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Comprehensive laboratory diagnosis of Fanconi anaemia: comparison of cellular and molecular analysis

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ABSTRACT

Background Fanconi anaemia (FA) is a rare inherited bone marrow failure disease caused by germline pathogenic variants in any of the 22 genes involved in the FA-DNA interstrand crosslink (ICL) repair pathway. Accurate laboratory investigations are required for FA diagnosis for the clinical management of the patients. We performed chromosome breakage analysis (CBA), FANCD2 ubiquitination (FANCD2-Ub) analysis and exome sequencing of 142 Indian patients with FA and evaluated the efficiencies of these methods in FA diagnosis.

Methods We performed CBA and FANCD2-Ub analysis in the blood cells and fibroblasts of patients with FA. Exome sequencing with improved bioinformatics to detect the single number variants and CNV was carried out for all the patients. Functional validation of the variants with unknown significance was done by lentiviral complementation assay.

Results Our study showed that FANCD2-Ub analysis and CBA on peripheral blood cells could diagnose 97% and 91.5% of FA cases, respectively. Exome sequencing identified the FA genotypes consisting of 45 novel variants in 95.7% of the patients with FA. *FANCA* (60.2%), *FANCL* (19.8%) and *FANCG* (11.7%) were the most frequently mutated genes in the Indian population. A *FANCL* founder mutation c.1092G>A; p.K364=was identified at a very high frequency (~19%) in our patients.

Conclusion We performed a comprehensive analysis of the cellular and molecular tests for the accurate diagnosis of FA. A new algorithm for rapid and cost-effective molecular diagnosis for~90% of FA cases has been established.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Diagnosis of Fanconi anaemia (FA) is challenging as the disease-associated pathogenic variants are present in a large number of genes.

⇒ Patients with FA present with progressive bone marrow failure and are at risk of developing malignancies in the later stages of life.

⇒ Therefore, early diagnosis is extremely important for proper clinical management of the disease.

WHAT THIS STUDY ADDS

⇒ We performed comprehensive cellular and molecular analysis of peripheral blood and fibroblasts of a large number of patients with FA to establish a novel robust algorithm for laboratory diagnosis.

⇒ FANCD2 ubiquitination analysis, compared with chromosome breakage analysis, will provide a more accurate FA diagnosis.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ The designed algorithm will aid in the early diagnosis of FA, which may improve treatment outcomes.

INTRODUCTION

Fanconi anaemia (FA) is a rare inherited bone marrow failure (BMF) disease, with an estimated incidence of 1 per 360 000 live births.[1] This disease is caused by germline pathogenic variants in any of the 22 genes of the FA DNA repair pathway,[2] which consists of core complex proteins encoded by the FA upstream pathway genes (*FANCA*, *FANCB*, *FANCC*, *FANCE*, *FANCF*, *FANCG*, *FANCL* and *FANCM*) that monoubiquitinate FANCD2/I complex, which recruits the proteins encoded by the downstream pathway genes (*BRCA2*, *BRIP1*, *PALB2*, *RAD51C*, *SLX4*, *ERCC4*, *RAD51*, *BRCA1*, *XRCC2*, *REV7* and *RFWD3*) for DNA interstrand crosslink (ICL) repair. FA is predominantly an autosomal recessive disorder, with the exceptions of *FANCB* and *FANCR*, which show X linked and autosomal dominant inheritance, respectively.[3 4]

BMF leading to pancytopenia, with variable latency, is the most common phenotype of FA and is observed in about 90% of the patients.[5] The characteristic congenital physical abnormalities associated with FA are observed in 75% of the patients.[6] Acute myeloid leukaemia (AML) and solid tumours develop in approximately 20% and 30% of the patients, respectively, and the incidence of myelodysplastic syndrome (MDS) is about 40% by the age

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of 50 years.[7] Some patients present with malignancies before the underlying FA is diagnosed.[8] More importantly, patients with FA do not tolerate the standard doses of DNA damaging chemotherapy drugs used for treating other haematological diseases and as part of the conditioning regimen for a curative allogeneic haematopoietic cell transplantation (allo-HCT) in aplastic anaemia (AA). It is also challenging to differentiate idiopathic AA from FA without physical abnormalities. For these reasons, an accurate laboratory diagnosis of FA is essential.[5 9]

