Original articles

Genetic linkage to the type VII collagen gene (COL7A1) in 26 families with generalised recessive dystrophic epidermolysis bullosa and anchoring fibril abnormalities

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Abstract

To strengthen the evidence for genetic linkage to COL7A1, we have studied 26 generalised recessive dystrophic epidermolysis bullosa (EB) families of British, Italian, Irish, and South African origin. We chose two linkage markers, a COL7A1 *PvuII* intragenic polymorphism and a highly informative anonymous microsatellite marker, D3S1100, which maps close to the COL7A1 locus at 3p21.1-3. Diagnosis was established by family history, clinical examination, immunofluorescence, and ultrastructural studies. The *PvuII* marker was informative in 16 families with a maximum lod score (Z_{max}) of 3.51 at recombination fraction (θ) = 0. The D3S1100 microsatellite was informative in 24 out of 25 families with Z_{max} = 6.8 at θ = 0.05 (Z = 4.94 at θ = 0) and no obligatory recombination events. These data strongly suggest that COL7A1 mutations cause EB in these families and, combined with previous studies, indicate locus homogeneity. The importance of anchoring fibrils for dermal-epidermal adhesion is further underlined. D3S1100 may later prove useful in prenatal diagnosis of this disease, if used in combination with other markers.

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Epidermolysis bullosa (EB) encompasses a group of inherited diseases characterised by inappropriate blistering of the skin after minor trauma. Clinically it varies from a limited, non-scarring form to a severe mutilating disorder with widespread loss of skin and scarring. Three major types are separated on the basis of the microscopic level of blistering within the skin¹: (1) EB simplex with cleavage through the basal epidermis; (2) junctional EB with cleavage through the lamina lucida of the basement membrane; and (3) dystrophic EB with cleavage below the lamina densa of the basement membrane. Dystrophic EB has autosomal dominant and recessive forms. Attempts at

further sub-categorisation² have been based on both clinical and microscopic variation in the phenotype, but there is overlap between categories causing difficulties in precise classification.

COL7A1 is the prime candidate gene for both autosomal dominant and recessive dystrophic EB. Anchoring fibrils, visible on electron microscopy as cross banded fanning structures extending from the lower lamina densa into the papillary dermis, are composed mainly of type VII collagen³ and have both qualitative and quantitative abnormalities in dystrophic EB.^{4,5} In generalised recessive dystrophic EB, recognisable anchoring fibrils may be missing and immunofluorescence staining with type VII collagen antibodies is markedly reduced or absent.⁶⁻⁸ Partial cloning of COL7A1⁹ and identification of an intragenic *PvuII* polymorphism¹⁰ enabled demonstration of tight linkage to dominant dystrophic EB¹⁰⁻¹³ and, in one study of 19 patients from France, to generalised recessive dystrophic EB with Z_{max} = 3.97 at θ = 0.¹⁴ Recently a homozygous insertion-deletion within the N-terminal non-collagenous (NC1) domain of COL7A1 has been recognised in a generalised recessive dystrophic EB patient¹⁵ and a methionine to lysine substitution within the C-terminal non-collagenous (NC2) domain of a patient with mitis type of recessive dystrophic EB.¹⁶ These data strongly suggest that COL7A1 mutations cause EB in these patients. Following the demonstration of increased synthesis and secretion of collagenase^{17,18} the collagenase genes became strong candidates. However, two independent studies have excluded genetic linkage between it and recessive dystrophic EB.^{19,20}

Informative markers which are tightly linked to a disease locus can be used for prenatal diagnostic testing of fetal DNA obtained from chorionic villus samples at 11 weeks' gestation.²¹ Currently prenatal diagnosis of EB is performed by electron microscopy and immunofluorescence of a fetal skin biopsy, taken at 16 weeks' gestation.²² Although there have been reports of prenatal diagnosis with the *PvuII* RFLP,^{23,24} this marker is only fully

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informative in some cases (heterozygosity in our population was 36%). A highly informative marker would therefore be very useful for this purpose.

