. 1997). Ten multiplex families were ascertained, with families 37001–37005 being of Indian ethnic origin, family 37009 being of Tunisian ethnic origin, and families 37010 and 37011 being of European ethnic origin. Samples from single affected individuals were collected from two additional families: 370012 (European) and 370013 (Tunisian). Affected individuals presented typically in the first or second decade, with the most constant symptom being photophobia. Patients presenting in the first decade showed punctate flat epithelial and subepithelial opacities in the central cornea. The peripheral cornea was unremarkable. The visual acuity in

such cases was typically between 20/80 and 20/200, and the symptoms of photophobia and tearing were almost always more impressive than the corneal signs in such early cases.

Patients with advanced lesions had visual acuity that was often reduced to counting fingers. Multiple protuberant multinodular yellow-gray subepithelial corneal nodules appeared opaque with direct illumination and had a distinctive translucent appearance on retroillumination. Variable degrees of anterior stromal opacification occurred in these advanced cases. Interestingly, the lesions in our patients did not have significant corneal vascularization or epithelial breakdown and involved the central interpalpebral area more than the periphery. The clinical characteristics of many of these patients have been reported previously (Buchi et al. 1994; El Matri et al. 1991; Kirk et al. 1973; Li et al. 1996; Mondino et al. 1981; Stock and Kielar 1976).

Family 37011 showed a typical clinical presentation for GDLD (Stock and Kielar 1976). The disease was limited to the cornea and characterized by the deposition of amyloid beneath the corneal epithelium. The deposits resembled droplets clinically, and they invariably recurred after a corneal graft. The family was also studied biochemically and histochemically, and the histopathology and immunohistochemical findings were identical to those of patients with GDLD, with amyloid deposits being localized beneath the epithelium (Klintworth et al. 1997). The corneal deposits in family 37011 contained abundant lactoferrin (Klintworth et al. 1997).

Linkage analysis

To determine the disease locus, linkage analysis initially was carried out by using ABI panel markers. Regions known to be involved in corneal amyloid deposition were considered as potential candidates, including the TGFBI and GSN gene regions. The lactoferrin (hLF) gene on 3p21 (Klintworth et al. 1997, 1998) was also excluded by linkage analysis in five of our eight families, although not in each individual family when analyzed alone. Two-point analysis of genotypes from chromosome 1 microsatellite markers was performed separately on families of each ethnic group. Families 37001 through 37005 (Indian) yielded significant lod scores ($Z>3.0$) with markers D1S519 ($Z_{max}=6.34$, at $\theta=0$), D1S2890 ($Z_{max}=3.94$, at $\theta=0.0$), D1S220 ($Z_{max}=4.62$, at $\theta=0$), D1S203 ($Z_{max}=4.35$ at $\theta=0$), and D1S2835 ($Z_{max}=3.66$ at $\theta=0.06$). Family 37009 (Tunisian) yielded significant lod scores with markers D1S519 ($Z_{max}=3.07$ at $\theta=0$) and D1S220 ($Z_{max}=3.38$, at $\theta=0$). However, families 37010 and 37011 (Caucasian) when analyzed together yielded no significant lod scores. When data from each of the two families were examined separately, positive lod scores were obtained with data from family 37010 for markers between D1S519 and D1S2835, whereas negative lod scores were obtained with all markers in the M1S1 region in family 37011. Summed lod scores at different recombination fractions for all markers from families 37001 through 37010 are listed in Table 1. Significant positive lod scores ($Z_{max}>3$) without recombinations were shown by markers between D1S519 and D1S230. The marker D1S519 showed the highest lod score ($Z_{max}=10.6$ at $\theta=0$). Lod scores for family 37011 obtained with these markers are shown in Table 2. It is noteworthy that family 37011 (referred to as Family

Table 1 Summed two-point Lod scores for GDLD in families 37001–37010 (*inf* infinity)

