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Localisation of a Fanconi anaemia gene to chromosome 9p

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Using homozygosity mapping in a large consanguineous family, we have localised to chromosome 9p a further gene for the autosomal recessive, genetically heterogeneous disease Fanconi anaemia (FA). This is the fourth of at least eight FA genes to be localised to a discrete chromosomal region. Previously localised genes are *FAA*, *FAC* and *FAD*. By analysis of assigned families we show that the gene localised to chromosome 9p is *FAF*, *FAG* or *FAH*, or a new *FA* gene, and refine the localisation to the 21 cM region between markers *D9S1678* and *D9S175*.

Keywords: Fanconi anaemia; genetic heterogeneity; homozygosity mapping; chromosome 9p

Introduction

The autosomal recessive disorder Fanconi anaemia (FA) is characterised clinically by progressive bone marrow failure, sometimes associated with skeletal abnormalities, and an increased cancer risk. Patient cells exhibit increased chromosomal breakage.[1] Particularly after exposure to DNA cross-linking agents, and it has generally been assumed that this reflects a defect in a DNA repair pathway, although other explanations have been proposed (see Digweed[2] and Auerbach[3] for reviews).

FA shows extensive locus heterogeneity[4] with currently eight complementation groups (FA-A to FA-H) determined by somatic cell fusion and analysis of cellular sensitivity to treatment with DNA cross-linking agents.[5] Three FA genes, *FAA*,[6] *FAC*[7] and *FAD*,[8] have been mapped and *FAC* and *FAA* have been cloned by expression and positional cloning strategies.[9–11] It was recently possible to demonstrate that the two proteins, FAA and FAC, associate to form a complex which is localised to both cell cytoplasm and cell nucleus.[12] Although the function of this protein complex is as yet unknown, the finding that FAC also associates with the cyclin dependent kinase $p34^{cdc2}$ suggests that cell cycle regulation may be involved.[13] Indeed, other studies have suggested an involvement of cyclins in the cellular phenotype of FA.[14,15]

The lack of significant global homology of FAA or FAC to known proteins has hampered the elucidation of the basic defect in this disorder. Thus the cloning of further FA genes is of considerable importance, not only for diagnostic purposes, but also in the expectation that they will show homology to known proteins and

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thus help define the primary pathway affected in FA. One approach to this goal is the chromosomal localisation of other FA genes.

We have established such a chromosomal localisation for an FA gene on chromosome 9p by homozygosity mapping in a consanguineous family which had not previously been assigned to a complementation group. By linkage analysis we show that this gene is not the gene for FA complementation groups A to E. Analysis of two further FA families, similarly excluded from groups A to E, is compatible with this localisation and allows refinement to a 21 cM region between markers *D9S1678* and *D9S175*.

Materials and Methods

Families

All patients described were diagnosed on the basis of their chromosomal hypersensitivity to DNA cross-linking agents. The five families analysed are referred to here as families I to V. The large consanguineous family I (patients FA15BER and FA23BER) has not been previously assigned to a complementation group. The Lebanese parents are first cousins once removed. Family II (patient EUFA178) has been assigned to group B.[16] The Turkish parents in family III are first cousins and their affected child (EUFA130) is the reference group E patient.[17,18]

In Family IV there are two affected children (patients EUFA348 and EUFA349). Family V has two affected children (EUFA316); fibroblasts of an affected aborted foetus, diagnosed prenatally on the basis of Mitomycin C induced chromosome breakage, were also available. Families IV and V have been excluded from groups A, B, C, D and E by cell fusion and complementation analysis (H Joenje 1998 unpublished results). Pedigrees of these five families are shown in Figures 2 and Figure 3.

Where possible, lymphoblastoid cell lines were established by EBV transformation at the Departments of Human Genetics in Amsterdam or Würzburg. DNA was extracted from blood or from lymphoblastoid cells by standard techniques.