Chromosome breakage analysis (CBA) is the most widely used diagnostic test for FA.[10] However, some rare haematological diseases can also cause chromosomal breakage and pose false positives.[10–12] Although increased G2/M cell-cycle arrest in FA cells treated with ICL agents can be used as a marker of FA,[13] it is also observed in AML cells.[14] Complementation analysis, which uses viral vectors to express wild type cDNAs of FA proteins in the FA cells and corrects the cellular phenotype,[15] is laborious and can take 4–5 weeks to establish a diagnosis. Pathogenic variants resulting in defects in the FA core complex proteins, which occur in >90% of patients with FA,[1 3] fail to monoubiquitinate the short form of FANCD2 to the active long form.[3] The analysis of defective FANCD2 ubiquitination (FANCD2-Ub) is a robust test;[16] however, this method is not routinely used for FA diagnosis. Due to somatic mosaicism[17] observed in 25% of patients with FA, which can yield false-negative results by CBA and FANCD2-Ub analysis in the blood cells,[18] non-haematopoietic cells (fibroblasts) are preferred to perform these tests.[19]

Identifying defective genes and pathogenic variants is crucial for carrier detection and prenatal diagnosis of FA in the affected families and genotype-phenotype correlation in the patients. A few whole exome sequencing (WES) studies have been carried out to determine the frequencies of defective genes and the spectrum of mutations in populations.[2 20–22] Strong associations between malignancies and biallelic pathogenic variants in *FANCD1/BRCA2* and *FANCN/PALB2*[7 23–26] and monoallelic pathogenic variants in *FANCS/BRCA1*, *FANCJ/BRIP1* and *FANCO/RAD51C* have been established.[3 27] Genotyping a large number of patients from different populations, especially those with high consanguinity rates, helps better comprehend the genotype-phenotype correlation.[7 20 28–30] We performed CBA, FANCD2-Ub analysis and exome sequencing of 142 patients with FA from the Indian population and evaluated the efficacy of these methods in diagnosis. Based on our findings, we could establish an efficient algorithm for a faster and more cost-effective laboratory diagnosis of FA.

PATIENTS AND METHODS

Patients

Patients with FA in this study included those with pancytopenia, with or without FA-like physical abnormalities and higher CBA scores in the peripheral blood cells than normal controls or those with normal scores but had FA-like physical abnormalities. The patients were recruited from June 2009 to 2021 after their clinical evaluation at the Department of Haematology, Christian Medical College, Vellore (India). The idiopathic AA group included patients with pancytopenia with low CBA scores and who did not have FA physical abnormalities. Written informed consent was obtained before sample collection.

Chromosome breakage analysis

CBA was performed using a previously described protocol[31] as detailed in the online supplemental methods. A total of 40 well-spread metaphases of the cells with normal ploidy were analysed.

Culture of human dermal fibroblasts

Human dermal fibroblasts were isolated from skin biopsies and cultured using a previously described protocol.[32]

FANCD2-Ub analysis in the peripheral blood cells and dermal fibroblasts

Peripheral blood mononuclear cells (PBMNCs) isolated from 5 mL of blood were stimulated with phytohemagglutinin-A to culture T cells for the whole cell lysate preparation. Fibroblasts treated with MMC (mitomycin C) for 16 hours were harvested for lysate preparation. Western blot was carried out using standard protocols using a FANCD2 antibody (Santa Cruz Biotechnology; sc-20022) to detect the presence or absence of FANCD2 monoubiquitination.

Next-generation sequencing to identify the FA genotypes

Exon capture for 35 samples was performed using SureSelect Human All Exon V5 (Agilent), 35 samples with xGen Exome Research Panel v1 (Integrated DNA Technologies)), 42 samples with SureSelect Human All Exon V5+UTR (untranslated region) (Agilent), 25 samples with SureSelect Human All Exon V6+UTR (Agilent) and 5 samples with a focused exome sequencing panel that covers all the genes described in OMIM. Sequencing was performed on an Illumina HiSeq X system to generate 2×150 bp sequence reads with 8–10 GB of data per sample.

