Linkage studies of four fibrillar collagen genes in three pedigrees with Larsen-like syndrome

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Abstract

We report seven children from three families who had a set of common clinical features suggestive of Larsen-like syndrome, including unusual facies, bilateral dislocations of the knees and elbows, club foot, and short stature. All of the patients originated from the island of La Réunion in the Indian Ocean. The occurrence of several affected sibs in these families and the large number of consanguineous marriages on this island are consistent with autosomal recessive inheritance of the disease. Based on this hypothesis, the pedigrees were used for linkage analysis in a candidate gene assay.

Lod score calculations in a pairwise study with four different fibrillar collagen genes, COL1A1, COL1A2, COL3A1, and COL5A2, allowed us to exclude these genes as the mutant loci. Supporting this, electrophoretic analysis of collagens derived from fibroblast cultures failed to show defective molecules. We conclude that this syndrome is not a collagen disorder.

Larsen syndrome is a relatively rare genetic disease characterised by multiple dislocations, unusual facies, and ligamentous hyperlaxity.[1] Additional clinical and radiological features have also been described.[2-4] They include vertebral anomalies, palate defects, hydrocephalus, and a higher number of ossification centres. This clinical variability sometimes makes the recognition of this syndrome difficult. Evidence for two modes of inheritance (autosomal dominant and recessive) has been reported[5] which could be related to phenotypic variability. The milder form of the disease seems to be dominantly inherited whereas the most severe forms, which include dwarfism and major skeletal abnormalities, might be transmitted as recessives. Despite its rarity in European countries and North America, an unexpectedly large number of patients with a severe form of the disease has been registered on the island of La Réunion, situated in the Indian Ocean off the east coast of Africa. On this island of 600 000 inhabitants, more than 40 affected children have been identified during the last 20 years, giving an approximate incidence of 0·0008 (1 per 1500 births) which, according to our estimations, would be much higher than in France (approximately 1 per 100 000 births) or other western countries. All patients have short stature which distinguishes them from the original description of Larsen *et al.*[1] It could correspond to the most severe form of the disease since several of the affected children died in early childhood.

The underlying pathogenesis of Larsen syndrome is not yet known, although it has been proposed, in two previous studies, that defective aggregation of collagen fibrils and ultrastructural abnormalities in proteoglycan filaments could be implicated.[6,7] In an attempt to test the hypothesis of a mutation in a fibrillar collagen gene, linkage analyses were performed with the two type 1 collagen genes (COL1A1 and COL1A2). Two other collagen genes, COL3A1 and COL5A2, which form a cluster on the long arm of chromosome 2,[8] were also tested in a candidate gene assay.

Materials and methods

PATIENTS

Sixty-three members from three pedigrees (I, II, and III) comprising 130 subjects were investigated (fig 1). Four affected patients in pedigree I (Ia, Ib, Ic, and Id), two in pedigree II (IIa and IIb), and one in pedigree III (IIIa) were examined clinically. All of them exhibited a common set of clinical features. Unfortunately, two other children in pedigree III who died in early childhood were not available for further clinical and radiological examination. Blood samples from only six affected subjects (Ia, Ib Id, IIa, IIb, and IIIc) were obtained for DNA analysis.

DNA STUDIES

DNA extracted from peripheral blood was digested with appropriate enzymes and fractionated on 1% agarose gels. Transfer to nylon membrane (Hybond N, Amersham) was performed according to the manufacturer's instructions. Filters were hybridised with cDNA or genomic probes labelled with ^{32}P either by nick translation or by random priming. Washing was undertaken at room temperature with 2 × SSC/0·1% SDS, then at 65°C in the same buffer for 10 to 20 minutes.

The probes used for detection of seven different dimorphisms were kindly provided by Drs B Sykes,[10] F Ramirez,[11,12] and R Dalgleish[13] and are summarised in table 1. A recently described polymorphism in the COL3A1 gene was also used.[14] PCR amplification of a 300 bp genomic DNA fragment was followed by *Alu*I digestion and analysis on a 6% acrylamide gel. A single base change (G→A) produced an additional *Alu*I site.