Markers	0	0.01	0.05	0.1	0.2	0.3	0.4	Z_{max}	θ_{max}
D1S2797	–inf	–2.87	–0.11	0.69	0.98	0.73	0.31	0.98	0.2
D1S2742	–inf	0.23	2.19	2.38	1.78	0.9	0.21	2.39	0.09
D1S519	10.6	10.4	9.38	8.11	5.59	3.22	1.21	10.61	0.001
D1S2890	7.78	7.59	6.8	5.8	3.87	2.13	0.73	7.78	0
D1S220	9.22	9.02	8.18	7.12	5	2.95	1.19	9.22	0
D1S203	4.76	4.63	4.13	3.51	2.33	1.25	0.41	4.76	0
D1S230	4.82	4.7	4.26	3.75	2.61	1.46	0.52	4.82	0
D1S2835	–inf	5.54	6.24	5.98	4.56	2.77	1.07	6.24	0.05
D1S2841	–inf	–2.06	0.83	1.64	1.68	1.11	0.45	1.68	0.2

Table 2 Two-point LOD scores for GDLD in family 37011 (*inf* infinity)

Markers	0	0.01	0.05	0.1	0.2	0.3	0.4	Z_{max}	θ_{max}
D1S2797	–inf	–2.85	–1.47	–0.9	–0.39	–0.15	–0.03	0	0.5
D1S2742	–inf	–1.47	–0.78	–0.49	–0.22	–0.08	–0.02	0	0.5
D1S519	–inf	–1.52	–0.81	–0.51	–0.23	–0.09	–0.02	0	0.5
D1S2890	–inf	–2.68	–1.34	–0.81	–0.34	–0.13	–0.03	0	0.5
D1S220	–inf	–2.68	–1.34	–0.81	–0.34	–0.13	–0.03	0	0.5
D1S203	–inf	–1.8	–1.02	–0.65	–0.29	–0.11	–0.02	0	0.5
D1S230	–inf	–2.68	–1.34	–0.81	–0.34	–0.13	–0.03	0	0.5
D1S2835	–inf	–2.68	–1.34	–0.81	–0.34	–0.13	–0.03	0	0.5
D1S2841	–inf	–2.68	–1.34	–0.81	–0.34	–0.13	–0.03	0	0.5

10 by Klintworth et al. 1998) lacked the exon 2 polymorphisms in the lactoferrin gene, preventing us from drawing any conclusions about the presence or absence of intragenic linkage to the lactoferrin gene on chromosome 3 (Klintworth et al. 1998).

When linkage results from all families were subjected to the admixture test by using the HOMOG program for markers between D1S519 and D1S2835 with θ values of 0, 0.001, 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.1, 0.2, 0.3, and 0.4, evidence for heterogeneity was seen with markers D1S519 (α=0.9, χ^2=1.933, *P*=0.08), D1S2890 (α=0.85, χ^2=4.36, *P*=0.018), D1S220 (α=0.9, χ^2=5.343, *P*=0.01), D1S203 (α=0.8, χ^2=2.47, *P*=0.058), D1S230 (α=0.85, χ^2=4.84, *P*=0.014) and D1S2835 (α=0.7, χ^2=2.57, *P*=0.054).

Multipoint analysis of GDLD confirmed linkage of the GDLD locus to the 16 cM region between markers D1S2742 and D1S2835 in families 37001–37010 (data not shown). Analysis of family 37011 resulted in a negative lod score (Z<–2) throughout the entire interval, excluding the locus from the M1S1 gene region in this family. The multipoint data also give significant evidence of locus heterogeneity. When the multipoint lod scores at 1-cM intervals between markers D1S2890 and D1S220 were analyzed by using HOMOG, heterogeneity was suggested with an α=0.85, a maximum ln (likelihood) for linkage with heterogeneity (H2) of 25.05, and a maximum ln (likelihood) for linkage with homogeneity (H1) of 13.94 resulting in χ^2=22.21. These values gave a *P*<0.0001 favoring linkage with heterogeneity if an asymptotic χ^2 distribution was assumed. More conservatively, the likelihood ratio between the two hypotheses was R=6.7×10^4. On examination, the haplotypes of affected individuals from families 37001–37010 were consistent with GDLD residing in the M1S1 region, whereas in family 37011, two affected siblings had inherited four distinct haplotypes throughout this region (Fig. 1).

