European Journal of Human Genetics (2000) 8, 861–868

www.nature.com/ejhg

ARTICLE

Spectrum of mutations in the Fanconi anaemia group G gene, *FANCG/XRCC9*

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***FANCG* was the third Faconi anaemia gene identified and proved to be identical to the previously cloned *XRCC9* gene. We present the pathogenic mutations and sequence variants we have so far identified in a panel of FA-G patients. Mutation screening was performed by PCR, single strand conformational polymorphism analysis and protein truncation tests. Altogether 18 mutations have been determined in 20 families – 97% of all expected mutant alleles. All mutation types have been found, with the exception of large deletions, the large majority is predicted to lead to shortened proteins. One stop codon mutation, E105X, has been found in several German patients and this founder mutation accounts for 44% of the mutant *FANCG* alleles in German FA-G patients. Comparison of clinical phenotypes shows that patients homozygous for this mutation have an earlier onset of the haematological disorder than most other FA-G patients. The mouse *Fancg* sequence was established in order to evaluate missense mutations. A putative missense mutation, L71P, in a possible leucine zipper motif may affect FANCG binding of FANCA and seems to be associated with a milder clinical phenotype.** *European Journal of Human Genetics* (2000) **8**, 861–868.

Keywords: Fanconi anaemia; *FANCG/XRCC9*; mutation screening; founder mutation

Introduction

The autosomal recessive genetic disorder Fanconi anaemia (FA; MIM 227650) is genetically highly heterogeneous with seven complementation groups so far established and thus seven causative genes anticipated[1] (Joenje *et al* 2000, personal communication). The identification of these genes is an important goal for understanding the pathophysiology of the disease since the basic defect is still poorly understood.

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Received 24 March 2000; revised 6 July 2000; accepted 11 July 2000

The characteristic chromosomal breakage observed in patient cells, particularly after treatment with a bifunctional alkylating agent capable of forming DNA interstrand crosslinks, has led to the assumption that FA cells are deficient in the repair of this DNA lesion. However, alternative theories implicating cell cycle regulation, oxygen detoxification and apoptosis have been proposed.[2,3]

The clinical picture of FA is characterised by progressive bone marrow failure and an increased risk of neoplasia, particularly leukemia. In addition, a range of variable congenital defects such as growth retardation, skeletal abnormalities including radial aplasia and hyperpigmentation of the skin are observed in some patients.[4] Whilst increased

chromosomal breakage remains the critical diagnostic test for FA, recent studies have demonstrated reverse mosaicism in FA leading to a population of undamaged cells in peripheral blood lymphocytes.[5,6]

Of the seven FA genes, four have been identified: *FANCA*,[7,8] *FANCC*,[9] *FANCF*[10] and *FANCG*.[11] The localisation of *FANCG* on chromosome 9p13 was established by homozygosity mapping and linkage analysis in FA-G families,[12] its identification was achieved by functional complementation of FA-G lymphoblastoid cells after cDNA transfer.[11] The gene thus identified was identical to *XRCC9*, localised on 9p13, and previously identified by virtue of its ability to complement a crosslinker sensitive hamster cell mutant, UV40.[13] The gene covers some 6 kb with 14 exons and has a 2.5 kb mRNA encoding the FANCG protein of 622 amino acids and a calculated molecular weight of 68 kD. The protein shows no homology to known proteins and its function is thus unclear. However, several studies have shown that FANCG is found in the cells as a nuclear complex with the FANCA protein.[14,15]

Pathogenic mutations in *FANCG* were previously reported in four FA-G patients, we report here further mutations in 16 FA-G patients, including common German and Turkish founder mutations.

Materials and methods

Patients and samples

Patients were diagnosed on the basis of chromosomal breakage tests and clinical features. Many patients were recruited as part of the European Concerted Action on FA Research (EUFAR), others were referred to the contributing laboratories for diagnosis. Clinical data were collected on a standard EUFAR questionnaire. Where possible, lymphoblastoid cell lines were established by EBV transformation and fused to the 7 FA reference cell lines to establish complementation group.[1,16] In some cases, assignment to FA group G was indicated by the absence of detectable FANCG protein in immunoblots. DNA and RNA were extracted from LCLs and/or peripheral blood samples by standard techniques.