The LINKAGE program[16] was used for lod score calculations. The disease was assumed to be recessive and fully penetrant. The

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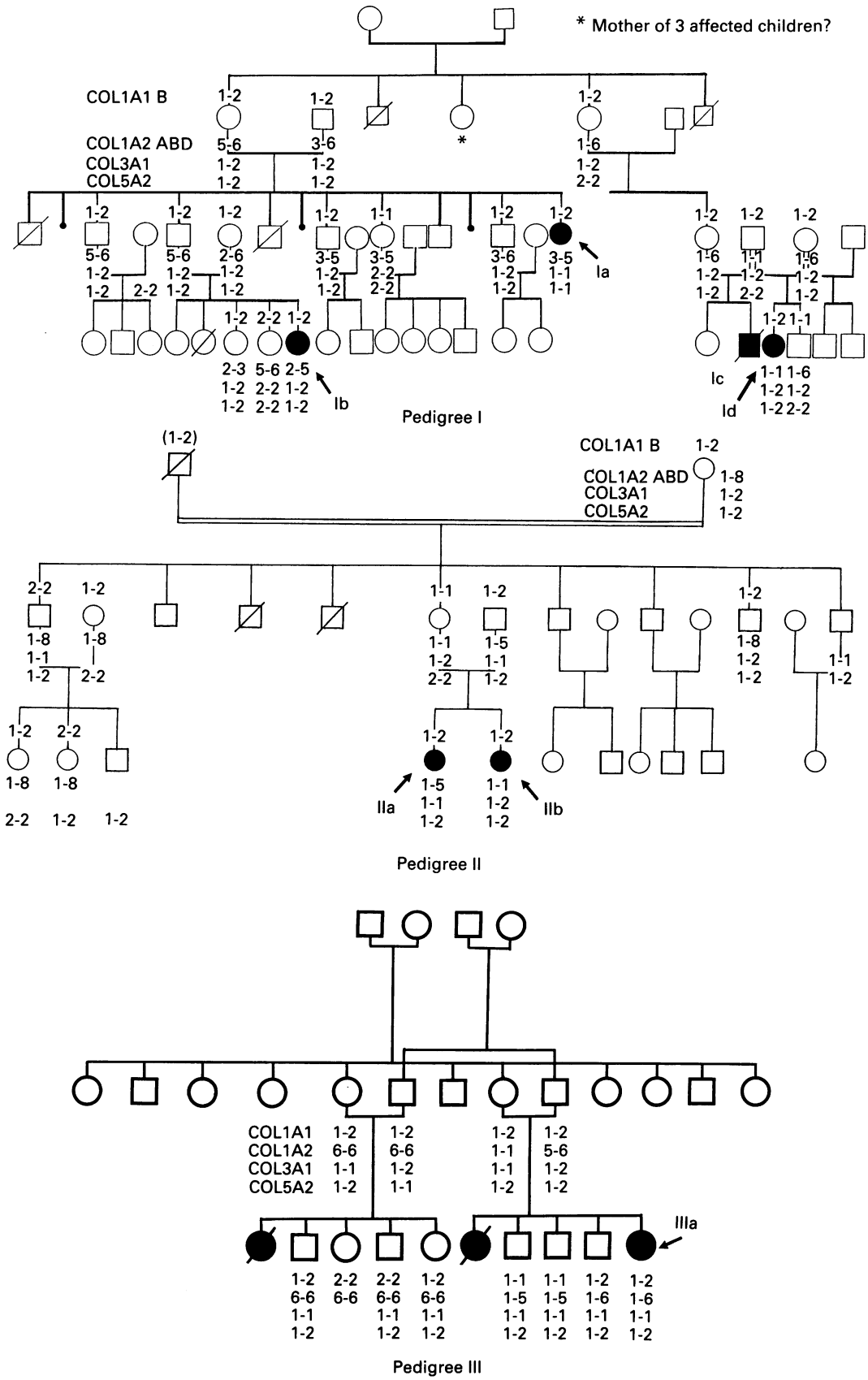