Detection of deletions using multiplex ligation-dependent probe amplification and gene dosage analysis

Deletions in *FANCA* were identified by multiplex ligation-dependent probe amplification (MLPA) using SALSA (Selective Adaptor Ligation, Selective Amplification) MLPA P031 and P032 Probe Mixes and SALSA MLPA EK1 reagent kit (MRC Holland) and Coffalyser software. *FANCT* deletion was determined by gene dosage analysis by capillary electrophoresis of fluorescently labelled PCR products.

Long-amplicon next-generation sequencing

Long-amplicon next-generation sequencing (LA-NGS) was performed for *FANCA* and *FANCG* genes, and the data were analysed using publicly available bioinformatic analysis tools using Galaxy (https://usegalaxy.org/) (refer to the online supplemental section).

Generation of lentiviral plasmids and complementation analysis

pLX301-*FANCA*, pLX301-*FANCG* and pLX301-*FANCC* plasmids were generated by Gateway cloning, and pCW-*FANCL* and pCW-*FANCF* plasmids were generated by cloning the cDNAs in pCW lentiviral plasmid.

The detailed methods are provided in the online supplemental methods.

RESULTS

Clinical phenotypes of patients with FA

As per the inclusion criteria described in the methods section, 142 patients (55 female and 87 male) were selected for the molecular diagnosis of FA. The majority of patients (78.9%) were from the southern states of India (Andhra Pradesh: 28.1%, Tamil Nadu: 24.6% and Kerala: 21.1%) (online supplemental table S1, online supplemental figure S1). The median age at diagnosis at our centre was 10 years (range: 1–45 years), with the predominant phenotype being cytopenia, estimated based

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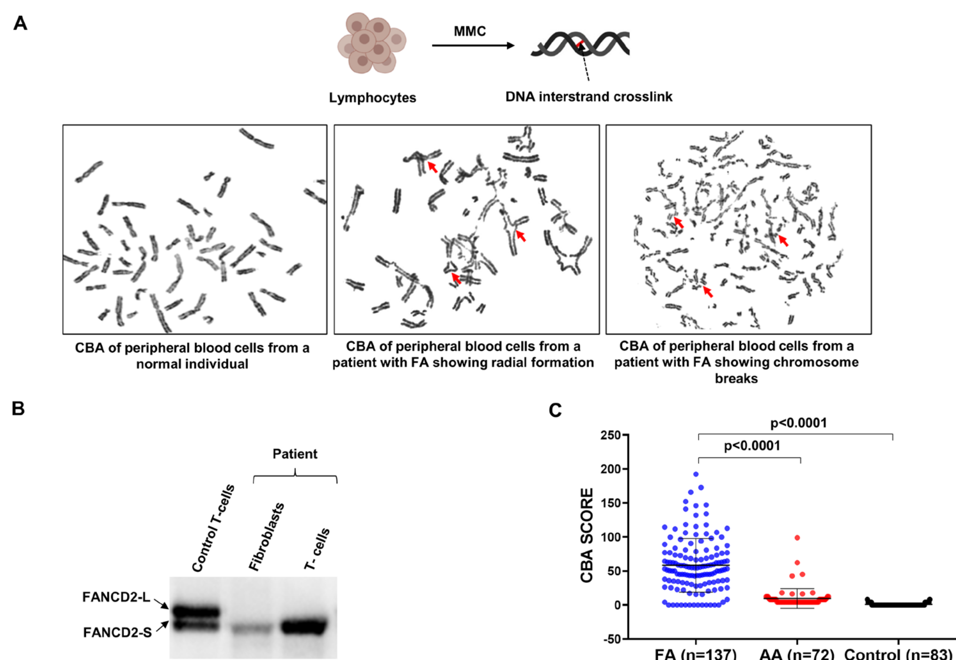

Figure 1 Chromosome breakage analysis (CBA) and FANCD2 ubiquitination (FANCD2-Ub) analysis in 142 patients with Fanconi anaemia (FA). (A) Representative microscopy images of chromosomes showing higher numbers of abnormalities in the T cells of patients with FA than in normal controls. (B) Representative western blot analysis for the detection of short form of FANCD2 (FANCD2-S) and long form of FANCD2 (FANCD2-L) isoforms. T cells and fibroblasts from a patient with FA have only FANCD2-S, whereas both the FANCD2-S and FANCD2-L forms are present in the cells of a normal control subject. (C) Comparison of CBA scores in patients with FA and aplastic anaemia (AA) and normal controls.