Mutation screening

Mutations in the *FANCG* gene were screened by amplification of all 14 exons from genomic DNA, using primers flanking the exon/intron boundaries as described previously,[11] or by amplification of *FANCG* cDNA from RNA. SSCP analysis under four different conditions was used to identify exon PCR products with an aberrant mobility pattern; bis-acrylamide:acrylamide ratios of 1:30 and 1:50, gel runs at 4°C and at 15°C. Fragments showing an aberrant SSCP pattern were sequenced using the ABI 310 (Foster City, CA, USA) sequencer to identify the mutations. Most patients showed mobility shifts in one or more exons, those mutant alleles not detectable by SSCP were sequenced directly. Some patient samples were screened by RT-PCR and *in vitro* translation using standard methods.[17] Appropriate *FANCG* exons were then amplified and sequenced to establish the underlying mutation.

Where possible, the identified mutations were confirmed by restriction enzyme digest of appropriate PCR products bearing the mutation, by RT-PCR and/or by examination of DNA from the patients' parents.

Mouse *Fancg* sequence

A BLAST v2.0 search with the entire *FANCG* cDNA sequence revealed high homology ($P = 7e{-}37$) to an anonymous murine BAC clone in the GenBank database (Lamerdin *et al*, accession no. AC005259). The murine coding sequence was verified by the amplification of the *Fancg* cDNA from mouse total RNA by nested RT-PCR using specific primers. The amplified product was 1.9 kb long, in agreement with estimates for the size of the *Fancg* transcript encoded by the BAC clone. The RT-PCR product was sequenced and the completed *Fancg* cDNA sequence has been submitted to GenBank with accession number AF112439.

Haplotype analysis at the *FANCG* locus

Examination of the sequence of the P1 clone, 11659 (Lamerdin *et al*, accession no. AC004472), containing the entire *FANCG* gene revealed the presence of a (CA) dinucleotide repeat at position 5531 of the clone, 48 kb from the 3' end of the *FANCG* coding sequence. Primers were designed which flank this repeat and 65 unrelated individuals were examined for polymorphism in the size of the CA repeat sequence; 81% of the individuals were heterozygous. This genetic marker has been given the assignment *D9S2176*.

DNAs from families in which the common 313G→T or 1649delC mutations segregate were examined for microsatellite markers *D9S2176*, *D9S1853*, *D9S1874*, *D9S1817*, and *D9S165* located at the *FANCG* locus. PCR products were analysed on Pharmacia ALF (Freiburg, Germany) sequencing apparatus.

Results

Table 1 details the *FANCG* mutations found in the 16 new FA-G patients examined here and for the four patients previously described.[11] The majority of mutated alleles (94%) are expected to result in protein truncation. Six mutations are small deletions (1, 2, or 10 bp) or insertions (2 bp) leading to frame shifts and premature termination of translation after, between 4 and 81 novel codons. Six mutations affect the invariable intronic bases of mRNA splice sites (donor sites of introns 2, 5 and 11; acceptor sites of introns 8, 9 and 13), and would be predicted to result in false splicing, frame shift and truncated proteins. The loss of exon 2 due to the IVS-2 + 1G→A mutation in patient FA1BER was verified by RT-PCR and sequencing (Figure 1). Interestingly, the second mutant allele, which has a dinucleotide deletion in exon 4, was not detectable in this RT-PCR, suggesting that the