Figure 1 COL1A1, COL1A2, COL3A1, and COL5A2 genotypes of the three pedigrees (I, II, and III). The haplotype notation used in this figure for COL1A1 and COL2A1 is the same as proposed by Sykes et al.[9] B=RsaI, A=EcoRI, B=MspI, D=RsaI. The haplotype in brackets was deduced from relatives. For COL3A1 only the results obtained with AluI are presented. The arrows indicate the patients whose blood samples were available.

HOMOG program[17] was used to test the hypothesis of heterogeneity in the three families. Evaluation of the Ln likelihoods with this program gave no evidence for genetic heterogeneity.

PROTEIN STUDIES

Skin biopsies obtained from patient Ib and from additional patients who had no other affected relatives were used to initiate fibroblast cultures. Confluent cells were labelled

Table 1 Probes and RFLPs used in this study.

Gene	Restriction enzyme	Probe	Allele fragment (kb)	Allele frequency* (Caucasians)
COL1A1	*Rsa*I	2FC6	3·6/2·6	0·21/0·79
COL1A2	*Eco*RI	NJ142	14/10·5+3·5	0·66/0·34
COL1A2	*Rsa*I	Hf1131	2·9/2·1	0·34/0·66
COL1A2	*Msp*I	Hf1131	2·1/1·6	0·14/0·86
COL3A1	*Eco*RI	Idf17	2·1/1·6	0·39/0·61
COL3A1	*Ava*II	pPB1	6·2/4·5	0·74/0·26
COL3A1	*Alu*I	0·208/0·113+0·095†		0·32/0·68
COL5A2	*Msp*I	DMC2	13/9·0	0·15/0·85

* Values drawn from references 10, 14, and 15.
† A genomic DNA fragment of 298 bp was amplified by PCR and digested with *Alu*I. A constant band of 86 bp was obtained.

Table 2 Clinical and radiological anomalies found in affected subjects.

	Ia	Ib	Ic*	Id	IIa	IIb	IIIa
Characteristic facies	+	+	+	+	+	+	+
Multiple dislocations							
Hips	Uni	Bi	?	Bi	Bi	Bi	Bi
Knees	Bi	Bi	Bi	Bi	Bi	Bi	Bi
Elbows	Bi	No	?	Bi	Bi	Bi	Bi
Equinovarus or valgus deformity	Bi	Bi	Uni	Uni	Bi	Bi	Bi
Joint laxity	+	+	+	+	+	+	+
Short stature†	+	+	+	+	+	+	+
Palate defect	+	−	−	−	−	−	−
Scoliosis	+	−	?	?	−	−	+
Hydrocephalus	−	−	+	−	−	−	−
Multiple ossification centres	+	?	?	+	+	+	−
Mental retardation	+	?	?	−	?	?	+

* This patient who died at 7 years was not available for additional clinical examination during the course of this study.
† The size at birth varied between 38 and 41 cm and weight was in the range of 2500 g.
Uni = unilateral, Bi = bilateral.

with ^{3}H proline and incubated for 18 hours. Labelled material was analysed by gel electrophoresis and total collagen secretion was evaluated according to previously described procedures.[18]

Results

CLINICAL REPORTS

The clinical features of seven affected subjects are summarised in table 2. Further examination of the patients confirmed the early description of two of them.[19] They all had severe dwarfism, both weight and height being affected. The face was small with a prominent forehead and widely set eyes (fig 2A). The nasal bridge was depressed and the mouth and ears were rather small, the latter exhibiting an abnormal rim. The teeth were decayed in early childhood. Generalised muscular hypotonia was found to be associated with skin and ligamentous hyperlaxity (fig 2B). Protrusion of the abdomen was particularly obvious.