on the peripheral blood cell counts, observed in 140 (98.6%) patients and hypocellular bone marrow observed in 126 (88.8%) patients. FA characteristic physical abnormalities were observed in 136 (95.7%) patients, with skin pigmentation in 119/142 (83.8%) patients, radial ray abnormalities in 62 (43.6%), short stature in 41 (28.8%) and microcephaly in 41 (28.8%) patients (online supplemental table S1). Haematological malignancies were observed in 21 (14.7%) patients (median: 27; range: 6–45). Of these 21 patients, 3 had AML and 16 (11.2%) had MDS at presentation, while 2 patients with MDS transformed to AML during the follow-up. Eighty-eight (61.9%) patients were treated with androgen therapy (danazol and stanozolol), while 42 (29.6%) received an allo-HCT (online supplemental table S1). Eighteen (12.6%) patients had family members diagnosed with FA.

Chromosome breakage and FANCD2-Ub analysis

We performed CBA (figure 1A) and FANCD2-Ub analysis (figure 1B) to compare the sensitivities of these two tests to distinguish FA cases from non-FA cases. CBA was performed in the peripheral blood of 137 of the 142 patients with FA. As expected, the patients with FA had overall higher CBA scores (median: 54.7; range: 0–192.2) compared with the 72 patients with idiopathic AA (median: 4.1; range: 4.1–98.9) who did not have FA characteristic physical abnormalities and 83 normal individuals (median: 0; range: 0–8.2) (figure 1C). There were 16/137 (11.6%) patients with FA with very low CBA scores (median: 0; range: 0–8.2) overlapping with those of normal controls and patients with AA (figure 1C and online supplemental table S2). As these patients had FA-related physical abnormalities in addition to pancytopenia, they were further analysed as described below. FANCD2-Ub analysis was performed for 134 patients with FA. Depending on the type of samples available, this test was carried out in peripheral blood cells of 53 patients, fibroblasts of 26 patients, and both cell types of 55 patients (figure 1B and online supplemental figure S7). Lack of FANCD2-Ub (FANCD2-Ub−) was observed in 51/53, 25/26 and 52/55 patients in each group. To identify patients with mosaicism, we compared the results of FANCD2-Ub analysis in both T cells and fibroblasts of 55 patients. Only 3/55 patients were FANCD2-Ub+ in the peripheral blood cells and FANCD2-Ub− in fibroblasts (online supplemental figure S7), suggestive of mosaicism in these patients. Of these patients, one was lost to the follow-up, and two presented with MDS and were evaluated for FA due to their marginally elevated CBA scores and FA physical abnormalities. Overall, 128/134 (95.5%) were FANCD2-Ub− (online supplemental figure S7), which suggested that FA in the Indian population is predominantly caused by pathogenic variants in the FA upstream pathway genes.[1 33] NGS confirmed the FA pathway upstream genes, *FANCA*, *FANCC*, *FANCE*, *FANCF*, *FANCG*, *FANCI*, *FANCL* and *FANCT*, in these patients who were FANCD2-Ub−.

We could compare the sensitivities of CBA and FANCD2-Ub analysis in the peripheral blood cells of 106 patients for whom both the tests were performed. CBA scores were >15 in 94 (88.6%) patients and FANCD2-Ub− in 98 (92.5%) patients. There were three patients with CBA >15 and FANCD2-Ub+, caused by downstream pathogenic variants. NGS confirmed pathogenic variants in *FANCJ* in two patients and *BRCA2* in one patient. Of 16 patients with low CBA scores (<15), 7 were FANCD2-Ub−. In the five patients with low CBA scores and FANCD2-Ub+ in the peripheral blood, two were FANCD2-Ub− in the fibroblasts suggesting mosaicism in these cases (online supplemental table S2). The overall sensitivity of FANCD2-Ub analysis in the non-mosaic FA cases was 97% and that of CBA was 91.5% when peripheral blood cells were analysed (online supplemental table S8).