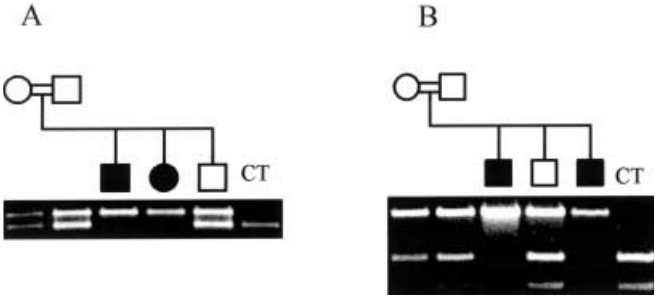

Fig. 2A, B Agarose gel electrophoresis of restriction-endonuclease-digested PCR products of the M1S1 gene. **A** *Nco*I digestion of amplified products from family 37001 cuts control but not mutant alleles. **B** *Apa*I digestion of amplified products from family 37005 cuts control but not mutant alleles

Mutation analysis of the M1S1 gene

The critical region identified for the GDLD locus in nine families included the M1S1 gene shown by Tsujikawa et al. (1999) to be responsible for GDLD in Japanese families. A 1200-bp DNA fragment including the entire coding region of the M1S1 gene was sequenced in affected members of each family and in 50 unrelated ethnically matched controls (100 total chromosomes, see above).

Four different mutations were identified in the five Indian families. Single-base changes T308G (resulting in an amino acid substitution of M1R at the initial methionine) and T887 A (causing an amino acid substitution of V194 E) were identified in families 37001 and 37005, respectively. The mutations disrupted restriction endonuclease recognition sites, viz., *Nco*I for M1R (T308G) and *Apa*II for V194 E (T887 A), and both resulted in a single band (1.2 kb) on agarose gel electrophoresis after restriction enzyme digestion (Fig. 2). Another single-base change at C658G resulting in an amino acid substitution of Q118 E was found in families 37003 and 37004 (Table 3). Family 37002 showed an 8-bp insertion at nucleotide 799 resulting in amplification of a 244-bp fragment in homozygous affected individuals, and 236-bp and 244-bp fragments in heterozygous carriers when PCR was carried out with primers 5′TGCTGGTGCGTGAACTCGGTG3′ and 5′ATCTGGATGGTGGGCTGCTCG3′ (controls gave a 236-bp fragment; Fig. 3A). All of the mutations co-segregated with the disease phenotypes (Fig. 1) and were not observed in 100 unaffected individuals of various ethnic backgrounds.

Two different mutations were identified in the three families of Tunisian origin. A single-base change T661A, resulting in a C119 S missense mutation, was detected in families 37009 and 37013. A single-base deletion, 1117delA, was found in family 37012 (Table 3). These sequence

Table 3 Summary of M1S1 mutations detected in nine GDLD families

Family ID	Protein change	Nucleotide change	Ethnic origin
37001	Met1Arg	T308G	India
37002	164 frameshift	799 +CCACCGCC (8 bp insertion)	India
37003	Gln118Glu	C658G	India
37004	Gln118Glu	C658G	India
37005	Val194Glu	T887 A	India
37009	Cys119Ser	T661 A	Tunisia
370010	188 frameshift	870 CCC to CC– (1 bp del C)	Europe
370012	271 frameshift	1117 AAG to –AG (1 bp del A)	Tunisia
370013	Cys119 Ser	T661 A	Tunisia

