European Journal of Human Genetics (1999) 7, 52–59

http://www.stockton-press.co.uk/ejhg

ARTICLES

Heterogeneous spectrum of mutations in the Fanconi anaemia group A gene

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Fanconi anaemia (FA) is a genetically heterogeneous autosomal recessive disorder associated with chromosomal fragility, bone-marrow failure, congenital abnormalities and cancer. The gene for complementation group A (*FAA*), which accounts for 60–65% of all cases, has been cloned, and is composed of an open reading frame of 4.3 kb, which is distributed among 43 exons. We have investigated the molecular pathology of FA by screening the *FAA* gene for mutations in a panel of 90 patients identified by the European FA research group, EUFAR. A highly heterogeneous spectrum of mutations was identified, with 31 different mutations being detected in 34 patients. The mutations were scattered throughout the gene, and most are likely to result in the absence of the FAA protein. A surprisingly high frequency of intragenic

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Received 23 December 1997; revised 20 May 1998; accepted 11 June 1998

deletions was detected, which removed between 1 and 30 exons from the gene. Most microdeletions and insertions occurred at homopolymeric tracts or direct repeats within the coding sequence. These features have not been observed in the other FA gene which has been cloned to date (*FAC*) and may be indicative of a higher mutation rate in *FAA*. This would explain why FA group A is much more common than the other complementation groups. The heterogeneity of the mutation spectrum and the frequency of intragenic deletions present a considerable challenge for the molecular diagnosis of FA. A scan of the entire coding sequence of the *FAA* gene may be required to detect the causative mutations, and scanning protocols will have to include methods which will detect the deletions in compound heterozygotes.

Keywords: Fanconi anaemia; *FAA* gene; mutations

Introduction

Fanconi anaemia (FA) is an autosomal recessive inherited disorder which is associated with a variety of congenital anomalies. These include morphometric abnormalities involving mainly the head and face, skeletal malformations particularly of the radial ray, growth retardation, abnormal skin pigmentation, deafness, renal, ocular, genital and cardiac defects.[1] The cardinal clinical feature, however, is a severe, progressive pancytopaenia, with a mean age of onset of 7–8 years, with death generally occurring within 5 years. The development of acute nonlymphoblastic leukaemia is a common occurrence. The clinical diagnosis is supported by demonstration of hypersensitivity to DNA cross-linking agents[2] such as diepoxybutane (DEB) and mitomycin C (MMC), or by delayed passage through the G2 phase of the cell cycle.[3] The increased chromosomal instability in cells from FA patients has led to its classification as a chromosome breakage disorder, but the basis of this presumed failure in DNA repair is not understood (reviewed by Buchwald *et al*[1] and Joenje *et al*[4]). FA is an attractive model for gene therapy, because of the accessibility of the most critically affected tissue (bone marrow), and because proliferating cells which take up a normal copy of the appropriate gene would be expected to have a significant growth advantage over untransfected cells. A functionally active retrovirus vector has been used to transduce CD34+ progenitor cells with the *FAC* gene.[5]

FA is genetically heterogeneous, with eight complementation groups having been described to date.[6–8] The group C gene was cloned by functional complementation,[9] and localised to chromosome 9q22.3.[6] Only 10 mutations have been detected in the *FAC* gene,[9–15] including one missense mutation of proven functional significance.[16] The group A gene (*FAA*), which accounts for 60–65% of FA in most populations,[17,18] was mapped to chromosome 16q24.3 by linkage analysis in FA-A families,[19] and subsequently identified by both functional complementation[20] and positional cloning.[21] The gene has an open reading frame of 4365 bp, and encodes a protein with no significant homology to sequences in public databases. The cloning of the major Fanconi anaemia gene has created the opportunity to carry out a detailed investigation of the molecular pathology of the condition. This has been assisted by the characterisation of the genomic structure of the gene, which is composed of 43 exons spread over about 80 kb of genomic DNA.[22] Specific issues which can now be investigated include the degree of heterogeneity in the mutational spectrum, the classes of mutation which inactivate the gene, whether specific mutations are associated with a particular geographic or ethnic origin, and whether specific mutations can be correlated with particular clinical phenotypes. Such information will be very useful in the future design of a rational molecular diagnostic strategy for FA and, if genotype/phenotype correlations exist, could influence patient management. The location and nature of the mutations may also shed light on functionally important regions in the gene.