Microsatellite Analysis

In the initial homozygosity search, the parents and the two affected children of family I were analysed. The haplotypes of the homozygous regions of chromosome 7 and chromosome 9 were then examined for the seven unaffected children, and the other four families. The microsatellite markers were from the MDC-Généthon mapping panel with an average spacing of 11 cM. Markers were amplified by PCR using fluorescently labelled primers and analysed on the Pharmacia ALF (Freiburg, Germany) or ABI 377 (Weiterstadt, Germany) sequencers. Length determination was carried out using an internal standard with the Genescan 1.2 and Genotyper software.

Linkage Analysis

Version 5.1 of the LINKAGE software was used to calculate pairwise lod scores between the disease and marker loci assuming a fully penetrant autosomal recessive mode of inheritance and uniform allele frequencies. All genotypes were checked for Mendelian inheritance using the LINKRUN program. For the two consanguineous families, I and III, loops were broken at the fathers.

Results

Exclusion of FA-A, FA-C and FA-D

The starting point for the localisation reported here was a locally resident, consanguineous family of Lebanese origin with two children affected with FA (Figure 2, pedigree I). Initial diagnosis was based on clinical and haematological findings and was confirmed by the analysis of chromosomal breakage after treatment with a trifunctional alkylating agent (data not shown). Three attempts to establish a permanently transformed lymphoblastoid cell line from the affected children failed,

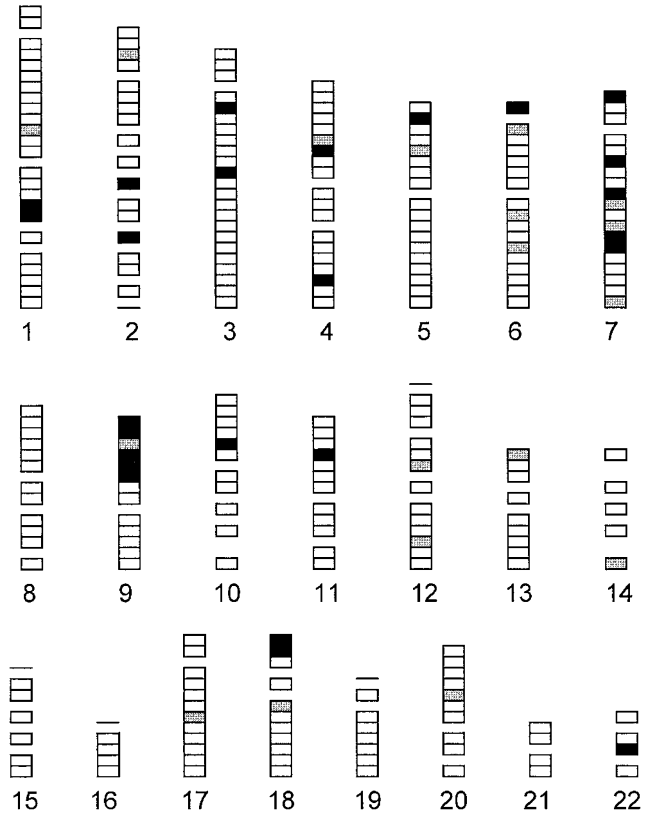

Figure 1 *Homozygosity mapping of 269 microsatellite markers spaced at 11 cM intervals in Fanconi anaemia family I. Each box represents a microsatellite marker located on the 22 autosomes; black boxes indicate markers that were informative and homozygous in both affected children; shaded boxes are markers that were homozygous in both affected children but not informative; white boxes were markers that were heterozygous in at least one of the two affected children; missing boxes are markers that were not analysed.*