Skeletal abnormalities were consistently observed. They included dislocations of the knees, elbows, and hips (fig 2C,D) as well as micromelia of the forearms and internal curvature of the hands. Equinovarus or equinovalgus deformity of the feet was always present.

Radiological anomalies were highly relevant for the diagnosis. Generalised osteoporosis was associated with early bone maturation and an increase in the number of ossification centres (fig 2C). Metaphyses were enlarged and diaphyses were bowed (fig 2C,D). All these skeletal, muscular, and ligamental disturbances resulted in walking disability.

Mental retardation was mentioned in two cases,[19] but the influence of psychosocial problems on the development of intelligence was not clearly delineated in three others. Hearing loss, hypertelorism, and respiratory distress were not reported in any of these patients, though they have been mentioned in a few studies.[20 21] Cardiovascular defects were noticed in patients IIa and IIb who had hypertrophy of the left ventricle; this had been previously described in two other Italian sibs of consanguineous parents.[22] Palate defect and hydrocephalus, which have been reported in a few cases,[4] were found in only one subject. Karyotypes were normal.

RFLP STUDIES

Sixty-three subjects were investigated for eight DNA polymorphisms in four different collagen genes. The size of the fragments generated by restriction enzymes and the allele frequencies for unrelated Caucasians[10 15] are presented in table 1. The values obtained from 10 unaffected and unrelated inhabitants of the island of La Réunion did not differ significantly (not shown). Combination of results from the three restriction site dimorphisms in COL1A2 allowed us to construct haplotypes (fig 1). Six of the eight possible combinations were observed. Segregation analysis was carried out on the basis of recessive transmission of the disease, which corresponded to the most likely hypothesis. Results of the pairwise linkage study are given in table 3. Evidence against linkage was obtained for the COL1A1 and the COL1A2 markers (with two different polymorphisms in the case of COL1A2).

The COL3A1 gene was first tested with two polymorphic sites (*Eco*RI and *Ava*II) situated in the 3′ end of the gene. A maximum likelihood value of 1·62 at $\theta = 0$ for *Eco*RI and 0·82 at $\theta = 0{\cdot}01$ for *Ava*II was obtained. Since these values were inconclusive, a recently described polymorphism found in exon 31 of the type III collagen gene was tested. Discordance between this marker and the disease locus was found. Similarly, the COL5A2 marker showed evidence against linkage. Since COL3A1 and COL5A2 map to the same region of chromosome 2 and are closely linked,[8] it became obvious that this gene cluster could be excluded as the mutant locus.

COLLAGEN STUDIES

Confluent fibroblasts from patient Ib were labelled for 18 hours. Total collagen synthesis in the culture medium (CM) and cell layer (CL) were in the same range as controls: 12·2% *v* 12% ± 2·4 of total proteins in the CM. Types I and III procollagen and collagen chains had normal electrophoretic mobility and their CNBr peptide maps were the same as controls (not shown). Type V, which represents 1 to 3% of total collagen in normal fibroblasts, was not detected on the electrophoretograms. Type I (α_1I + α_2I) represented 90% of total collagen and 10% corresponded