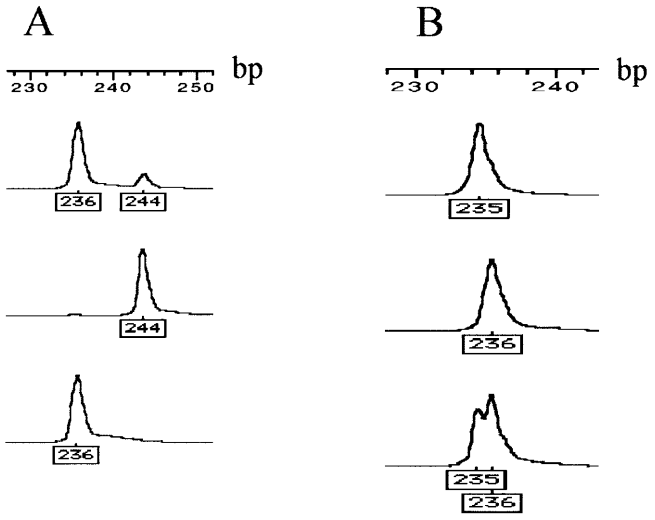

Fig. 3A, B DNA sequencer tracings of PCR products from normal and mutant alleles. **A** PCR products from a control individual (*bottom*), homozygous affected individual (*middle*) and heterozygote individual (*top*) from family 37002 demonstrating the 8-base insertion. **B** PCR products from a control individual (*middle*), homozygous affected individual (*top*), and heterozygote individual (*bottom*) from family 37010 demonstrating the 1-base deletion

changes co-segregated with affected individuals in their families and were not observed in 60 control individuals of various ethnic backgrounds (Fig. 1).

In one of the two Caucasian families, a single-base deletion, 870delC, was detected in family 37010. This gave a 235-bp fragment in homozygous affected individuals, 236-bp and 235-bp fragments in heterozygous carriers, and a 236-bp fragment in control individuals when the region was amplified by using the above primers (Fig. 3B).

Locus heterogeneity of GDLD

Bi-directional sequencing of the entire coding region of the M1S1 gene from three individuals (two affected and one unaffected) in family 37011 showed no changes when compared with the published sequence and that of controls. Haplotype analysis exhibited no similarity between the haplotypes of affected individuals in this and other families in this study, and haplotypes of the two affected siblings were different for markers in the M1S1 region (Fig. 1). In addition, the haplotypes of affected individual 3 and unaffected individual 11 were identical in the region between markers D1S2797 and D1S2841, which includes the M1S1 gene.

Because protein isolated from corneal tissue from an affected individual from family 37011 had been shown to contain abundant lactoferrin, the lactoferrin gene was examined for mutations in individual 4 of this family. All exons of the lactoferrin gene were sequenced in affected individual 4 and in two unrelated and unaffected controls. The only sequence difference from the lactoferrin genomic sequence from Genbank NT 005997 was a polymorphic A185G transition seen in exon 2 from both individual 4 and one control. This results in a polymorphic T30 A amino acid sequence change when using sequence numbers from the lactoferrin cDNA sequence with Genbank accession no. U07643, in which this sequence variant is also documented.

Discussion

This study demonstrates genetic heterogeneity of GDLD. We have mapped the disease locus, in seven unrelated families, to a 16 cM interval on the short arm of chromosome 1 between markers D1S519 and D1S2835 including the M1S1 gene. Seven novel mutations have been identified in affected individuals from various ethnic backgrounds in seven unrelated families and two isolated individuals. These include four missense and three frame-shift mutations. In addition, the mutations are distinct not only between each ethnic group, but also within the same ethnic group (Table 3), which suggests multiple independent origins for mutations causing GDLD in these families.