We have tested linkage to COL7A1 in 26 further generalised recessive dystrophic EB families to confirm the previous study and to seek evidence of locus heterogeneity. We chose the intragenic *PvuII* COL7A1 RFLP and a second, microsatellite repeat marker, D3S1100.²⁵ This marker has a heterozygosity of 77% and has been mapped close to 3p21.3 by the NIH/CEPH collaborative mapping group.²⁶ We have also examined the suitability of this marker for prenatal diagnosis by its segregation patterns in our families.

Materials and methods

PATIENTS

Families with generalised recessive dystrophic EB were recruited from clinics in London at St Thomas's Hospital, Great Ormond Street Hospital, and through the three charitable organisations, DEBRA UK, DEBRA Ireland, and DEBRA Italy. One family was also included from South Africa. Clinical details were catalogued and a skin biopsy, taken from unblistered skin after gentle rubbing, was examined by transmission electron microscopy using standard methods.²⁷ Indirect immunofluorescence using LH7:2,²⁸ GB3,²⁹ and anti-type IV collagen antibodies was performed on UK patients. Blood samples were taken from all available family members with ethical committee approval.

DNA ANALYSIS

Standard methods were used for phenol/chloroform extraction of lymphocyte genomic DNA. Two variable DNA markers were used. (1) *PvuII* RFLP. The PCR was used to amplify

a 431 bp fragment containing the relevant *PvuII* site. (Primer 1: 5'GTGGCCAGGAACAGTCCCGGGTCC3'; primer 2: 5'CGAGGTGTCCACCACACGTAGTTC3'). After five minutes denaturing at 95°C, 30 cycles of denaturing at 95°C, annealing at 68°C and elongation at 72°C were performed. Amplified fragments were digested with *PvuII* (Northumbria Biologicals Limited) and separated on 2% agarose gels (fig 1A). (2) D3S1100 microsatellite. PCR amplification of this microsatellite using ³²P end labelled primer 1 (5'GGTTTCATATACCATCAATCCCAC3') and cold primer 2 (5'GTACACCATCATGAGGAGTCTGG3') was performed.²⁵ PCR product (10 µl) and 4 µl of loading buffer (98% formamide, 1% xylene cyanol, 1% bromophenol blue) were heated to 95°C for five minutes, cooled on ice, and the single stranded products were immediately electrophoresed on 6% polyacrylamide gels containing 8 mol/l urea at 37 W for three to four hours. The dried gel was autoradiographed at -70°C for two to seven days (fig 1B).

LINKAGE ANALYSIS

Two point lod scores were calculated using the MLINK programme in the LINKAGE package.³⁰ Allelic frequencies were calculated on the basis of 47 unrelated persons from our study population. Autosomal recessive inheritance and complete penetrance were assumed.

Results

CLINICAL AND ULTRASTRUCTURAL FINDINGS

The structure, consanguinity, and origins of the 26 families are shown in table 1 and fig 2. All patients had extreme skin fragility with blistering, scarring, and milia formation from birth. Nail loss, pseudosyndactyly, con-

Figure 1 Marker results. Pedigrees are shown with subjects above their corresponding lane. Open box: unaffected male; open circle: unaffected female; filled box: affected male; filled circle: affected female. (A) *PvuII* RFLP in family 13. The affected child is homozygous for the + allele and shows two digested fragments of 326 bp and 105 bp. (B) D3S1100 microsatellite in family 12. (C) D3S1100 microsatellite in family 15. Arrows show the number of repeats (rpt).

Table 1 Structure, consanguinity, and origin of families with generalised recessive dystrophic epidermolysis bullosa