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Table 1 Deletions identified in the *FANCA* and *FANCT* genes in patients with Fanconi anaemia (FA)

Patient ID	Gene	Deleted exons	Zygosity
FA04	*FANCA*	Exons 10–36	Heterozygous
FA11	*FANCA*	Exons 32–36	Homozygous
FA16	*FANCA*	Exon 27	Homozygous
FA21	*FANCA*	Exons 32–38	Homozygous
FA24	*FANCA*	Exons 21–30	Heterozygous
FA513	*FANCA*	Exons 1–3	Homozygous
FA529	*FANCA*	Exon 27	Homozygous
FA561	*FANCA*	Exon 27	Homozygous
FA631/18	*FANCA*	Exons 32–36	Heterozygous
FA636/18	*FANCT*	Exon 7	Homozygous
FA672/18	*FANCA*	Exons 32–36	Homozygous
FA554	*FANCA*	Exons 39–43	Heterozygous
FA674/18	*FANCA*	Exons 10–36	Heterozygous
FA592	*FANCA*	Exons 1–20 and Exon 27	Compound heterozygous
FA614	*FANCA*	Exons 16–17	Homozygous
FA622	*FANCA*	Exons 30–31	Heterozygous
FA17/19	*FANCA*	Exon 7	Homozygous
FA18/19	*FANCA*	Exon 7	Homozygous
O-117	*FANCA*	Exons 4–6	Homozygous
O-126	*FANCA*	Exon 11	Homozygous
FA-08/19	*FANCA*	Exon 27	Homozygous
FA-30/21	*FANCT*	Exon 7	Homozygous

Detection of pathogenic variants by exome sequencing

We performed NGS for all the 142 patients with FA recruited in this study using the DNA extracted from the PBMNCs of 63 patients and the fibroblasts of 79 patients. The bioinformatics pipeline for identifying the pathogenic variants is shown in online supplemental figure S2. Single nucleotide variant (SNV) and short insertions/deletions were identified either in homozygous or compound heterozygous states in the FA pathway genes of 114 (80.3%) patients (online supplemental table S3). Of the remaining 28 patients, 16 without any SNVs and 12 heterozygous for SNVs were analysed for CNVs using ExomeDepth,[34] which compares the test exome reads to a reference set data from the same batch to normalise the read depths to detect the CNVs. As CNVs are less frequent in FA, we compared the reads of each patient's exome data with those of other patients that we analysed in the same batch (online supplemental figures S3A,C). The CNVs were predicted in 22 patients by ExomeDepth (online supplemental figure S3B, table 1), 5 with heterozygous *FANCA* deletions, 1 compound heterozygous with two different *FANCA* deletions and 15 with homozygous *FANCA* and 1 with homozygous *FANCT* deletions. MLPA analysis confirmed the presence and zygosity of the *FANCA* deletions in the 19 patients who were predicted to have deletions by ExomeDepth (online supplemental figures S3A and S4). The predicted homozygous *FANCT* exon 7 deletion was confirmed by a quantitative PCR using fluorescently labelled primers (online supplemental table S6) and capillary electrophoresis (online supplemental figure S3D,E).

By combining SNVs and CNVs, the disease-associated genotypes were identified in 136 out of 142 (95.7%) patients by exome sequencing (online supplemental table S3). As reported earlier, FA in the Indian population was caused by pathogenic variants in the upstream genes, *FANCA*, *FANCC*, *FANCG*, *FANCL* and *FANCT*. Pathogenic variants in the downstream genes were found in *FANCJ* and *BRCA2*. A total of 93 unique variants were identified in nine genes of the FA-pathway, and 45

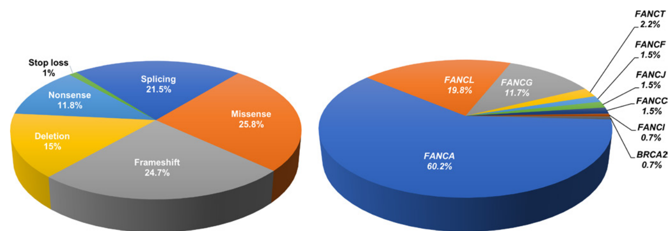

Figure 2 Genotyping of 142 patients with Fanconi anaemia (FA). Left: The percentages of different types of mutations identified. Right: The frequencies of the defective genes in homozygous and compound heterozygous states.