The European Fanconi Anaemia Research Group (EUFAR) is undertaking a major survey of mutations in the *FAA* gene in an extensive panel of FA patients from Europe, Asia and the Middle East. We now report the existence of a very heterogeneous mutational spectrum in *FAA*, and the identification of 29 novel mutations.

Methods

Patients and Samples

The clinical diagnosis of FA was based on typical findings including a progressive pancytopaenia and characteristic congenital abnormalities such as radial defects.[1,4] The diagnosis was confirmed in all cases by the demonstration of

either hypersensitivity to MMC or DEB, or by observation of a delayed G2 phase in the cell cycle, as previously described.[3] Patients were recruited to the study via the framework of the European Concerted Action on FA Research (EUFAR), or referred by haematology departments in the constituent countries. Lymphoblastoid cell lines (LCLs) were prepared from peripheral blood cells, subjected to cell fusion with the prototype FA-A cell line HSC720T,[23] and tested for complementation.[6,7] About 25% of LCLs could not be classified, because they were resistant to MMC.[24] In some cases, samples were only available as fibroblast cell lines of unknown complementation group, or as DNA extracted from peripheral blood lymphocytes. In cases of unknown complementation group in which samples from other affected and/or unaffected family members were available, DNAs were typed with microsatellite markers closely linked to the *FAA* gene,[21] to select cases most likely to be from group A. DNA and RNA were extracted from cell lines as described.[10] Genomic DNA was extracted from peripheral blood samples of FA patients with no cell lines, but cell counts were generally too low to obtain usable yields of RNA.

Complementation Analysis

Cell fusions to genetically marked reference cell lines from known FA complementation groups was carried out, as previously described.[8]

Mutation Screening

The methods used by the three laboratories to scan the *FAA* coding sequence for mutations were as follows:

1) In Amsterdam, screening was carried out on cell lines from 36 FA patients classified as FA-A by complementation analysis. Total RNA was extracted and the *FAA* mRNA reverse-transcribed and amplified by PCR (RT-PCR) in nine overlapping fragments using specific primers with T7 and SP6 extensions, sized by gel electrophoresis, and purified with Qiagen kits. PCR products were then digested with restriction enzymes to generate two fragments of about 300 bp, suitable for single-stranded conformational polymorphism (SSCP) and heteroduplex (HD) analysis. PCR primer and restriction enzyme combinations were (primers written in 5'-3' orientation):

   1) AATTGTCTCTCAGCAAAGTGAT and CGTGAAGATGCCACAA/*Bg1*II;
   2) TGTTCTCCCGTCTTTCCTTC and GTGAGCAGAGGGTGTGTC/*Bg1* II;
   3) GTTCGGAGTGTTCAGTGGAC and GGGTGGGTGGAGAATGTG/TaqI;
   4) GCCCTGGTCTTCCTGTTTA and CCTCAGCAGAGTTGGGTTCT/*Pvu*II;
   5) GACTCCCGTGTGGCGTTTAT and CAGCACATGTGGGGCACTCA/*EcoR*I;
   6) CTGCCAGCTGCTCCGTCACC and GTGGAAGTCCTGCCGTTCAG/*Pvu*II;
   7) TCAGATACTGAACGGCAGGACT and CTCAGAGTTGACCAAGTGGAAG/*BamH*I;
   8) GCAGCAGCTTCCAGGCAGAACA and CTGTGGTGCTATTTGAGGTCAG/*Pst*I;
   9) GATGGGCCTGCTGTCGTCACAT and CAGGTCCCGTCAGAAGAGATGA/*Pvu*II;

   Restriction fragments were then mixed with the same products generated from the wild type cDNA, heated for 2 min in a boiling waterbath, cooled on ice, and analysed on a 20% acrylamide gel with silver staining to visualise the bands (Pharmacia Phast system). Fragments showing aberrant SSCP or heteroduplex patterns were sequenced. Alternatively the 43 exons of *FAA* were amplified from genomic DNA with primers located 40–60 bp from exon boundaries.[25,26] Fragments were analysed on the Pharmacia Phast system. The effect of mutations predicted to cause premature termination of translation were confirmed by an *in vitro* transcription/translation protein truncation assay of cDNA as described.[20]