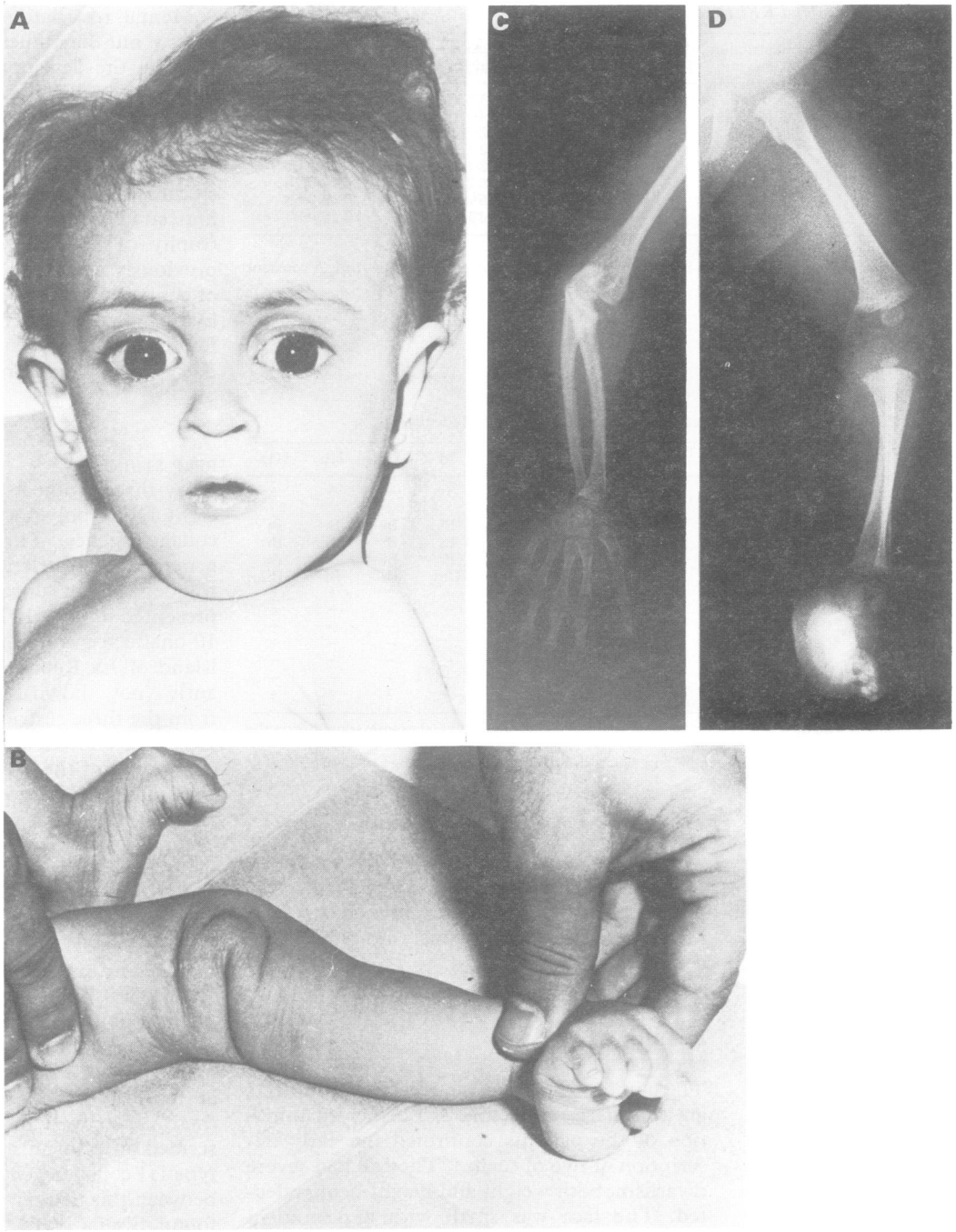

Figure 2 Clinical and radiological features of patients. (A) Facial appearance of patient Id. (B) Hyperextensibility of the lower limb in patient Id. (C) Dislocation of the elbow, bowing of the cubitus, and abnormal number of ossification centres in patient Ia. (D) Subluxation of the knees in patient IIa. Note the enlargement of the metaphyses.

Table 3 Lod scores for two point linkage analysis of Larsen syndrome and fibrillar collagen genes.

Locus	Enzyme	Recombination fraction (θ)						
		0·0	0·01	0·05	0·1	0·2	0·3	0·4
COL1A2	*Eco*RI	−∞	−3·12	−1·69	−1·08	−0·49	−0·20	−0·05
COL1A2	*Rsa*I	−5·34	−1·98	−1·13	−0·71	−0·31	−0·12	−0·03
COL1A1	*Rsa*I	−∞	−0·65	−0·01	0·17	0·18	0·09	0·03
COL3A1	*Eco*RI	1·62	1·57	1·36	1·11	0·68	0·33	0·09
COL3A1	*Alu*I	−∞	−2·39	−1·33	−0·80	−0·31	−0·10	−0·02
COL5A2	DMC2	−2·86	0·01	0·53	0·57	0·36	0·15	0·03

The three pedigrees were combined for lod score calculations.

to type III. These values were equivalent to normal ones.