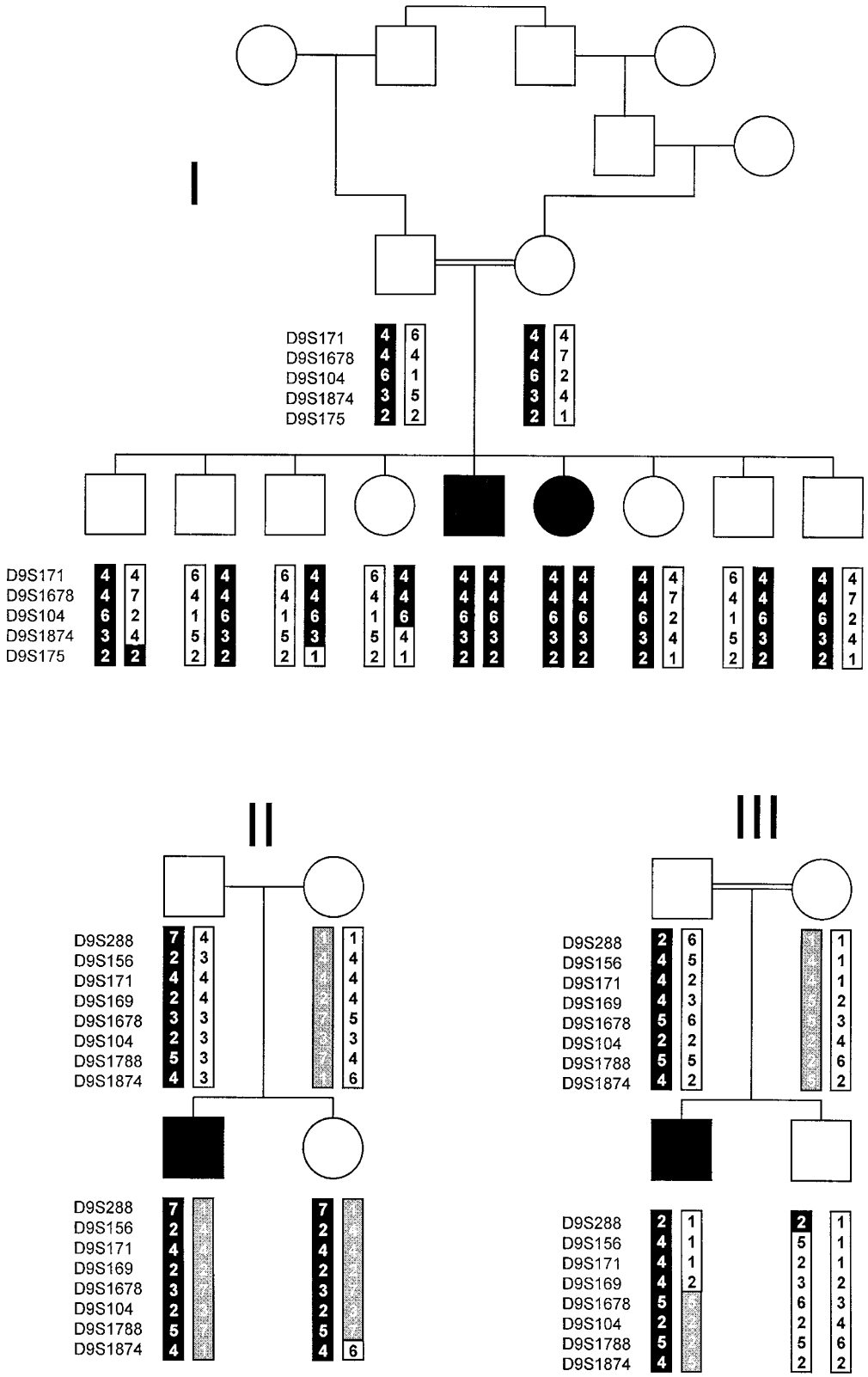

Figure 2 *Most likely haplotypes for microsatellite markers on chromosome 9p. I: the large consanguineous Lebanese family not previously assigned to a complementation group; II: a family assigned to complementation group B;*[16] *III: the reference group E family;*[17] *symbols of affected individuals are filled.*

