Prenatal Exclusion of Lamellar Ichthyosis Based on Identification of Two New Mutations in the Transglutaminase 1 Gene

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Lamellar ichthyosis is a severe, generalized, autosomal recessive genodermatosis characterized clinically by large, parchment-like scales and histologically by acanthosis and marked hyperkeratosis. Genetic heterogeneity in lamellar ichthyosis has been recognized with reports of two linked loci (on chromosomes 14q11 and 2q33–35). In a cohort of four small families with lamellar ichthyosis we found confirmatory evidence for linkage ($p \leq 0.01$) to D14S275, a microsatellite marker close to transglutaminase 1 on chromosome 14q11. We also identified two novel transglutaminase 1 mutations in an affected sibling pair from one of these families. The paternal mutation in exon 3, 1387insCAGC, causes a frameshift predicted to result in premature termination of translation within the same exon. The maternal mutation in exon 8, 4561delAC, also causes a frameshift and a premature stop codon in this exon. The mother of these siblings recently became pregnant with twins. Genotyping and direct sequencing of DNA isolated from fetal amniotic fluid cultures revealed the presence of the paternal but the absence of the maternal mutation, thus predicting a normal skin phenotype. Both twins were born with normal-appearing skin. Our findings demonstrate that mutations of both alleles of the transglutaminase 1 gene are the cause of lamellar ichthyosis in this family, and illustrate an emerging clinical application of molecular genetics in dermatology. Key words: genodermatoses/human genetics/linkage analysis/prenatal diagnosis. *J Invest Dermatol 110:179–182, 1998*

Lamellar ichthyosis (LI, MIM 242100) is a clinically heterogeneous congenital disorder of keratinization (Phillips and Baden, 1993). The disease has an autosomal recessive mode of inheritance and an estimated incidence of 1:250,000. Affected individuals are usually erythrodermic and encased in a collodion membrane at birth and go on to develop large, parchment-like, brown scales covering the entire body with flexural predominance. Although nonspecific, the most prominent histologic findings in LI are acanthosis and hyperkeratosis.

Two separate studies have mapped the disease gene for autosomal recessive LI to chromosome 14q near the transglutaminase 1 locus (TGM1) in some of the families studied (Russell *et al*, 1994; Huber *et al*, 1995). Individuals affected at this locus display a marked reduction in keratinocyte transglutaminase (TGK or transglutaminase 1) activity, and several laboratories have identified deleterious mutations in the TGM1 gene (Huber *et al*, 1995; Parmentier *et al*, 1995; Russell *et al*, 1995). Recently, the identification of a second disease locus on chromosome 2q33–35 has confirmed the long-suspected presence of genetic heterogeneity in LI (Parmentier *et al*, 1996). The existence of additional kindreds apparently unlinked to both TGM1 and 2q suggests that mutations in at least one additional gene can cause this genodermatosis (Bale *et al*, 1996; Parmentier *et al*, 1996).

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Abbreviations: LI, lamellar ichthyosis; TGM1, transglutaminase 1 gene.

We investigated genetic linkage of LI to TGM1 in four families with two or more affected siblings. A confirmatory lod score ($p \leq 0.01$) was found for D14S275, a microsatellite marker close to TGM1. We also identified two novel TGM1 mutations in one of these families and report the use of these findings to provide prenatal testing of LI in a recent twin pregnancy.

MATERIALS AND METHODS

Clinical evaluation and sample collection Study subjects were identified from the billing records of the University of Michigan Dermatology clinic. All pedigrees were Caucasian and nonconsanguineous. After receiving informed consent under a protocol approved by the Institutional Review Board, subjects were examined by a dermatologist and a blood sample (30 ml) was obtained. The diagnosis of LI was established through total body skin examination using standard criteria (Phillips and Baden, 1993). Ten milliliters of blood was used for establishment of lymphoblastoid cell lines and the remainder was used to isolate genomic DNA as previously described (Nair *et al*, 1995). Most experiments were performed using lymphoblastoid cell line DNA.