Table 1 *FANCG* mutations detected in Fanconi anaemia patients

Patient code	*Ancestry*	*Consanguineous*	*Mutations*[a]	*Predicted effect*	*Exon*	*State*[c]
EUFA313	Germany	no	IVS9-1G→C/1310-1311insGA	R359S+20X/D437E+80X	i9/10	HET
F99/112	Germany	no	313G→T/not determined	E105X	4	HET
FA1BER	Germany	no	IVS2+1G→A/346-347del	V29G+11X/Q116G+36X	i2/4	HET
EUFA1093	Germany	no	313G→T	E105X	4	HOM
EUFA282	Germany	no	652C→T/1183-1192del	Q218X/E395W+3X	6/10	HET
F00/49	Germany	no	313G→T/1183-1192del	E105X/E395W+3X	4/10	HET
F99/186	Turkey	yes	1649delC	T550I+7X[g]	13	HOM
EUFA636	Turkey	no	1649delC	T550I+7X	13	HOM
EUFA569	Turkey	yes	212T→C	L71P[b]	3	HOM
EUFA624	Turkey	no	1642C→T	R548X	13	HOM
F99/121	Turkey	yes	IVS5+1G→T	S171V+3X[g]	i5	HOM
DF3	Arabia	yes	1636G→C	A495-G546del	12	HOM
PRU63622	Arabia	yes	1749delA	D584M+8X	13	HOM
EUFA0334	Italy	no	1715G→A	W572X	13	HOM
EUFA0872	Portugal	–	IVS8-2A→G	R359S+20X and G361L+2X[f]	i8	HOM
PRU104210	Iran	yes	109-110del	L37E+17X	2	HOM
FA15BER[d]	Lebanon	yes	IVS13-1G→C	truncated protein	i13	HOM
EUFA143[d]	Germany	no	313G→T	E105X	4	HOM
EUFA316[d]	Germany	no	313G→T/1183-1192del[e]	E105X/E395W+3X	4/10	HET
EUFA349[d]	Germany	no	313G→T/IVS11+1G→C	E105X/C479G+4X	4/i11	HET

[a]Numbering from the initiation codon of the cDNA; [b]Not confirmed experimentally; [c]HOM=homozygous mutation; HET=compound heterozygous mutation; [d]Previously published (de Winter *et al*.[11]); [e]This is a correction of the mutation previously published as 1184–1194del; [f]Two abberantly spliced mRNAs differing in length by 25 bases are detected by RT-PCR (data not shown); [g]Confirmed by *in vitro* translation of RT-PCR products (data not shown).

mutated mRNA is unstable. One further splice mutation affected the last base of exon 12 and was first detected as an aberrant RT-PCR product lacking the entire exon 12.

The splice mutation IVS8-2A→G leads to two aberrant mRNAs. One RNA species has exon 8 joined to exon 10, as expected if the acceptor of intron 8 is inactivated. This is predicted to result in protein truncation after 20 amino acids. The other RNA has exon 8 joined to a cryptic acceptor site, 25 bases into exon 10, leading to truncation after two amino acids (data not shown).

Four base change mutations lead to premature stop codons, in exon 4, exon 6 and exon 13, but only one missense mutation has been found so far. The transition 212T→C, for which the patient EUFA569 is homozygous, leads to the non-conservative amino acid substitution of proline for leucine at position 71. This mutation was not found in 100 ethnically matched chromosomes and no other sequence variation was found in this patient's DNA. Examination of the mouse cDNA sequence presented here (Figure 2) shows that the amino acid leucine is conserved at this position in the mouse sequence. The 18 mutations found so far show no obvious clustering within the gene (Figure 3).

Three sequence variants, all affecting amino acid coding, were found in the *FANCG* gene of FA-G patients. These are not considered to be disease related, since in each case a truncating mutation was found in the same allele. Analysis of 100 ethnically matched chromosomes resulted in the frequencies for these polymorphisms given in Table 2. The base change, 77A→G, leading to Q26R was not found in 70 ethically matched chromosomes and affects an amino acid conserved in the mouse sequence; however, it does not disturb complementation of Mitomycin C sensitivity after transfection into FA-G cells (data not shown) and thus presumably represents a rare variant.