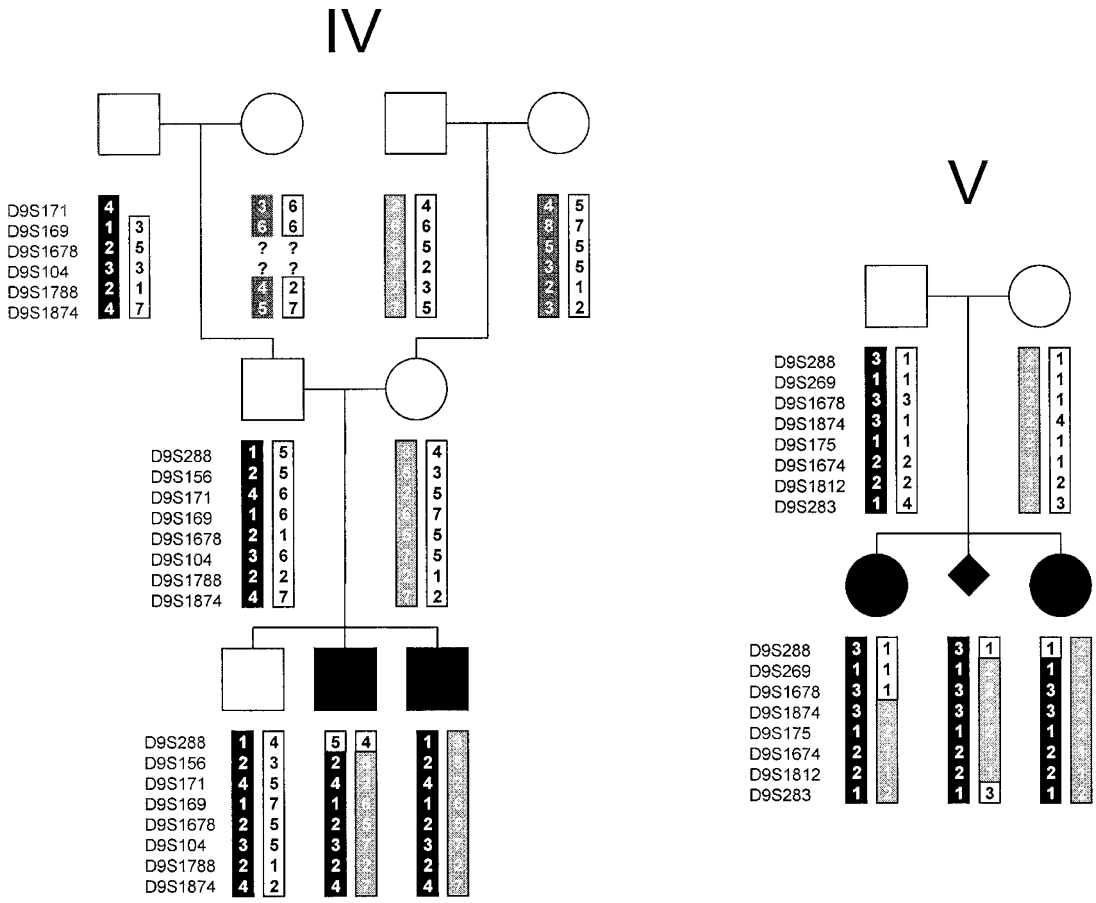

Figure 3 *Most likely haplotypes for microsatellite markers on chromosome 9p: Non-A, B, C, D, E families. IV and V are families assigned to complementation group F, G or H by somatic cell fusion; symbols of affected individuals are filled. ?: not typed.*

so that somatic cell fusion for complementation group analysis, as usually carried out for FA patients, was not possible. An assignment to one of the known complementation groups, A–H, could therefore only be made based on the chromosomal locations of FA genes.