Family	Origin (race)	Consanguinity	No of parents	No of affected offspring	Total no of offspring
1	UK (White)	No	2	1	2
2	UK (White)	No	2	1	2
3	UK (White)	No	2	1	2
4	UK (White)	No	2	1	2
5	UK (White)	No	2	1	2
6	UK (White)	No	2	1	2
7	UK (White)	No	2	2	2
8	UK (White)	No	2	1	3
9	UK (White)	No	2	1	3
10	UK (White)	No	2	1	4
11	UK (White)	1st degree	4*	2	2
12	UK (African)	No	2	1	2
13	UK (African)	1st degree	2	1	2
14	UK (Asian)	No	2	1	2
15	UK (Asian)	1st degree	2	2	3
16	UK (Asian)	1st degree	2	1	6
17	Eire (White)	No	2	1	2
18	Eire (White)	1st degree	2	2	3
19	Eire (White)	No	2	1	4
20	SA (Asian)	No	2	1	2
21	Italy (White)	No	2	2	2
22	Italy (White)	No	2	1	2
23	Italy (White)	No	2	1	2
24	Italy (White)	No	2	1	2
25	Italy (White)	1st degree	4*	2	2
26	Italy (White)	No	2	2	3

SA=South Africa. * Pedigrees for the two families with four parents were identical (fig 2).

Table 2 Lod score table between generalised recessive dystrophic epidermolysis bullosa phenotype and (A) *PvuII* marker and (B) D3S1100 microsatellite

(A) Family	Recombination fraction (θ)						
	0.0	0.01	0.05	0.1	0.2	0.3	0.4
1	-0.18	-0.17	-0.14	-0.10	-0.06	-0.02	-0.01
3	-0.18	-0.17	-0.14	-0.10	-0.06	-0.02	-0.01
4	0.12	0.12	0.10	0.08	0.05	0.02	0.01
6	0.12	0.12	0.10	0.08	0.05	0.02	0.01
7	0.3	0.29	0.26	0.21	0.13	0.06	0.02
9	0.25	0.24	0.21	0.17	0.10	0.05	0.01
11	0.2	0.19	0.14	0.08	0.01	-0.02	-0.01
13	0.24	0.23	0.18	0.13	0.05	0.01	-0.01
15	0.10	0.10	0.08	0.06	0.03	0.01	0.00
16	0.5	0.49	0.43	0.37	0.24	0.13	0.05
18	0.84	0.82	0.71	0.58	0.33	0.13	0.01
20	0.12	0.12	0.10	0.08	0.05	0.02	0.01
22	0.12	0.12	0.10	0.08	0.05	0.02	0.01
23	0.12	0.12	0.10	0.08	0.05	0.02	0.01
25	0.67	0.65	0.55	0.44	0.25	0.11	0.04
26	0.12	0.12	0.09	0.06	0.02	0.00	0.00
Total	3.51	3.38	2.89	2.3	1.3	0.56	0.13

(B) Family	Recombination fraction						
	0.0	0.01	0.05	0.1	0.2	0.3	0.4
2	0.12	0.12	0.09	0.06	0.02	0.00	0.00
3	0.12	0.12	0.09	0.06	0.02	0.00	0.00
4	0.12	0.12	0.10	0.08	0.05	0.02	0.01
5	0.12	0.12	0.09	0.06	0.02	0.00	0.00
6	0.12	0.12	0.09	0.06	0.02	0.00	0.00
7	0.60	0.58	0.52	0.43	0.27	-0.13	0.03
8	0.25	0.24	0.19	0.14	0.06	0.02	0.00
9	-0.05	-0.05	-0.04	-0.03	-0.02	-0.01	0.00
10	0.37	0.36	0.29	0.22	0.12	0.05	0.01
11	1.33	1.30	1.16	1.00	0.66	0.36	0.12
12	0.12	0.12	0.12	0.11	0.08	0.04	0.01
13	0.59	0.57	0.50	0.41	0.25	0.13	0.05
14	0.12	0.12	0.09	0.06	0.02	0.00	0.00
15	-1.48	-0.78	-0.23	-0.03	0.07	0.05	0.02
16	1.30	1.27	1.16	1.02	0.73	0.44	0.18
17	0.12	0.12	0.09	0.06	0.02	0.00	0.00
18	1.59	1.55	1.42	1.25	0.89	0.55	0.24
19	0.37	0.37	0.33	0.28	0.19	0.10	0.03
20	0.12	0.12	0.12	0.11	0.08	0.04	0.01
21	0.6	0.58	0.52	0.43	0.27	0.13	0.03
22	0.12	0.12	0.12	0.11	0.08	0.04	0.01
23	0.12	0.12	0.12	0.11	0.08	0.04	0.01
24	0.12	0.12	0.09	0.06	0.02	0.00	0.00
25	-2.03	-0.79	-0.20	-0.02	0.06	0.04	0.01
Total	4.94	6.64	6.8	6.01	4.04	2.2	0.78

tractures, and oesophageal strictures developed in older children. Electron microscopy confirmed the structural and numerical abnormality of anchoring fibrils, with a level of