2) In London, screening of the *FAA* coding sequence was initiated in five patients classified as group A by complementation analysis. The coding sequence was amplified in seven overlapping fragments by RT-PCR and automated sequencing performed on a Perkin-Elmer/Applied Biosystems 377 DNA sequencer with fluorecent dye-terminator sequencing kits, as recommended by the suppliers. Intragenic deletions detected in the cDNA were confirmed in homozygotes by exon amplification from genomic DNA, and in compound heterozygotes by a quantitative fluorescent gene dosage assay which measures the relative intensity of *FAA* gene fragments compared to an *FAC* gene control[27] (and Morgan NV *et al*, in preparation). A further 27 FA patients from whom no cell lines were available, and who were therefore unclassified with respect to complementation group, were screened by amplification of the individual exons and SSCP/HD analysis of ^{32}P-labelled PCR products as previously described,[10] using published primers.[25,26] Fragments with band shifts were characterised by sequencing independent PCR products on both strands and mutations confirmed by restriction site analysis.

3) In Wurzburg, the *FAA* coding sequence was screened in 22 FA cell lines of unknown complementation group. The exons were amplified, screened for mutations by SSCP analysis on the Pharmacia Phast system, and band shifts characterised by direct sequencing. The effect of the mutations detected was analysed by RT-PCR and sequencing. Deletions of genomic DNA were confirmed by the detection of aberrant bands on Southern blots using the *FAA* cDNA as a probe.

The total number of patients screened was 90, of whom 41 were FA-A by complementation analysis. Since about 60% of all FA patients are from group A,[17,18] there are approximately $41 + (49) \times 0.6 = 70$ FA-A patients in the total screening panel.

In order to distinguish rare polymorphic variants from pathogenic mutations, control chromosomes were screened for the missense mutations found in FA patients, and the segregation of all mutations was analysed in FA families from whom other family members were available.

Results

A total of 31 different mutations which are likely to be pathogenic were detected in this study (see Figure 1 and Table 1), 29 of which are novel mutations. Most classes of mutation were detected, including missense mutations, stop codons, microdeletions or insertions which generally caused frameshifts, splice site mutations and large intragenic deletions. Most of the large deletions were detected by consistent lack of amplification of the relevant exons in patients who were homozygous for the deletion; the exons immediately proximal and distal to the deletion amplified successfully. In compound heterozygotes the endpoints were fully or partially defined by quantitative fluorescent PCR.[27] In seven of the nine microdeletions or insertions, the mutation occurred either at homopolymeric tracts or by removal of one or two di- or tri-nucleotide direct repeats. The four putative missense mutations, D598N, S858R, R1055W and S1088F, are non-conservative substitutions which were not seen in 100 control chromosomes. The most common mutation was 1263delF, which was present in five apparently unrelated patients from five European countries; 401insC has also been described by Levran *et al*.[25] The mutations were scattered throughout the coding sequence of the *FAA* gene (Figure 1). Mutations were detected in 34 patients from the screening panel, which contained approximately 70 patients from FA-A (see Methods). This indicates an overall detection rate of 49%, which was very similar in all three participating laboratories. The heterogeneity of the mutational spectrum is evident from the fact that 19 of the 25 patients from non-consanguineous matings were compound heterozygotes for two different mutant alleles. The total number of independent mutant alleles in the 34 mutation-positive patients is 59 ($25 \times 2 + 9$). We have now detected 47 (79.7%) of these alleles, (Table 1 and Lo Ten Foe Jr *et al*,[20] Fanconi Anaemia/Breast Cancer Consortium.[21]) which comprise 31 different mutations.

The geographic and ethnic spectrum of *FAA* mutations is shown in Table 2. These data are a collation of the mutation data from the present study, and three other studies from the EUFAR group,[20,21,26] and comprise a total of 43 different mutations. The largest numbers of patients screened from single population groups have been from Italy and Germany, and these data reinforce the picture of extensive genetic heterogeneity even within a single country.