Chromosomal localisations for the *FAA*, *FAC* and *FAD* genes have been reported.[6–8] An analysis of linkage in family I with marker *D16S413*, located 4 cM from the *FAA* gene, excluded a mutation in the *FAA* gene for this family (Table 1). Similarly, analysis with markers *D9S283* and *D9S53*, which flank the *FAC* gene,

Table 1 Pairwise LOD scores for an FA locus in family I and microsatellite markers on chromosome 3p, 9q and 16q

	Recombination fractions (θ)								
Marker	*0.000*	*0.001*	*0.010*	*0.050*	*0.100*	*0.200*	*0.300*	Z_{max}	θ_{max}
D3S1297	–99.999	–4.968	–1.535	–0.343	0.010	0.132	0.070	0.134	0.187
D3S1560	–99.999	–3.727	–1.713	–0.966	–0.625	–0.288	–0.119	–	–
D3S1597	–2.324	–2.172	–0.709	–0.097	0.100	0.179	0.125	0.179	0.192
D3S1286	–99.999	8.667	–3.223	–1.291	–0.589	–0.096	0.023	–	–
D3S1266	0.573	0.573	0.560	0.507	0.439	0.303	0.175	0.573	0.000
D3S1298	–99.999	–5.269	–1.829	–0.608	–0.209	0.022	0.047	–	–
D9S283	–99.999	–2.999	–0.988	–0.364	–0.153	–0.021	0.004	–	–
D9S53	–99.999	–9.424	–3.459	–1.507	–0.786	–0.240	–0.051	0.011	0.437
D16S413	–99.999	–6.297	–2.821	–1.457	–0.895	–0.386	–0.147	–	–

excluded membership of this group for this family (Table 1). The localisation of the *FAD* gene to chromosome 3p was relatively imprecise so that we elected to look for linkage by homozygosity mapping.[9] Six markers covering 70 cM on chromosome 3p, including the critical *FAD* region,[8] were examined. The pairwise lod scores in Table 1 show the results of this analysis, which clearly excluded group D.

Homozygosity Mapping

Since no further chromosomal localisations for FA genes were known, we ran a genome-wide analysis for homozygosity expecting to find a large overlapping region of homozygous markers covering around 30 cM in the two affected children. After excluding approximately 87% of the genome by analysing 269 microsatellite markers, we found two regions to be homozygous in the two affected children (Figures 1). One region of maximally 53 cM on chromosome 7q, between *D7S479* and *D7S661*, could be excluded by haplotype analysis of the seven unaffected children (data not shown). The other region, 73.5 cM on chromosome 9, between pter and marker *D9S167*, proved to be homozygous only in the two affected children, except for *D9S175* which was also homozygous, due to a crossover, in an unaffected child (Figure 2, pedigree I). This reduces the critical region to 61.2 cM from pter to *D9S175*. The maximum overall pairwise lod score is $Z_{max} = 2.282$ at $\theta = 0.00$ for marker *D9S1874* (Table 2a).

For homozygosity mapping, calculation of lod scores for entire haplotypes is appropriate.[19] For this, the haplotype, 4–4–6–3, at *D9S171–D9S1678–D9S104–D9S1874* was considered as a single marker with a frequency of 0.00003 calculated from the individual frequencies of alleles at these markers. The maximum lod score based on this haplotype is $Z_{max} = 2.68$ at $\theta = 0.00$. This convincingly suggested that the FA gene mutated in this family is located on chromosome 9p.

Analysis of FA-B and FA-E Families

In order to establish which complementation group is involved we examined whether the hitherto unmapped *FAB* and *FAE* genes might be localised in this region.

Complementation group B is represented worldwide by just two families, one is the German family shown in Figure 2, pedigree II. As shown by the haplotypes in the figure, the *FAB* gene can be clearly excluded from the majority of the critical region of chromosome 9p, since both the affected and unaffected child have inherited the same chromosomes from marker *D9S288* to *D9S1788*. A recombination event in the maternal meiosis of the unaffected child results in a different allele combination at *D9S1874* so that this last marker cannot be excluded (Table 2b).

The reference patient for the group FAE is EUFA130, the affected child of a consanguineous family of Turkish origin[18] (Figure 2, pedigree III and Table 2b). As shown in Figure 2, only two markers on chromosome 9p are homozygous in the affected child. This gives a homozygous region of 9.1 cM, much less than the 30 cM expected for progeny of first cousins.[20] Furthermore, the region of common haplotype is no larger in the parents, where an uninterrupted common region of 60 cM could be expected for first cousins. This makes it highly unlikely that a gene in this region is responsible for FA in this family. We are currently extending the search for homozygous regions in this family.