Tsujikawa et al. (1999, 2000) have previously have reported four mutations detected in the Japanese population: Q118X, 623delA, Q207X and S170X, the most frequent being in the nonsense mutation Q118X. Similarly, at the same site, we have identified a single-base change (C658G) leading to the codon substitution of Q118 E in two of the Indian families. Although this seems consistent with the possibilities that either it is a mutational hot spot or that this part of the M1S1 sequence encodes a functionally important part of the protein, Q118X is a founder mutation in the Japanese population so that the cause of the high frequency of this mutation might lie in the population genetics of Japan and India, rather than in the M1S1 protein itself.

The M1S1 gene product is a type I transmembrane protein of 323 amino acids. The single transmembrane region extends from amino acid residue 275 to 298. The M1S1 protein also contains an epidermal growth factor (EGF)-like repeat, a thyroglobulin repeat and a phosphatidylinositol (PIP2)-binding site containing serine, and tyrosine phosphorylation sites (residues 302–308) near its carboxyl terminus (Tsujikawa et al. 1999). This 40-kDa protein was originally identified as gastrointestinal carcinoma antigen by monoclonal antibody GA733 (Fornaro et al. 1995) and is highly expressed in normal human trophoblast cells, multistratified epithelia, and human carcinomas (Alberti et al. 1992; Fradet et al. 1984; Lipinski et al. 1981; Miotti et al. 1987). Although it has suggested to function as a cell-cell adhesion receptor in cancer cells, thereby regulating cell growth (Zutter 1998), its gene structure is consistent with a function in cell signal transduction (Tsujikawa et al. 1999). In this regard, Tsujikawa et al. (2000) have recently shown that M1S1 mutations associated with GDLD result in epithelial permeability several orders of magnitude higher than control samples, suggesting that this might contribute to the pathogenesis of

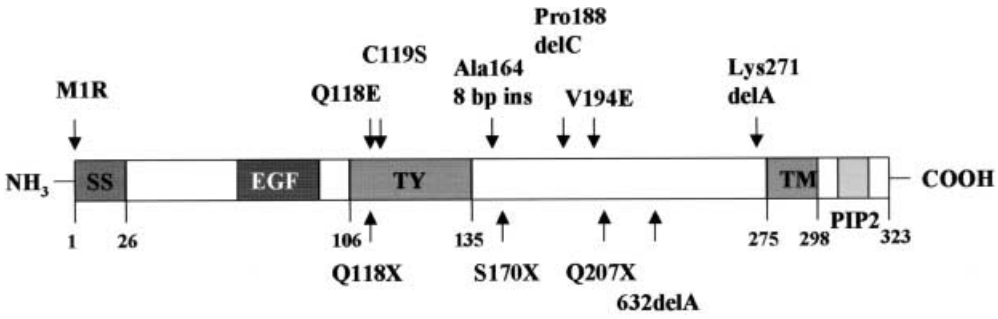

Fig. 4 Schematic diagram of the M1S1 gene and sequence changes identified with GDLD. Sequence changes identified in this study are shown *above* the gene, and previously identified mutations are shown *below* the gene (*SS* signal sequence, *EGF* epidermal growth factor-like repeat, *TY* thyroglobulin-like repeat, *TM* transmembrane region, *PIP2* phosphatidylinositol binding site)

amyloid deposition in this disease. All the mutations that we have identified in families 37001–37010 are located within the coding sequence of the M1S1 gene. The M1R mutation eliminates the initiation site, two mutations occur in the thyroglobulin repeat, and the remaining four mutations are distributed between the thyroglobulin repeat and the transmembrane sequence (Fig. 4). The mutations previously described by Tsujikawa et al. (1999) show a similar distribution. The lack of a specific pattern in these mutations makes it difficult to propose a single hypothesis to explain the mechanism of altered membrane permeability and amyloid deposition in this disease.