Discussion

Because of its unexpected clinical heterogeneity, Larsen syndrome is sometimes difficult to diagnose accurately.[3] The minimal diagnostic criteria include multiple dislocations of joints and a typically flat face. However, many additional features can occur[4 23 24] which are highly variable and should be only regarded as contributory.[3] The patients reported here exhibited multiple congenital dislocations and

facial abnormalities suggesting that the diagnosis of Larsen syndrome might be considered. They also all had short stature which has only been described in the most severe forms of the disease.[7 25]

Genetic heterogeneity is generally recognised in Larsen syndrome. Both autosomal recessive and dominant transmission have been suggested,[4 24 26 27] but a clear correlation with phenotypic variations remains to be delineated, although, according to Hall,[5] severe forms with short stature would be more frequently recessive. Recessive inheritance is likely in the pedigrees reported here for the following reasons. (1) In two of these pedigrees (II and III) normal parents had two affected sibs and in pedigree III two sisters who had married two brothers gave birth to three affected children. (2) All members of the three pedigrees originated from the same mountainous part of the island. The geographical isolation of this region for about 200 years has favoured consanguineous marriages. The mother of patients IIa and b was born to consanguineous parents. Many persons in this area bear the same name, for instance the grandmother of patient Ia has the same name as the grandmother of patient IIIa, and a further patient not reported here, whose parents are consanguineous, also has this name. (3) The child mortality on the island was high until 1960. The mother of patient Ia had four miscarriages and three of her sibs died before the age of 6 months, but no accurate diagnosis was given. One of her sisters who married a man with the same name as the father of patient Ic and Id had three children with mental retardation and club foot. They died before the age of 20 years without detailed clinical and radiological assessment. This suggests that some patients with this Larsen-like syndrome could have remained undiagnosed especially before 1960. If this assumption were true this would indicate that the frequency of the disease has been underestimated.

Therefore, recessive inheritance appears to be more likely than dominant transmission with low penetrance since it would better explain the abnormally high incidence of the disease. Heterozygous carriers of the mutation could have originated from a common ancestor. Indeed, such a founder effect was shown on this island with Werdnig-Hoffmann disease.[28]

Linkage studies have allowed us to eliminate the direct involvement of two genes, COL1A1 and COL1A2, of type I collagen. This result was rather unexpected since two previous studies performed at the histological level suggested that ultrastructural abnormalities of collagen fibres could be responsible for the phenotype.[6 7] However, these assumptions were based only on light and electron microscopy and were restricted to three isolated cases. No biochemical studies had been carried out. In a recent paper, increased hydroxylation of type I collagen was observed in the skin of two patients with Larsen syndrome and an abnormal karyotype.[29] Deletion of a gene located on the proximal part of chromosome 6 was proposed to explain the phenotype, but so far neither collagen gene nor enzyme involved in the maturation of collagen fibres have been mapped to this region. It needs to be emphasised that no chromosomal abnormalities were found in our patients like in most previously reported cases.

The COL3A1 and COL5A2 genes exist as a gene cluster on chromosome 2.[30] Lod score calculations have provided exclusion of these markers as the mutant loci. This confirms that this syndrome is not the result of a type III collagen defect, which is consistent with the normal electrophoretic mobility of type III collagen chains and with the lack of any sign of vessel fragility in the patients.

Elucidation of the defective gene in this syndrome will require further linkage studies either with anonymous DNA markers or with other candidate genes. On the basis of ultrastructural findings (not shown), a possible involvement of an elastin associated microfibrillar component in the Larsen phenotype should not be excluded.

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