As with other FA genes, most mutations have been found only once. However, the truncating mutation, 313G→T, was found in six of nine unrelated German patients examined. Of the 18 alleles expected among these German patients, eight carry 313G→T. Analysis of microsatellite markers linked to *FANCG* indicated a common ancestral haplotype on which the 313G→T mutation occurred (Table 3). The most tightly linked markers, *D9S2176* (approximately 50 kb from *FANCG*) and *D9S1817* each have the same size repeat on the mutant haplotype in all patients. The *D9S2176* allele 335 bp has a population frequency of 0.038. *D9S1853* and *D9S165* have the same alleles in most patients but also show evidence of haplotype erosion due to mutation and/or recombination. The transversion 313G→T thus represents a common *FANCG* founder mutation in Germany. A further common mutation among German patients is the 10 bp deletion, 1183–1192del. This is present only together with the sequence variant 1182T→C, not found among 100 matched chromosomes (data not shown), thus indicating a common origin.

Evidence for a founder chromosome among Turkish patients is given by two patients homozygous for the deletion 1649delC. Again there is a common haplotype at *D9S2176* (327 bp) and *D9S1817* (267 bp) in these patients (data not shown). These patients also share the sequence variant, S7F (Table 2).

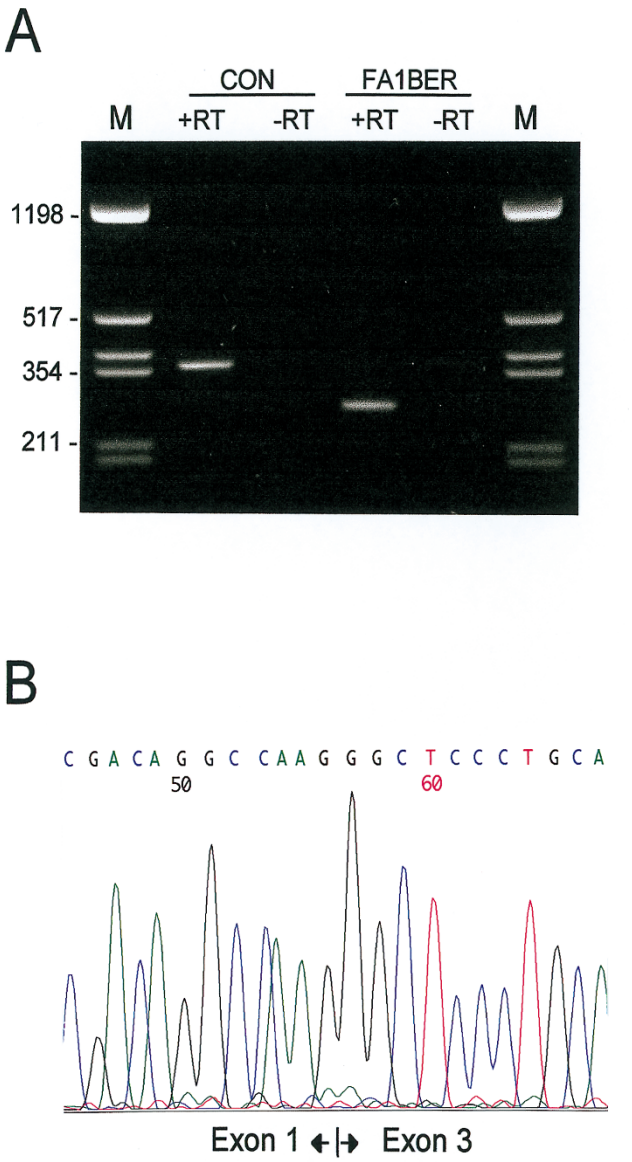

Figure 1 Detection of an aberrantly spliced mRNA in patient FA1BER **A** RT-PCR was used to amplify a cDNA fragment of 365 bp extending from exon 1 to exon 3 from FA1BER RNA and control RNA. The PCR product from FA1BER is about 90 bp shorter, compatible with the loss of exon 2 due to the splice site mutation, IVS2 + 1G→A **B** Sequence analysis of the aberrantly spliced mRNA in FA1BER showing the loss of sequence from exon 2.