Linkage and haplotype analysis of family 37010 show no obligate recombinants, although the two-point lod scores generated by this family alone do not reach statistical significance. Sequence analysis of this family has identified a nonsense mutation (870delC) in all affected members, consistent with M1S1 being the GDLD locus in this family. In contrast, the GDLD locus in family 37011 can be excluded from the M1S1 region with negative lod scores throughout. Heterogeneity tests and examination of the haplotypes support these findings. Consistent with these data, the absence of sequence changes in the M1S1 coding region in this family also suggest that GDLD in this family results from mutations at a separate locus. To our knowledge, this is the first time that locus heterogeneity has been described for GDLD.

Potential candidates for an alternative locus causing GDLD include TGFBI (Munier et al. 1997; Yamamoto et al. 1998) and GSN (de la Chapelle et al. 1992a, 1992b; Hiltunen et al. 1991; Steiner et al. 1995), the two other genes known to be mutated in amyloid deposition in the cornea. However, the diseases associated with these genes are autosomal dominant traits having distinctly different phenotypes from GDLD. Furthermore, linkage analysis of family 37011 has excluded the TGFBI and GSN regions (data not shown). Thus, mutations in these genes are unlikely to cause GDLD in family 37011.

Because lactoferrin was specifically isolated from corneal tissue in one affected member of family 37011 (Klintworth et al. 1998), the lactoferrin gene was felt to be a potential candidate in this family. In an earlier study, the lactoferrin gene was excluded as a candidate for GDLD in most families with GDLD, but family 37011 (referred to as Family 10 by Klintworth et al. 1998) was not informative for the exon 2 polymorphisms in the lactoferrin gene and thus did not allow any conclusions to be made about the presence or absence of linkage of GDLD to the lactoferrin gene on chromosome 3 (3p21). However, no mutations were seen on sequencing the exons of the lactoferrin gene in an affected individual from family 37011. The only sequence variant identified, a polymorphic a-to-G transition in codon 30, was one of those used previously by Klintworth et al. (1998) for exclusion. Although there could be sequence changes in the regulatory regions of this gene, these results argue against lactoferrin being the site of the causative mutation. Identification of a new locus for GDLD may provide additional insights into the genetic basis of amyloidosis in the cornea.

Acknowledgement The authors are grateful to patients and their family members for their participation in this study. We should also like to acknowledge funding by the Swiss National Science Foundation grant no. 65250.01.

References

Akiya S, Furukawa H, Sakamoto H, Takahashi H, Sakka Y (1990) Histopathologic and immunohistochemical findings in gelatinous drop-like corneal dystrophy. Ophthalmic Res 22:371–376

Akiya S, Nagaya K, Fukui A, Hamada T, Takahashi H, Furukawa H (1991) Inherited corneal amyloidosis predominantly manifested in one eye. Ophthalmologica 203:204–207

Alberti S, Miotti S, Stella M, Klein CE, Fornaro M, Menard S, Colnaghi MI (1992) Biochemical characterization of Trop-2, a cell surface molecule expressed by human carcinomas: formal proof that the monoclonal antibodies T16 and MOv-16 recognize Trop-2. Hybridoma 11:539–545

Buchi ER, Daicker B, Uffer S, Gudat F (1994) Primary gelatinous drop-like corneal dystrophy in a white woman. A pathologic, ultrastructural, and immunohistochemical study. Cornea 13:190–194

Chapelle A de la, Kere J, Sack GH, Jr., Tolvanen R, Maury CP (1992a) Familial amyloidosis, Finnish type: G654 – a mutation of the gelsolin gene in Finnish families and an unrelated American family. Genomics 13:898–901

Chapelle A de la, Tolvanen R, Boysen G, Santavy J, Bleeker-Wagemakers L, Maury CP, Kere J (1992b) Gelsolin-derived familial amyloidosis caused by asparagine or tyrosine substitution for aspartic acid at residue 187. Nat Genet 2:157–160

Cottingham RW, Idury RM, Schaffer AA (1993) Faster sequential genetic linkage computations. Am J Hum Genet 53:252–263