Genotyping and linkage analysis Four LI kindreds were genotyped for four polymorphic microsatellite markers on chromosome 14q11. The map order was cen–MYH7–(5.1 cM)–TGM1–(0 cM)–D14S264–(4.2 cM)–D14S275–q ter (Gyapay *et al*, 1994; Russell *et al*, 1994). The TGM1 microsatellite marker is located in intron 14 of the TGM1 gene. Genotyping methods have been described in detail elsewhere (Nair *et al*, 1995). Allele sizes were ascertained by comparison with sizes obtained for CEPH individual no. 134702, a known standard (Gyapay *et al*, 1994). Linkage analysis of the genotype data was performed using LINKAGE 5.1 (Ott, 1991) under an autosomal recessive model, assuming full penetrance and a disease allele frequency of 0.002, calculated according to Hardy–Weinberg equilibrium

Table I. Linkage analysis of 14q11 loci in four lamellar ichthyosis kindreds

		Recombination fraction (θ)			
Marker	0	0.1	0.2	0.3	0.4
MYH7	−2.1526	0.2547	0.3942	0.2523	0.0792
TGM1	−0.4173	0.2123	0.1572	0.0720	0.0162
D14S264	−0.2634	0.3681	0.2924	0.1670	0.0610
D14S275	0.8565	1.1544	0.7452	0.3447	0.0794

using the mendelian inheritance in man value for the prevalence of LI (1/250,000).

DNA fragment amplification The complete TGM1 gene contains 15 exons, ranging from 89 to 403 bp, interspersed by 14 introns of highly variable size (Kim *et al*, 1992). DNA fragments containing most TGM1 exons were amplified by polymerase chain reaction (PCR) using the primers described by Russell *et al* (1995). The fragment containing exons 5–6 was amplified using primers 5′-GGCTGGGGGTTCTTAGGAATCC-3′ and 5′-GGTGAGGCCCAGGGAGAGAAC-3′, exon 12 using primers 5′-GGAA-TTGGAACCTCACCCTTGAC-3′ and 5′-TCCATGTCCACAGCCCTG-AG-3′, and exon 13 using primers 5′-TAAGTGCTCCTTACCCAG-CCCTGCC-3′ and 5′-CGCCCACCTCTGATGTCCTTATC-3′. PCR was performed in a volume of 50 μl containing 200 ng of lymphoblastoid cell line DNA, 0.5 mM of each primer, 200 mM each of dATP, dCTP, dGTP, and dTTP, 50 mM KCl, 10 mM Tris-HCl pH 9.0, 1.5 mM $MgCl_2$, 5% dimethyl sulfoxide, 0.5% Triton X-100, and 1 U Taq polymerase (Promega, Madison, WI). The PCR reaction was run with an initial denaturation at 94°C for 3 min, followed by 30 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, and a 7 min extension at 72°C on an MJ Research PTC-100 thermal cycler (Watertown, MA). PCR products were run on 1.5% agarose gels and DNA was purified for sequencing by electro-elution.

Mutation detection Approximately 0.5 pmol PCR product was sequenced using the Sequenase™ PCR Product Sequencing Kit (Amersham Life Sciences, Arlington Heights, IL) with [^{35}S]-dATP label. PCR amplification primers were used as sequencing primers, except for the anti-sense primer 5′-TTCAGCACAGATGGGCAGT-3′ used for sequencing of exon 9. Mutations identified by direct sequencing of the PCR products were confirmed by sequencing of cloned PCR products. Several clones of each cloned PCR product, generated using the pCRII™ vector (Invitrogen, San Diego, CA) were sequenced on an ABI DNA sequencer (Applied Biosystems, Foster City, CA, model 373 A).

Prenatal testing Following genetic counselling and obtaining informed consent with the approval of the Institutional Review Board of the University of Michigan, amniotic fluid was obtained in the fourteenth week of pregnancy from each amniotic cavity of the twin pregnancy (amniocentesis was indicated due to advanced maternal age and a history of Down's Syndrome in a previous pregnancy). Genomic DNA was isolated from ≈2 × 10^6 fetal cells cultured from the amniotic fluid. Genotyping was performed using microsatellite markers MYH7 and TGM1, and direct sequencing of exons 3 and 8 was performed as described above.