The phenotypes of 23 of the patients for whom *FANCG* mutations have been established here are given in a standardised format for a selection of particularly common clinical features in Table 4. The heterogeneity characteristic for FA is clearly demonstrated, even the sibling pairs show discordance for several symptoms. The average age of haematological onset in this group of patients is 6.1 years (range 0 to 15 years), not significantly different from that found in other FA complementation groups (Faivre *et al*, 2000 personal communication); growth retardation and café au lait spots are common (78% and 75% of patients, respectively). Thumb malformations and microphthalmia are present in less than half the patients (36% and 36%, respectively).

Discussion

Mutation analysis by SSCP of amplified exons has proved to be highly efficient for detection of mutations in *FANCG*. In the 20 patients screened for mutations in the *FANCG/XRCC9* gene, a total of 18 different mutations which are likely to be pathogenic were identified. Of these, >90% were initially detected by SSCP or similar indirect methods. Thirteen of the patients proved to have homozygous mutations and this was expected in seven of these, due to consanguinity. For the compound heterozygous patients, only one mutation could not be determined, although this allele is expressed at the mRNA level as shown by RT-PCR (data not shown). This equates to a detection rate of 97% using the methodology described here, contrasting strongly with the situation in *FANCA*, where frequent partial deletions make mutation analysis difficult.[18,19]

The majority of the mutations described are expected to yield truncated FANCG proteins varying from 7% to 95% of the wild type 68 kD. FANCG is known to form a complex with FANCA, and it has been shown that this binding is due to sequences both in the amino terminal and the carboxy terminal ends of the protein[20] (Kuang *et al*, 2000 personal communication). The carboxy terminal binding region has been shown to involve sequences coded by exon 10 and exon 14,[15] thus offering a pathogenic mechanism even for those expected mutant proteins truncating in exons 13 and 14. The mutation in patient DF3 leads to an interstitial deletion of exon 12 and a deduced protein of 58 kD, it is likely that the relative positioning of the binding regions coded by exon 10 and exon 14 is thus disturbed.

Only one missense mutation has been found and its physiological relevance is revealed by comparison with the mouse cDNA sequence presented here. A comparison of the Fancg sequence with that of FANCG is shown in Figure 2. The two orthologues show 71.8% identity and 88% similarity at the amino acid level. Direct comparison of the murine and human protein sequences allows the identification of totally conserved and conservatively substituted residues which might be essential for biological function. Attention has been drawn to leucine zipper motifs in the FANCA and FANCG proteins.[7,8,13] In the case of murine Fancg, this motif (a heptad repeat of leucine residues of the form L-X_6-L) is not totally conserved (Figure 2). Interestingly, the murine protein also contains another shorter leucine zipper motif from residues 58–79 which is not totally conserved in the human protein. The missense mutation L71P in patient EUFA569 is located directly in the second putative leucine zipper at a conserved leucine residue. Substitution of leucine by proline