In addition to the pathogenic mutations, 13 sequence changes which give rise to amino acid substitutions were detected which are either common polymorphisms or rare variants (Table 3). Five of these are clearly polymorphisms, since they were present in 9–50% of the control chromosomes. M717I is present in the *FAA* cDNA which was cloned by functional complementation,[20] and is therefore unlikely to affect the function of the protein. E878Q does not segregate in the single consanguineous family in which it was detected, since it was present in the heterozygous state in two of three affected siblings, and absent in the third. V6D is one of three mutations in a multiplex family in which the other two mutations would produce a truncated protein product. V295L, R296K and S303T were all detected in the same Black South African FA patient, who has subsequently been shown to be non-A by complementation analysis. R245K was also detected in a non-A patient from this population. The complementation group of the patient with the S309N mutation cannot be resolved as the cell-line is resistant to MMC. A large number of other polymorphisms which do not alter the coding sequence or which occur in non-coding regions not involved in mRNA splicing were detected. These have been submitted to the Fanconi Anaemia Mutation Database (http://www.rockefeller.edu/fanconi/mutate).

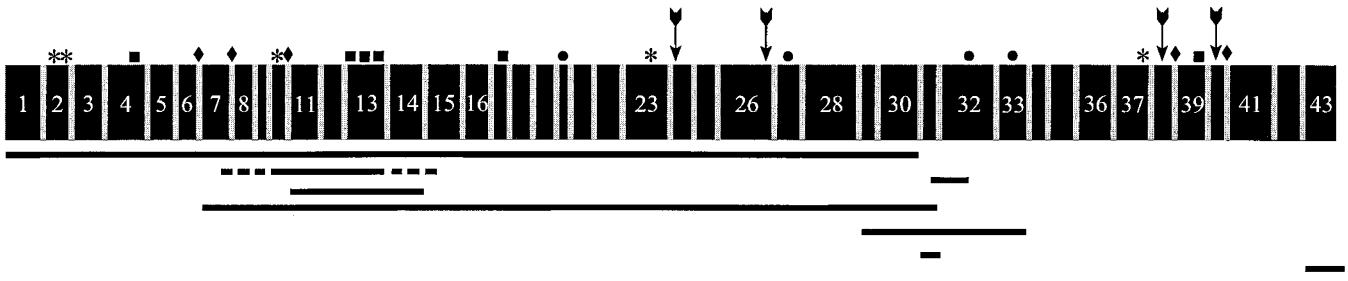

Figure 1 *Mutations detected in the FAA gene in Fanconi anaemia patients. The figure is a schematic representation of the 43 exons of the FAA gene: * = nonsense mutation; ■ = frameshift; ◆ = splice site; ● = missense; □ = microdeletion; – = large deletion.*

Table 1 Mutations detected in the *FAA* gene of Fanconi anaemia patients

Patient code	*Mutation*	*Effect*	*Exon*	*State*	*N*	*Other mutation*
7PIR	1–2981del	deletion	1–30	HOM	2	–
EUFA353	154C→T	R52X	2	HET	1	Not detected
EUFA127	163C→T	Q55X	2	HET	1	1263delF
EUFA426	401insC[a]	frameshift	4	HET	1	Not detected
EUFA123	IVS6–2A→G	del13AA	i6	HET	1	Not detected
PRU80665	597–3066del	deletion	7–31	HOM	2	–
EUFA444	827–1225del[b]	deletion	10–13[b]	HET	1	1115–1118del[d]
12CWL	856C→T	Q286X	10	HET	1	Not detected
PRU78236	IVS10–1G→A	exon 10 skip	i10	HOM[c]	1	–
EUFA586	894–1359del	deletion	11–14	HOM[c]	1	–
EUFA418	1115–1118del	frameshift	13	HET	2	Not detected
16VRO	1164–1165del	frameshift	13	HET	1	4010delG+18
EUFA763	1191–1194del	frameshift	13	HET	1	Not detected
EUFA704	1615delG	frameshift	17	HOM	2	–
EUFA134	1792G→A	D598N	20	HET	2	Not detected
EUFA245	1792G→A	D598N	20	HET	2	Not detected
PRU65248	2107C→T	Q703X	23	HOM[c]	1	–
PRU91730	2167–2169del	723delL	24	HET	1	IVS38–1G→C
PRU79892	2495–2497del	832delF	26	HOM[c]	1	–
EUFA186	2574C→G	S828R	27	HET	1	Not detected
3VHE	2779–3348del	deletion	29–33	HOM	2	–
BD11	2982–3066del	deletion	31	HOM[c]	1	–
EUFA043	3061–3154del	frameshift	31–32	HOM	1	–
PRU77959	3163C→T	R1055W	32	HOM[c]	1	–
PRU66279	3263C→T	S1088F	33	HOM[c]	1	–
EUFA275	3760G→T	E1254X	37	HET	1	IVS7+5G→A[e]
EUFA006	3788–3790del	1263delF	38	HET	1	1360–1826del[e]
EUFA483	3788–3790del	1263delF	38	HET	5	Not detected
EUFA507	3788–3790del	1263delF	38	HET	5	Not detected
EUFA689	3788–3790del	1263delF	38	HOM[c]	5	–
PRU91730	IVS38–1G→C	exon 38 skip	i38	HET	1	2167–2169del
EUFA255	3920delA	frameshift	39	HET	1	Not detected
16VRO	4010delG+18[f]	exon 40 skip	40/i40	HET	1	1164–1165del
EUFA139	IVS40+1–18del	exon 40 skip	i40	HOM[c]	1	–
EUFA528	4267–4404del	deletion	43	HET	3	1263delF
EUFA598	4267–4404del	deletion	43	HOM	3	–