(48.4%) were novel variants (online supplemental table S3). Of these, 119 patients were homozygous (104 with SNVs and 15 with deletions) and 17 were compound heterozygous (10 with two different SNVs, 6 with SNVs and large deletions and 1 with two different large deletions). In six patients whose FA genotypes could not be established, a heterozygous pathogenic variant was identified in one patient, likely benign in four patients and was a variant of uncertain significance (VUS) in one patient as per the ACMG classification (online supplemental table S4. These six samples were also analysed by Golden Helix VarSeq 2.2.0 (Golden Helix, Bozeman, Montana, USA), the clinical genomics interpretation and reporting platform, to detect the SNVs and CNVs that could probably be missed due to the low read counts and by the filtration strategies in our pipeline. We could not detect any additional variants in these samples. Excluding the variants detected in more than one family, there were 93 unique variants: 20 (21.5%) splicing, 24 (25.8%) missense, 23 (24.7%) frameshift, 11 (11.8%) nonsense, 1 (1%) stop loss and 14 (15 %) large deletion (figure 2). Contrary to the previous studies that showed compound heterozygous pathogenic variants in the majority of patients with FA,[2,35] 83.8% of our patients were homozygous (figure 2) due to the high rate of consanguinity in the population.

The FA physical abnormalities in the patients with pathogenic variants in the three predominant defective genes, *FANCA*, *FANCL* and *FANCG*, were evaluated (online supplemental table S1). Kidney abnormalities were observed in 7.1% (6/83) patients with *FANCA*, 7.4% (2/27) with *FANCL* and 37.5% (6/16) with *FANCG* pathogenic variants. The major FA physical abnormalities observed in patients were radial ray abnormalities (56.3% patients with *FANCG*, 33.3% *FANCL* and 42.2% *FANCA*), short stature (37.5% in *FANCG*, 29.6% in *FANCL* and 26.5% in *FANCA*), microcephaly (25% in *FANCG*, 44.4% in *FANCL* and 22.9% in *FANCA*) and facial abnormalities (56.3% in *FANCG*, 37.7% in *FANCL* and 40.9% in *FANCA*). There were 21 patients who developed AML and MDS. Of these 21 patients, 16 had MDS and 3 had AML at presentation, while 2 patients with MDS transformed to AML during the follow-up. Of the 21 patients who developed MDS/AML, 12/21 (57.1 %) had *FANCA* pathogenic variants, and *FANCA* exon 29 pathogenic variants were found in 4/21 (8%) patients. Other defective FA pathway genes in those with MDS/AML were *FANCT* (2/21; 9.5%), *FANCG* (2/21; 9.5%), *FANCJ* (2/21; 9.5%), *BRCA2* (1/21: 4.8%), *FANCF* (1/21; 4.8%) and *FANCL* (1/21; 4.8%).

Highly frequent pathogenic variants in the Indian population

FANCA was the most frequently mutated gene (60.2%) in our study as reported in other populations (60%–80%)[2,20,36](online supplemental table S3, figure 2). In the 82 patients with *FANCA* pathogenic variants, 96 pathogenic variants were identified, out

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Table 2 Determination of pathogenicity of missense mutations