Further FA Families Link to Chromosome 9p

Since the gene localised by linkage in the large family I is not one of the genes FA-A to FA-E, we analysed two further families similarly excluded from groups A to E on the basis of cell fusion and complementation analysis. In Figure 3, pedigrees IV and V, two families are shown together with the deduced haplotypes for this chromosomal region. Clearly both these families are compatible with an FA gene on chromosome 9p. The maximum pairwise lod scores are $Z_{max} = 0.727$ at $\theta = 0.00$ in family IV and $Z_{max} = 1.204$ at $\theta = 0.00$ in family V, in both cases for marker *D9S1874* (Table 2a). Figure 4 shows chromosome 9p with the maximal region for the FA gene localised in this report.

Discussion

Fanconi anaemia is a rare disorder with an extreme degree of genetic heterogeneity, consequently for some complementation groups only a few families will be identified and localisation of the causative genes will only be possible by employing highly informative techniques such as homozygosity mapping. This method exploits the observation that an affected child of consanguineous parents will be homozygous by descent for a region of many centimorgans surrounding the disease gene.[20] We have examined a large consanguineous family in this way and demonstrate that an FA gene must be located on chromosome 9p. Although the maximal lod score achieved with this family is below

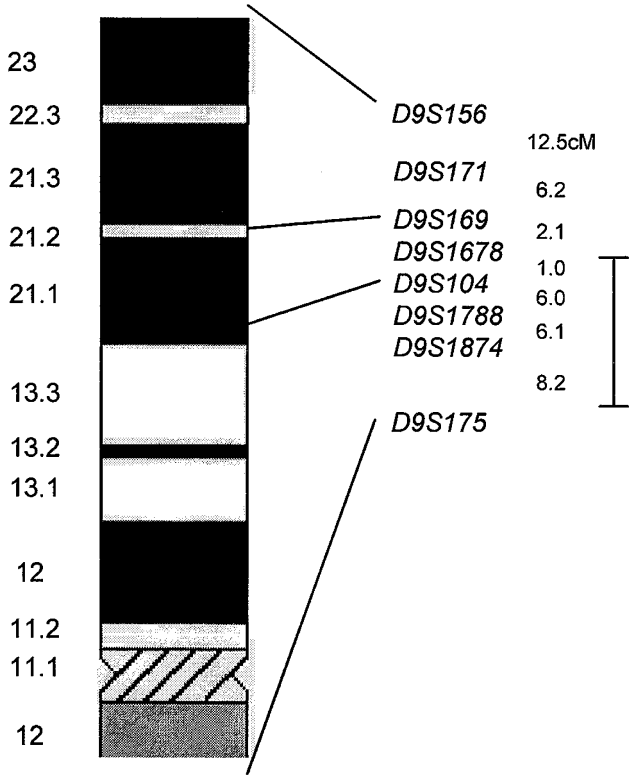

Figure 4 *Map of microsatellite markers on chromosome 9p. Genetic distances are indicated in centimorgans (cM).*[24] *Physical locations of key markers are taken from the consensus map of chromosome 9p.*[23]

the level normally required for significance in linkage studies, it must in fact be compared with the much lower likelihood of the random meeting of two independent alleles. The parents are first cousins once removed. This equates to a fraction of 1/32 of each child's genome being homozygous. In comparison, the FA allele frequency is approximately q = 0.002.[21]

A homozygous region of approximately 30 cM would be expected at this level of consanguinity.[20] Only one such homozygous chromosomal region could be identified in both affected children after excluding over 87% of the genome in this family. Interestingly, a previous study, using eight consanguineous FA families which did not link to markers around the FA gene also suggested an FA gene on chromosome 9p.[22]