[a25]; [b]Deletion endpoints not defined; [c]Consanguineous; [d21]; [e20]; numbering of cDNA from initiation codon; [f]18bp of IVS40; *N* = No. of *FAA* alleles containing the mutation (consanguineous alleles are counted as 1); HOM = homozygous; HET = heterozygous.

Discussion

The most striking feature of the mutation screening data reported here is the extensive heterogeneity of the mutational spectrum of the *FAA* gene. Mutations were detected in 34 patients, and 47 of the predicted 59 mutant alleles were identified in this group (Table 1). These 59 alleles were comprised of no fewer than 31 different mutations, which indicates that the majority of FA-A patients who are not the offspring of related parents will be compound heterozygotes for two different mutant alleles. This heterogeneity was not only a result of the variety of the geographic and ethnic origins of the patients screened, since it was also found within populations of a particular country, with 12 different mutations already defined in the Italian population,[21,26] and eight mutations in the German patients (Table 2). There was no evidence for common mutations in any particular population, although some mutations such as 1263delF and 1115–1118del were found in multiple unrelated patients from different populations.

The heterogeneity also extended to the class and location within the gene of the mutations detected. Almost all classes of mutations were observed, including a surprisingly high number of large intragenic deletions which removed one or more exons; these accounted for eight of the 31 mutations (26%) in this study (Table 1). No deletions of this sort have yet been observed in the *FAC* gene,[13,14] which suggests that there

Table 2 Countries of origin of Fanconi anaemia patients with *FAA* gene mutations

Country	*Mutations detected*
Britain	401insC, 1115–1118del, D598N, 723delL IVS38–1G→C
Czech Republic	1164–1165del, 1263delF, 4010delG+18
Egypt	IVS7+5G→T, 3061–3154del
France	1115–1118del, 1191–1194del, 1263delF
Germany	Q55X, IVS6–2A→G, Q286X, D598N, S858R, ex29–33del, 1263delF, IVS40+1–18del
Hungary	1263delF
India	ex7–31del, IVS10–1G→A, 823delF, R1055W
Iran	ex1–30del, S1088F, ex43del
Ireland	ex11–14del
Italy	Q264X, IVS9+3delA, IVS10+1G→T, IVS14+1G→C, ex18–21del, Q669X, Q772X, IVS28+83C→G, 2831dup2812–2830, 3559insG, 3638–3639del, D1359Y
Lebanon	Q703X
Netherlands	3920delA
Pakistan	ex31del
Poland	R52X, 1615delG
Spain	IVS7+5G→A, ex10–13del*, 1115–1118del, E1254X, 1263delF
Turkey	ex43del

*Deletion endpoints not defined.

Table 3 Polymorphisms and variants in the coding sequence of the Fanconi anaemia *FAA* gene

Mutation	*Exon*	*Effect*	*Frequency*[1]
17T→A	1	V6D	0.01
734G→A	8	R245K	0.00[2]
795G→A	9	A266T	0.50
883G→C	10	V295L	0.00[2]
887G→A	10	R296K	0.00[2]
908G→C	11	S303T	0.00[2]
926G→A	11	S309N	0.00[2]
1235C→T	14	A412V	0.09
1501A→G	16	S501G	0.34
2151G→T	23	M717I	0.01
2426G→A	26	G809D	0.45
2632G→C	28	E878Q	0.00[3]
3982A→G	40	T1328A	0.09