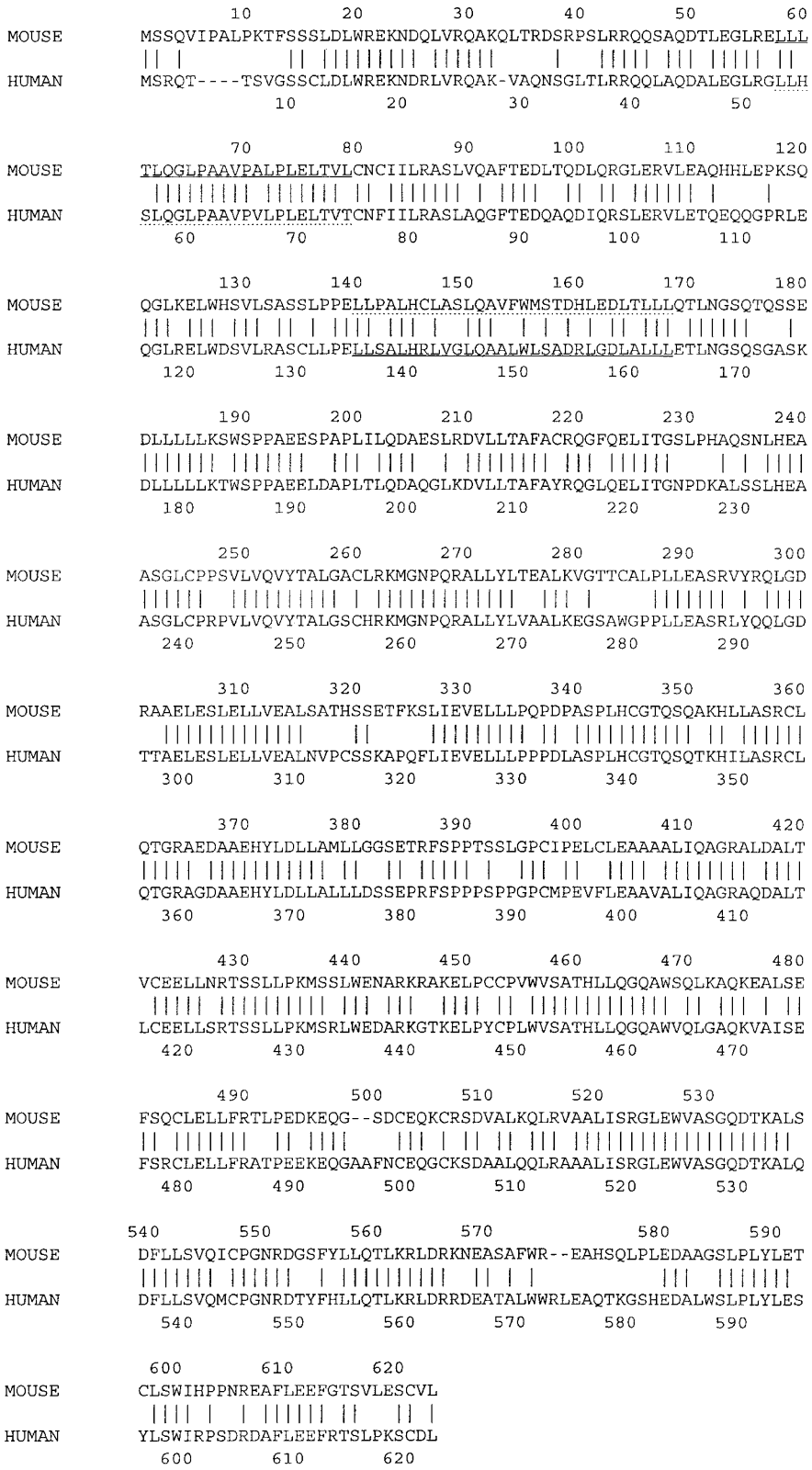

Figure 2 Mouse Fancg sequence and alignment to the human FANCG. Conserved residues are marked by |. The positions of the two possible leucine zippers are underlined.