RESULTS

Genotyping and linkage analysis Using the criteria recently recommended for genome-wide linkage analysis (Lander and Kruglyak, 1995), marker D14S275 provided confirmatory evidence for linkage of LI to the TGM1 region ($p \leq 0.01$), even in this relatively small sample of four kindreds (**Table I**). Genotype data are only shown for family 2 in which further mutation analysis was pursued (**Fig 1**).

Sequence analysis of TGM1 exons in family 2 Direct sequencing of the 764 bp PCR fragment containing exons 2 and 3 revealed a heterozygous CAGC insertion in exon 3 after position 1387 [sequence numbering according to Kim *et al* (1992)] in affected individuals 2–1 and 2–2, and the unaffected father 2–3 (**Fig 2**). The insertion was not found in the unaffected mother 2–4. The cloned PCR fragment from individual 2–1 was sequenced, and clearly carried the observed insertion (data not shown).

Direct sequencing of the 498 bp PCR fragment containing exons

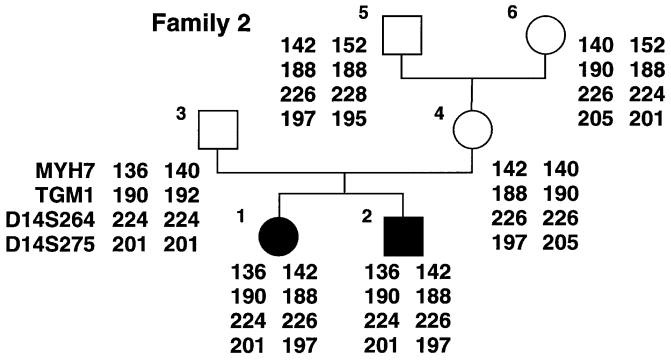

Figure 1. Genotype data of microsatellite markers MYH7, TGM1, D14S264, and D14S275 for family 2. □, males; ○, females; *solid symbols* denote affected individuals, *open symbols* denote unaffected individuals. Marker allele sizes are arrayed vertically as the most likely haplotypes based on the available parental data.

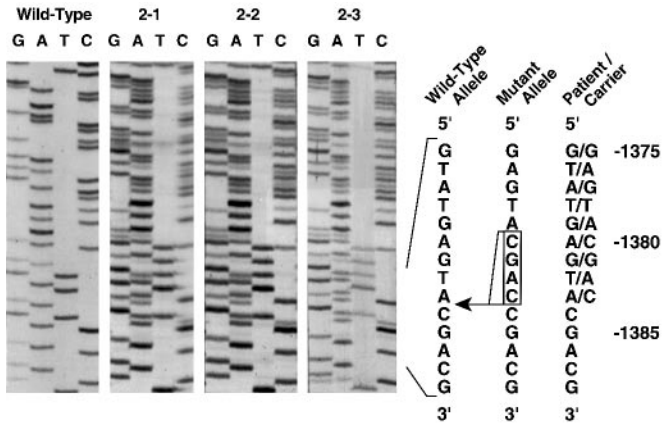

Figure 2. TGM1 exon 3 contains a 4 bp insertion in the paternal chromosome shared by affected siblings of LI family 2. The autoradiograph shown demonstrates a CAGC insertion after position 1387 (1387insCAGC) in the mutant allele present in the affected siblings (2–1 and 2–2) and father (2–3) of family 2, and not in the wild-type control.