El Matri L, Bachtobji A, Ghorbal M, Maamri J, Kamoun M, Ouertani A, Bardi R, Triki MF (1991) Forme familiale de dystrophie gélatineuse en gouttes de la cornée. J Fr Ophtalmol 14: 125–129

Fornaro M, Dell'Arciprete R, Stella M, Bucci C, Nutini M, Capri MG, Alberti S (1995) Cloning of the gene encoding Trop-2, a cell-surface glycoprotein expressed by human carcinomas. Int J Cancer 62:610–618

Fradet Y, Cordon-Cardo C, Thomson T, Daly ME, Whitmore WF, Lloyd KO, Melamed MR, Old LJ (1984) Cell surface antigens of human bladder cancer defined by mouse monoclonal antibodies. Proc Natl Acad Sci USA 81:224–228

Hiltunen T, Kiuru S, Hongell V, Helio T, Palo J, Peltonen L (1991) Finnish type of familial amyloidosis: cosegregation of Asp187-Asn mutation of gelsolin with the disease in three large families. Am J Hum Genet 49:522–528

Jiao X, Munier FL, Iwata F, Hayakawa M, Kanai A, Lee J, Schorderet DF, Chen MS, Kaiser-Kupfer M, Hejtmancik JF (2000) Genetic linkage of Bietti crystallin corneoretinal dystrophy to chromosome 4q35. Am J Hum Genet 67:1309–1313

Kirk HQ, Rabb M, Hattenhauer J, Smith R (1973) Primary familial amyloidosis of the cornea. Trans Am Acad Ophthalmol Otolaryngol 77:OP411-OP417

Klintworth GK (1999) Advances in the molecular genetics of corneal dystrophies. Am J Ophthalmol 128:747–754

Klintworth GK, Valnickova Z, Kielar RA, Baratz KH, Campbell RJ, Enghild JJ (1997) Familial subepithelial corneal amyloidosis – a lactoferrin-related amyloidosis. Invest Ophthalmol Vis Sci 38:2756–2763

Klintworth GK, Sommer JR, Obrian G, Han L, Ahmed MN, Qumsiyeh MB, Lin PY, Basti S, Reddy MK, Kanai A, Hotta Y, Sugar J, Kumaramanickavel G, Munier F, Schorderet DF, El Matri L, Iwata F, Kaiser-Kupfer M, Nagata M, Nakayasu K, Hejtmancik JF, Teng CT (1998) Familial subepithelial corneal amyloidosis (gelatinous drop-like corneal dystrophy): exclusion of linkage to lactoferrin gene. Mol Vis 4:31–38

Kwiatkowski DJ, Westbrook CA, Bruns GA, Morton CC (1988) Localization of gelsolin proximal to ABL on chromosome 9. Am J Hum Genet 42:565–572

Lasram L, Rais C, El Euch M, Ouertani A (1994) Gelatinous dystrophy of the cornea. Apropos of 5 cases. J Fr Ophtalmol 17: 24–28

Li S, Edward DP, Ratnakar KS, Reddy M, Tso MO (1996) Clinicohistopathological findings of gelatinous droplike corneal dystrophy among Asians. Cornea 15:355–362

Lipinski M, Parks DR, Rouse RV, Herzenberg LA (1981) Human trophoblast cell-surface antigens defined by monoclonal antibodies. Proc Natl Acad Sci USA 78:5147–5150

Miotti S, Canevari S, Menard S, Mezzanzanica D, Porro G, Pupa SM, Regazzoni M, Tagliabue E, Colnaghi MI (1987) Characterization of human ovarian carcinoma-associated antigens defined by novel monoclonal antibodies with tumor-restricted specificity. Int J Cancer 39:297–303

Mondino BJ, Rabb MF, Sugar J, Sundar Raj CV, Brown SI (1981) Primary familial amyloidosis of the cornea. Am J Ophthalmol 92:732–736