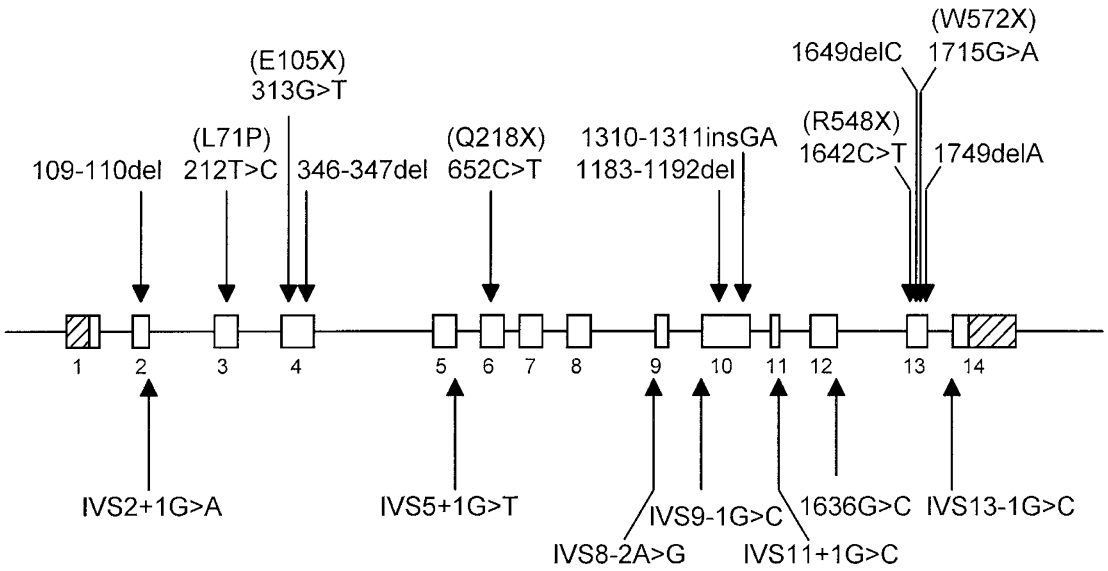

Figure 3 Positions of mutations detected in *FANCG*. The exons and introns of the *FANCG* gene are shown together with the positions of the mutations presented here. Splice mutations are shown below the gene, insertions, deletions and amino acid substitutions, above the gene.

Table 2 *FANCG* sequence variants detected in Fanconi anemia patients

Patient code	*Variant*	*Effect*	*Mouse sequence*	*Frequency*	*Exon*	*State*[1]
EUFA313	890C→T	T297I	A	<0.01	7	HET
EUFA636	20C→T	S7F	K	0.01	1	HOM
F99/186						HOM
PRU63622	77A→G	Q26R	Q	–	1	HOM

[1]HOM=homozygote; HET=compound heterozygote.

is likely to disrupt secondary structure in this region of the protein. It seems plausible that the disturbance of this leucine zipper prevents an essential protein:protein interaction required for FANCG function. Interestingly, LCLs from patient EUFA569 show neither FANCA nor FANCG signals in immunoblots (data not shown) suggesting that the L71P mutation destabilises the protein, perhaps due to a failure to complex with its partner, FANCA. We have observed interdependence of protein signals in immunoblots in other patients of both FA-A and FA-G complementation groups.[14]

Of 35 assigned German families, nine belong to complementation group FA-G making this the second largest group in Germany after FA-A (68%). The 313G→T mutation accounts for 44% of the alleles in German FA-G patients and is clearly due to a founder effect. Whilst there is generally no clear correlation between the mutations described here and the clinical symptoms, the two patients homozygous for the common 313G→T mutation do show a relatively early onset of the haematological disorder at 1.4 and 2.4 years in comparison with an average of 6.1 years for the 23 FA-G patients as a whole (Table 4). The putative missense L71P mutation found in patient EUFA569 would seem to lead to a disease with later onset (13 years) and milder clinical course. This patient is currently haematologically healthy at 26 years of age. The founder mutation, 1649delC, among Turkish patients is not obviously associated with a particular clinical phenotype.

One of the patients described here, FA1BER, was originally diagnosed with Estren Dameshek anaemia, considered to be a subtype of FA without malformations.[21,22] However, this patient has mutations in the *FANCG* gene as do other patients with a more classical phenotype. There is, therefore, no genetic basis for the Estren Dameschek subtype. Fibroblasts from the patient FA1BER, which would have, if at all, a truncated FANCG protein of 4.6 kD, have been examined extensively in various laboratories and have been shown to have several biochemical defects. Analysis of psoralen interstrand DNA crosslinks in these cells using isopyknic centrifugation of denatured genomic DNA provided one of the few demonstrations of a crosslink removal defect in Fanconi anaemia.[23] Persisting DNA crosslinks are expected to prevent semiconservative DNA replication, and indeed a permanently reduced DNA synthesis rate after a crosslinking treatment has been shown for these cells.[24] Nevertheless, the repair of UV-induced thymine dimers is normal in these cells,[25] reflecting the fact that lesions affecting just one DNA strand are repaired by the independent pathways of base-excision and nucleotide-excision repair.