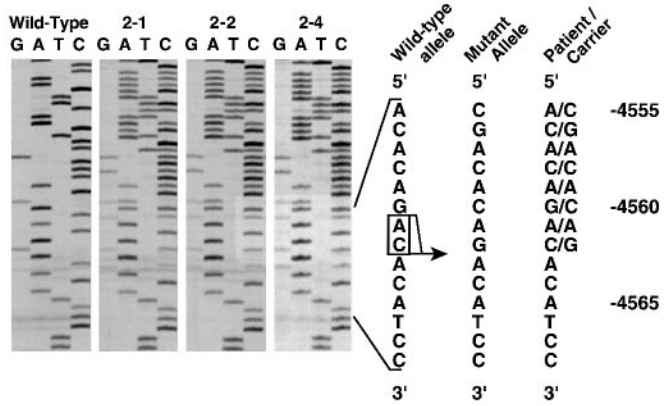

Figure 3. TGM1 exon 8 contains a 2 bp deletion in the maternal chromosome shared by affected siblings of LI family 2. The autoradiograph shown demonstrates a CA deletion at position 4561–4562 (4561delAC) in the mutant allele present in the affected siblings (2–1 and 2–2) and mother (2–4) of family 2, and not in the wild-type control

8 and 9 revealed a heterozygous AC deletion in exon 8 at position 4561–62 [sequence numbering according to Kim *et al* (1992)] in affected individuals 2–1 and 2–2. The identical mutation was found in the unaffected mother 2–4 and maternal grandfather 2–5, but not in the unaffected father (**Fig 3**). The deletion was also confirmed by sequencing of the cloned PCR fragment (data not shown). None of the previously reported TGM1 mutations (Huber *et al*,

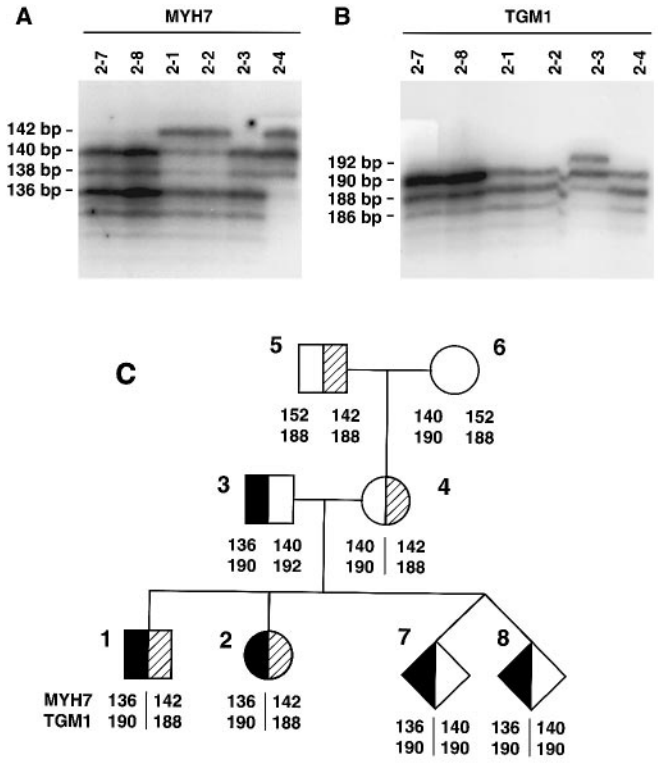

Figure 4. Prenatal testing reveals that only the paternal LI allele is transmitted to unborn twins in family 2. Except for the maternal grandparents (2–5 and 2–7), all family members were typed for microsatellite markers MYH7 (*A*) and TGM1 (*B*). Allele sizes are indicated to the left of the autoradiographs. The maternal TGM1 alleles are 190 and 192 bp long (individual 2–4), whereas the paternal alleles are 188 and 190 bp long (individual 2–3). There is comigration between the 188 bp allele and an artifactual "shadow band" generated from the 190 bp allele by Taq polymerase. The fact that the 190 bp bands obtained for individuals 2–7 and 2–8 are much darker than the 188 bp bands indicates homozygosity for the 190 bp allele. (*C*) Pedigree diagram, showing transmission of disease haplotypes. MYH7 and TGM1 allele sizes are separated by vertical bars only when the haplotype could be determined with certainty. Striped halves of symbols represent the paternally inherited exon 3 mutation (1387insCAGC); solid halves represent the maternally inherited exon 8 mutation (4561delAC). The fetuses (of unknown sex and zygosity at the time of analysis) are represented by diamonds.