Analysis of assigned families has proven that the FA gene on chromosome 9p is not that of FA group A, C, D or E and unlikely to be that of group B. Two further FA families also show positive lod scores with markers on chromosome 9p. We have no further grounds for combining lod scores of these three families other than their exclusion from the same complementation groups either by linkage (family I) or by cell fusion analyses (families IV and V); considering the genetic heterogeneity of FA, such a combination must be viewed with caution. However, bearing in mind this caveat, the three families together yield a lod score of 4.214 at $\theta = 0$ for *D9S1874* in chromosomal band 9p12–13.3[23]

Table 2a Pairwise LOD scores for an FA locus and markers on chromosome 9p in unassigned family (I) and two families, IV and V, excluded from complementation groups A–E

		Recombination fractions (θ)					
Marker	*Family*	*0.000*	*0.010*	*0.050*	*0.100*	*0.200*	*0.300*
D9S156	I	0.398	0.384	0.329	0.264	0.149	0.064
	IV	0.727	0.705	0.618	0.510	0.306	0.140
D9S171	I	0.833	0.814	0.740	0.643	0.448	0.266
	IV	0.727	0.705	0.618	0.510	0.306	0.140
D9S1678	I	0.631	0.618	0.563	0.491	0.341	0.202
	IV	0.125	0.121	0.104	0.084	0.049	0.023
	V	–99.999	–1.402	–0.721	–0.444	–0.194	–0.076
D9S104	I	2.215	2.153	1.906	1.604	1.033	0.537
	IV	0.727	0.705	0.618	0.510	0.306	0.140
D9S1874	I	2.282	2.224	1.990	1.701	1.142	0.637
	IV	0.727	0.705	0.618	0.510	0.306	0.140
	V	1.204	1.178	1.071	0.931	0.636	0.341
D9S175	I	0.777	0.760	0.689	0.597	0.414	0.244
	V	0.602	0.589	0.535	0.465	0.318	0.170
D9S1674	V	0.602	0.589	0.535	0.465	0.318	0.170
D9S1812	V	0.602	0.589	0.535	0.465	0.318	0.170
D9S283	V	–99.999	–0.813	–0.186	0.022	0.124	0.095

Table 2b Pairwise LOD scores for an FA locus and markers on chromosome 9p in complementation group B family (II) and complementation group E family III

	Recombination fractions (θ)							
Marker	*Family*	*0.000*	*0.001*	*0.010*	*0.050*	*0.100*	*0.200*	*0.300*
D9S156	II	–0.176	–0.175	–0.168	–0.137	–0.104	–0.056	–0.024
	III	–2.924	–0.962	–0.344	–0.138	–0.012	0.013	0.012
D9S169	II	–99.99	–2.274	–1.282	–0.617	–0.360	–0.145	–0.053
	III	–2.773	–1.155	–0.527	–0.299	–0.120	–0.045	–0.010
D9S1678	II	–0.176	–0.175	–0.168	–0.137	–0.104	–0.056	–0.024
	III	0.901	0.877	0.782	0.665	0.442	0.251	0.104
D9S104	II	–0.176	–0.175	–0.168	–0.137	–0.104	–0.056	–0.024
	III	0.662	0.642	0.563	0.467	0.294	0.157	0.062
D9S1788	II	–99.99	–2.274	–1.282	–0.617	–0.360	–0.145	–0.053
	III	–2.773	–1.151	–0.509	–0.271	–0.089	–0.026	–0.004
D9S1874	II	0.125	0.124	0.116	0.086	0.056	0.018	0.004
	III	–2.732	–1.342	–0.690	–0.434	–0.223	–0.133	–0.072

and the recombination events in family V would reduce the critical region to the 21 cM between *D9S1678* and *D9S175*.

The localisation of a further FA gene to a discrete chromosomal region reconfirms the genetic heterogeneity of this disease. At this stage it is unclear what proportion of FA cases will be due to a mutation of the FA gene on chromosome 9p. If the analysis presented here in which two German and one Lebanese family localise to chromosome 9p is representative, then this may not be one of the rarest FA genes. The chromosomal localisation reported here will have consequences for diagnosis of FA and may provide a basis for identifying another FA gene.

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