Table 3 Haplotypes at the *FANCG* locus on 313G→T alleles

	EUFA143mat	*EUFA1093mat*	*EUFA316*	*EUFA143pat*	*F99/112*	*EUFA1093pat*	*EUFA348*	*cM*[a]
D9S1853	249	249	249	249	251	261	257	53.60
D9S165	212	212	212	212	212	212	216	58.26
D9S2176	335	335	335	335	335	335	335	
D9S1817	257	257	257	257	257	257	257	59.34
D9S1874	195	195	195	197	197	197	195	61.38

Sizes of PCR products containing (CA)n repeats at the listed loci are given in bp; [a]Genetic distance in cM from 6pter taken from the Marshfield Medical Research Foundation (http://www.marshmed.org/genetics/). Conserved alleles are shaded.

Table 4 Clinical data on FA-G patients

Patient code	*Sex*	*Age at present*	*Haematological onset (years)*	*Thumb malform.*	*Microcephaly*	*Micro-phthalmia*	*Cutaneous symptoms café au lait/ hypopigmentation*	*Growth retard-ation*	*Organ abnormalities*	*MDS/ AL*
{EUFA0281	F	12.4	5.5	–	+	–	+/–	+	none	–/–
EUFA0313}	F	17.4	10.5	–	+	+	+/–	+	none	–/–
F99/112	F	10.0	9.5	–	–	+	+/+	–	none	+/–
FA1BER	M	10.8[b]	6.0	–	–	–	–/–	+	liver	+/+
EUFA1093	M	5.8	2.4	+	+	+	+/+	–	GIT, kidney	–/–
F99/186	M	0.3	0.0	–	–	–	–/–	+	heart	–/–
{EUFA636	M	19.0[b]	15.0	–	+	–	+/–	+	hypogonadism	+/–
10981-95-3	F	9.0[b]	5.0	+	+	+	n.a.	+	kidney	–/+
10981-95-2}	M	10.0[b]	9.0	+	–	–	n.a.	–	n.a.	–/+
EUFA569	F	26.0	13.0	–	+	+	+/+	+	none	–/–
EUFA624	M	11.0[b]	7.0	–	+	+	+/+	+	none	–/+
F99/121	M	5.3	1.5	–	–	–	–/–	+	esophagus	–/–
DF3	M	9.4	7.0	–	+	–	+/–	+	kidney	+/–
PRU63622	F	n.a.	3.0	n.a.	n.a.	n.a.	n.a.	+	n.a.	n.a.
{EUFA0334	F	16.0[b]	7.0	–	–	–	+/–	+	none	–/–
SdC01}	F	9.0[b]	5.0	+	+	–	+/–	+	kidney	–/–
{FA15BER	M	17.3[b]	7.1	–	+	–	+/–	+	none	+/–
FA23BER}	F	10.6[b]	8.8	–	+	–	–/–	–	CNS	+/+[a]
EUFA143	M	14.2[b]	1.4	+	–	–	+/–	+	hypogenitalia	–/–
{EUFA316	F	9.3	3.5	+	+	+	+/+	+	heart, kidney	+/–
FA26BER}	F	2.8	–	–	–	–	–/–	–	none	–/–
{EUFA348	M	10.0	4.8	+	+	+	+/–	+	none	–/–[a]
EUFA349}	M	8.3	3.0	+	+	–	+/–	+	kidney	–/–

[a]Patient received transplant; [b]Age at death; MDS: myelodysplastic syndrome; AL: acute leukaemia; GIT: gastro intestinal tract; CNS: central nervous system; n.a.: no data available. Affected siblings are shown in parentheses ({).

Acknowledgements

We are indebted to the Fanconi anaemia patients, their families and the Deutsche Fanconi-Anämie Hilfe e.V. for their co-operation. The excellent technical assistance of Gabriele Hildebrand, Susanne Rothe, Janina Radszewski, Carola van Berkel, Nicolle Cool, Ingrid Gruijs, Anneke Oostra, Martin Rooimans and Jurgen Steltenpool is gratefully acknowledged. AJT is supported by the Fanconi Anemia Research Fund, USA; NVM is supported by the European Union BIOMED2 Grant No. BMH4-98-3784. This work was supported by the Fritz Thyssen-Stiftung.

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