1995; Parmentier *et al*, 1995; Russell *et al*, 1995) were detected in these individuals.

Prenatal testing Recently, individual 2–4 (**Fig 1**) became pregnant with twins. Amniocentesis was indicated for assessment of the genetic risk to the fetuses due to advanced maternal age and a history of Down's Syndrome in a previous pregnancy. The family expressed interest in prenatal testing for LI as well. DNA derived from fetal amniotic fluid cultures was genotyped for the MYH7 and TGM1 loci (**Fig 4*A*,*B***). DNA from other family members previously studied was re-genotyped in parallel. As shown in **Fig 4(*C*)**, the disease-associated paternal haplotype (MYH7 136/TGM 1190) is inherited by both fetuses; however, the normal maternal haplotype (MYH7 140/TGM1 190) is also inherited by both unborn twins. This finding was confirmed by sequencing DNA from amniotic fluid cells after PCR amplification of exons 3 and 8. As expected, sequencing revealed the presence of the paternal 1387insCAGC mutation in exon 3, and the absence of the maternal 4561delAC mutation in exon 8 in both fetuses (data not shown). We therefore predicted both twins to be phenotypically normal carriers of the paternal TGM1 mutation. Six months later both twins were born in good health with completely normal skin.

DISCUSSION

Transglutaminases catalyze the post-translational modifications of proteins by transamidation of available glutamine residues in a Ca^{2+}-dependent manner (Greenberg *et al*, 1991). Five related but distinct transglutaminases have been identified: keratinocyte transglutaminase (TGK or transglutaminase 1), tissue transglutaminase (TGC or transglutaminase 2), epidermal transglutaminase (TGE or transglutaminase 3), human blood coagulation factor XIII, and erythrocyte band 4.2. Transglutaminase 1 plays a key role in the formation of the cornified envelope, a highly insoluble structure that replaces the plasma membrane during terminal differentiation of keratinocytes. The enzyme cross-links cornified envelope precursors, such as involucrin and loricrin, by catalyzing the formation of isodipeptide Nε-(γ-glutamyl)lysine bonds, leading to the formation of a scaffold onto which other reinforcement proteins are attached (Hohl *et al*, 1991; Kim *et al*, 1992). The mechanism by which TGM1 function is impaired by the novel mutations presented here is not known; however, the frameshifts caused by the CAGC insertion in exon 3 and the AC deletion in exon 8 both lead to premature stop codons that would be predicted to create premature translation termination. The former would eliminate the active site and calcium-binding site of transglutaminase 1, whereas the latter mutation would eliminate only the calcium-binding site (Greenberg *et al*, 1991). Alternatively, by analogy to frameshift mutations in Herlitz junctional epidermolysis bullosa (Baudoin *et al*, 1994) and numerous other genetic disorders (McIntosh *et al*, 1993), these mutations may result in mRNA instability, which would effectively generate null alleles. Consistent with this possibility, the only frameshift mutation previously reported in LI was shown to strongly reduce the level of normal transcripts (Huber *et al*, 1995). Quantitation of mutant transcript levels from a skin biopsy or keratinocytes cultured from these patients will be required to address this issue.

Previous reports of prenatal diagnosis of LI were based on morphologic examination of amniotic fluid cells and fetal skin biopsies at a gestational age of 17–21 wk (Perry *et al*, 1987; Akiyama *et al*, 1994). In other genodermatoses such as epidermolytic hyperkeratosis and recessive dystrophic epidermolysis bullosa, several laboratories have used PCR-based assays with DNA obtained by chorionic villus sampling or amniocentesis to perform prenatal diagnosis at 10–15 and 12–15 wk, respectively (Rothnagel *et al*, 1994; Christiano *et al*, 1996). This noninvasive approach and early diagnosis of diseases with such significant morbidity and life-threatening complications are clearly important advancements in modern-day medicine. In this paper we present for the first time a DNA-based prenatal testing of LI at a gestational age of 14 wk. Here we demonstrate that in individual cases the detection of mutations in TGM1 (or other genes yet to be recognized) can have significant clinical applications in families affected with LI.

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