Figure 2 The pedigree structure for families 11 and 25. Double line indicates consanguineous marriage.

cleavage, if present, below the lamina densa. Immunofluorescence in UK patients showed no visible staining with LH7:2 antibody except in a few cases where staining was present although greatly reduced in intensity and in a patchy distribution compared to controls. GB3 and type IV collagen staining were consistently normal.

DNA ANALYSIS AND LINKAGE

The lod scores for the *PvuII* RFLP and the microsatellite marker are shown in table 2(A) and (B) respectively. All families were informative for at least one of the markers. The 16 informative families for the *PvuII* RFLP gave a combined maximum lod score of 3.51 at $\theta=0$ with no obligatory recombinations. Our observed allelic frequencies were 0.28 (+ allele) and 0.72 (- allele). Heterozygosity in 50 unrelated persons was 36%. Zmax for the microsatellite was 6.8 at $\theta=0.05$ with $Z=4.94$ at $\theta=0$. Although there were no obligatory recombinations, in two consanguineous families (15 and 25) the affected patients were heterozygous for the marker, suggesting a probable recombination event in the grandparents or great grandparents (fig 1C). The calculated two point lod score between the two markers estimates the proximity of D3S1100 to COL7A1 (full lod table not shown). Thirteen families were informative for both markers with 28 informative meioses and one obligatory recombination. The disease showed no clustering of any particular allele for either marker.

Discussion

The lod score of 3.52 for the *PvuII* RFLP in our generalised recessive dystrophic EB families is highly significant and confirms the results of Hovnanian *et al.*¹⁴ The *PvuII* polymorphism lies in the coding sequence for a fibronectin-like domain (FN-8) in the NC-1 region of the type VII collagen molecule.^{9,14,31} The combined evidence of positive gene linkage, abnormalities of anchoring fibrils and type VII collagen protein expression in epidermal basement membrane, and the observation of two recessive dystrophic EB patients with separate COL7A1 mutations^{15,16} strongly suggests that all generalised recessive dystrophic EB families have allelic mutations.

Since the *PvuII* marker was only informative in 16 out of 26 families we used the microsatellite repeat D3S1100 at 3p21.1-3.^{9,26,32} This was informative for 24 out of 25 families tested. There were two possible recombinations

(consanguineous families 15 and 25) probably occurring in the grandparents or great grandparents with consequent heterozygosity in affected children. Alternatively, but less likely, the children are compound heterozygotes for mutations in COL7A1. Both families gave positive lod scores with the *PvuII* RFLP. Zmax for the microsatellite marker was at $\theta=0.05$. Linkage analysis between the microsatellite and *PvuII* marker site showed only one recombination in 28 informative meioses. These observations, combined with NIH/CEPH collaborative mapping group data,²⁶ suggest a maximum distance of 5 cM between COL7A1 and D3S1100. If used alone for prenatal diagnosis this predicts a 5% recombination (misdiagnosis) rate which we consider unacceptably high. However, in combination with a second suitable microsatellite located at the opposite flank of COL7A1, it could form the basis of an accurate and informative test for the majority of families.

Other workers have tested the hypothesis that the collagenase gene participates in the pathogenesis of recessive dystrophic EB, but two independent reports have excluded gene linkage.^{19,20}

Finally, despite strict criteria for clinical selection, we have noted significant interfamilial variation of clinical severity. Very probably this is related to the site and type of mutation within COL7A1. The lack of linkage disequilibrium for particular RFLP genotypes supports the hypothesis that many different COL7A1 mutations produce the EB clinical phenotype and is consistent with observations of other diseases such as osteogenesis imperfecta, Ehlers-Danlos syndrome type IV, and certain chondrodysplasias.

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