Munier FL, Korvatska E, Djemai A, Le Paslier D, Zografos L, Pescia G, Schorderet DF (1997) Kerato-epithelin mutations in four 5q31-linked corneal dystrophies. Nat Genet 15:247–251

Nakaizumi K (1914) A rare case of corneal dystrophy. Acta Soc Ophthalmol Jpn 18:949–950

Ohnishi Y, Shinoda Y, Ishibashi T, Taniguchi Y (1982) The origin of amyloid in gelatinous drop-like corneal dystrophy. Curr Eye Res 2:225–231

Ott J (1983) Linkage analysis and family classification under heterogeneity. Ann Hum Genet 47:311–320

Ren Z, Li A, Shastry BS, Padma T, Ayyagari R, Scott MH, Parks MM, Kaiser-Kupfer M, Hejtmancik JF (2000) A 5-base insertion in the g-crystallin gene is associated with autosomal dominant variable zonular pulverulent cataract. Hum Genet 106:531–537

Schaffer AA, Gupta SK, Shriram K, Cottingham RW (1994) Avoiding recomputation in genetic linkage analysis. Hum Hered 44:225–237

Shimazaki J, Hida T, Inoue M, Saito H, Tsubota K (1995) Long-term follow-up of patients with familial subepithelial amyloidosis of the cornea. Ophthalmology 102:139–144

Skonier J, Neubauer M, Madisen L, Bennett K, Plowman GD, Purchio AF (1992) cDNA cloning and sequence analysis of beta ig-h3, a novel gene induced in a human adenocarcinoma cell line after treatment with transforming growth factor-beta. DNA Cell Biol 11:511–522

Steiner RD, Paunio T, Uemichi T, Evans JP, Benson MD (1995) Asp187Asn mutation of gelsolin in an American kindred with familial amyloidosis, Finnish type (FAP IV). Hum Genet 95: 327–330

Stock EL, Kielar RA (1976) Primary familial amyloidosis of the cornea. Am J Ophthalmol 82:266–271

Takahashi M, Yokota T, Yamashita Y, Ishihara T, Uchino F, Imada N, Matsumoto N (1985) Unusual inclusions in stromal macrophages in a case of gelatinous drop-like corneal dystrophy. Am J Ophthalmol 99:312–316

Tsujikawa M, Kurahashi H, Tanaka T, Okada M, Yamamoto S, Maeda N, Watanabe H, Inoue Y, Kiridoshi A, Matsumoto K, Ohashi Y, Kinoshita S, Shimomura Y, Nakamura Y, Tano Y (1998) Homozygosity mapping of a gene responsible for gelatinous drop-like corneal dystrophy to chromosome 1p. Am J Hum Genet 63:1073–1077

Tsujikawa M, Kurahashi H, Tanaka T, Nishida K, Shimomura Y, Tano Y, Nakamura Y (1999) Identification of the gene responsible for gelatinous drop-like corneal dystrophy. Nat Genet 21: 420–423

Tsujikawa M, Tsujikawa K, Maeda N, Watanabe H, Inoue Y, Mashima Y, Shimomura Y, Tano Y (2000) Rapid detection of M1S1 mutations by the protein truncation test. Invest Ophthalmol Vis Sci 41:2466–2468

Weber FL, Babel J (1980) Gelatinous drop-like dystrophy. A form of primary corneal amyloidosis. Arch Ophthalmol 98:144–148

Yamamoto S, Okada M, Tsujikawa M, Shimomura Y, Nishida K, Inoue Y, Watanabe H, Maeda N, Kurahashi H, Kinoshita S, Nakamura Y, Tano Y (1998) A kerato-epithelin (betaig-h3) mutation in lattice corneal dystrophy type IIIA. Am J Hum Genet 62:719–722

Zutter MM (1998) Gastrointestinal carcinoma antigen GA733: target for immunodestruction and potential modifier of invasiveness and chemoresponsiveness. J Natl Cancer Inst 90:642–644