[1]Frequency of rare allele in 100 European control chromosomes.
[2]Conservative substitution in Black South African patients.
[3]Variant does not segregate with FA phenotype.

may be structural features of the *FAA* gene, such as repetitive sequence elements, which promote unequal recombination and the generation of deletions. This apparent genetic instability of the *FAA* gene suggests that it may have a higher mutation rate than the genes for the other FA complementation groups, which would explain why FA-A accounts for about two-thirds of all FA patients. Since most of the mutations are deletions, frameshifts or stop codons, they would be predicted to generate null alleles, although residual function for part of the protein cannot be excluded. This would be interesting to analyse, particularly for mutations such as 4267–4404del, which removes only the final exon of the *FAA* gene. The status of the missense mutations can be resolved by site-directed mutagenesis and analysis by functional complementation, as was done for several mutations in the *FAC* gene.[15,28] The mutations are scattered throughout the coding sequence (Figure 1), with no compelling evidence of mutation 'hotspots' at present.

In this study, mutations were detected in 34 patients, which is 49% of the predicted number of FA-A cases which were screened. Also, 47 of 59 mutant alleles were identified in these 34 patients. In the other two published studies, Savino *et al*[26] detected mutations in 29% of probable FA-A patients, and Levran *et al*[25] in 69% of patients. However, 14 of 40 mutations in the latter study were amino acid substitutions, and their effect on the function of the FAA protein is not yet known. These data indicate that a substantial proportion of mutations were not detected by current scanning protocols. This may be partly explained by the limited sensitivity of the SSCP technique, which detects 70–90% of mutations, depending on the size of fragment being screened and the gel conditions used.[29] However, an important additional factor in the context of the *FAA* gene is the relatively large number of intragenic deletions superimposed upon high mutational heterogeneity. Thus, if compound heterozygotes for a 'micro' mutation and an intragenic deletion are screened only by SSCP of exons, the deletion would not be detected since all exons would be amplified from the intact allele. This would also apply if two non-overlapping deletions were present. A rational screening strategy must therefore be devised which screens for 'micro' mutations by methods such as SSCP, and for deletions of entire exons by, for example, Southern blot analysis. The latter will be facilitated by the availability of a detailed restriction map of the entire 80 kb of genomic sequence of the *FAA* gene.[22] This will be of

considerable diagnostic importance, since knowledge of the causative mutation will permit rapid prenatal diagnosis on the basis of a molecular test. It will also allow rapid classification of patients as FA-A or non-A in cases where an MMC-sensitive cell line is not available.

In addition to the heterogeneous spectrum of pathogenic mutations, there is also an extensive degree of polymorphism in the *FAA* gene. The pathogenic status of an additional 13 missense mutations (see Table 3) was assessed by

(1) scanning the entire coding sequence for other mutations,

(2) screening a panel of control chromosomes,

(3) checking for co-segregation with the clinical phenotype, and

(4) complementation analysis.

This combination of approaches indicated that most of these mutations are unlikely to affect the function of the FAA protein, and emphasises the need for thorough evaluation of the significance of individual missense mutations.

This study and two other very recent publications[25,26] represent the start of a concerted effort to describe the molecular pathology of the major Fanconi anaemia gene. The current data indicate that a great deal more work needs to be done to define the mutation spectrum of the *FAA* gene in the major populations. The great heterogeneity of mutations will not facilitate correlation of clinical phenotype with specific mutations, since these will be rare, and will generally be found in compound heterozygotes. However, future work will be directed towards an analysis of the position of mutations within the gene in relation to phenotype, since this approach has yielded functional insights for genes such as *BRCA1*.[30]

Acknowledgements

We thank the families with Fanconi anaemia for their cooperation in providing blood samples, Dr D Schindler for cell lines, and Drs S Ball, J Evans, E Gordon-Smith, F Breatnach, A Newland, S Rassam, I Roberts, S Temtamy, W Friedrich, B Gautier, and many other physicians of the EUFAR collaboration for referring patients. This study was supported by EUFAR, the Fanconi Anaemia Research Fund Inc. (USA), Fanconi Anaemia Breakthrough (UK), Deutsche Fanconi Anaemia Hilfe e.V., the Italian Association for Fanconi Anaemia Research, Association Française de Maladie de Fanconi, Nederlands Stichting Fanconi Anemie, the Italian Ministry of Health and Telethon (E.364).

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