Sample ID	Gene	cDNA change	Amino acid change	Zygosity	ACMG	ClinVar	Varsome	EVE prediction	Final verdict*
FA-02	*FANCJ (BRIP1)*	NM_032043.3:c.1878A>T	p.Glu626Asp	Homozygous	Likely pathogenic	VUS	Pathogenic	Pathogenic	Pathogenic
FA-02/21	*FANCA*	NM_000135.4:c.3788T>C	p.Phe1263Ser	Compound heterozygous	Likely pathogenic	VUS	Likely pathogenic	VUS	VUS/likely pathogenic
FA-02/21	*FANCA*	NM_000135.4:c.1540G>A	p.Ala514Thr	Compound heterozygous	VUS	NA	VUS	Pathogenic	Pathogenic
FA-03	*FANCA*	NM_000135.4:c.2786A>C	p.Tyr929Ser	Compound heterozygous	Likely pathogenic	VUS	VUS	Pathogenic	Pathogenic†
FA-05	*FANCA*	NM_000135.4:c.1304G>A	p.Arg435His	Homozygous	Pathogenic	Pathogenic	Pathogenic	Pathogenic	Pathogenic
FA-06/20	*FANCA*	NM_000135.4:c.4198C>T	p.Arg1400Cys	Homozygous	Likely pathogenic	Pathogenic	Pathogenic	Pathogenic	Pathogenic
FA-12	*FANCA*	NM_000135.4:c.1303C>T	p.Arg435Cys	Compound heterozygous	Pathogenic	Pathogenic	Likely pathogenic	Pathogenic	Pathogenic
FA-18	*FANCA*	NM_000135.4:c.2852G>C	p.Arg951Pro	Homozygous	Pathogenic	NA	Likely pathogenic	Pathogenic	Pathogenic
FA-18/21	*FANCA*	NM_000135.4:c.2290C>T	p.Arg764Trp	Homozygous	VUS	Pathogenic	Pathogenic	Pathogenic	Pathogenic
FA-21/21	*FANCA*	NM_000135.4:c.3239G>A	p.Arg1080Gln	Homozygous	Likely pathogenic	Likely pathogenic	Likely pathogenic	Pathogenic	Pathogenic
FA-31/21	*FANCA*	NM_000135.4:c.1430T>C	p.Leu477Ser	Homozygous	VUS	NA	Pathogenic	Pathogenic	Pathogenic
FA-38	*FANCG*	NM_004629.2:c.425T>C	p.Leu142Pro	Compound heterozygous	VUS	NA	VUS	Pathogenic	Pathogenic
FA-5/21	*UBE2T/FANCT*	NM_014176.4:c.232A>C	p.Asn78His	Homozygous	VUS	NA	VUS	Pathogenic	Pathogenic
FA-527	*FANCC*	NM_000136.3:c.1585A>C	p.Thr529Pro	Homozygous	VUS	VUS	VUS	Pathogenic	Pathogenic†
FA-533	*FANCA*	NM_000135.4:c.3934G>A	p.Asp1312Asn	Compound heterozygous	Likely pathogenic	NA	VUS	Pathogenic	Pathogenic
FA-573	*FANCA*	NM_000135.4:c.2T>A	p.Met1Lys	Compound heterozygous	Pathogenic	Likely pathogenic	Pathogenic	Not available	Pathogenic
FA-593	*FANCA*	NM_000135.4:c.2851C>T	p.Arg951Trp	Homozygous	Likely pathogenic	Pathogenic	Likely pathogenic	Pathogenic	Pathogenic
FA-637/18	*FANCJ (BRIP1)*	NM_032043.3:c.751C>T	p.Arg251Cys	Homozygous	VUS	Conflicting	Likely pathogenic	Pathogenic	Pathogenic
FA-641/18	*FANCF*	NM_022725.4:c.41T>G	p.Leu14Arg	Homozygous	VUS	NA	VUS	VUS	Pathogenic†
FA-646/18	*FANCA*	NM_000135.4:c.2852G>A	p.Arg951Gln	Homozygous	Likely pathogenic	Pathogenic	Likely pathogenic	Pathogenic	Pathogenic
FA-649/18	*BRCA2*	NM_000059.4:c.92G>C	p.Trp31Ser	Homozygous	Pathogenic	NA	Likely pathogenic	Not available	Pathogenic
FA-652/18	*FANCA*	NM_000135.4:c.3163C>T	p.Arg1055Trp	Homozygous	Pathogenic	Pathogenic	Likely pathogenic	Pathogenic	Pathogenic
FA-659/18	*FANCA*	NM_000135.4:c.3689T>C	p.Leu1230Pro	Homozygous	VUS	NA	VUS	Pathogenic	Pathogenic
P-177	*FANCA*	NM_000135.4:c.3350G>C	p.Arg1117Thr	Homozygous	Likely pathogenic	Pathogenic	Likely pathogenic	Pathogenic	Pathogenic

Pathogenic—disease-causing.
*Final verdict considering all the pathogenicity prediction methods.
†Confirmed by complementation.
EVE, evolutionary model of variant effect; NA, not applicable; VUS, Variants of uncertain significance.

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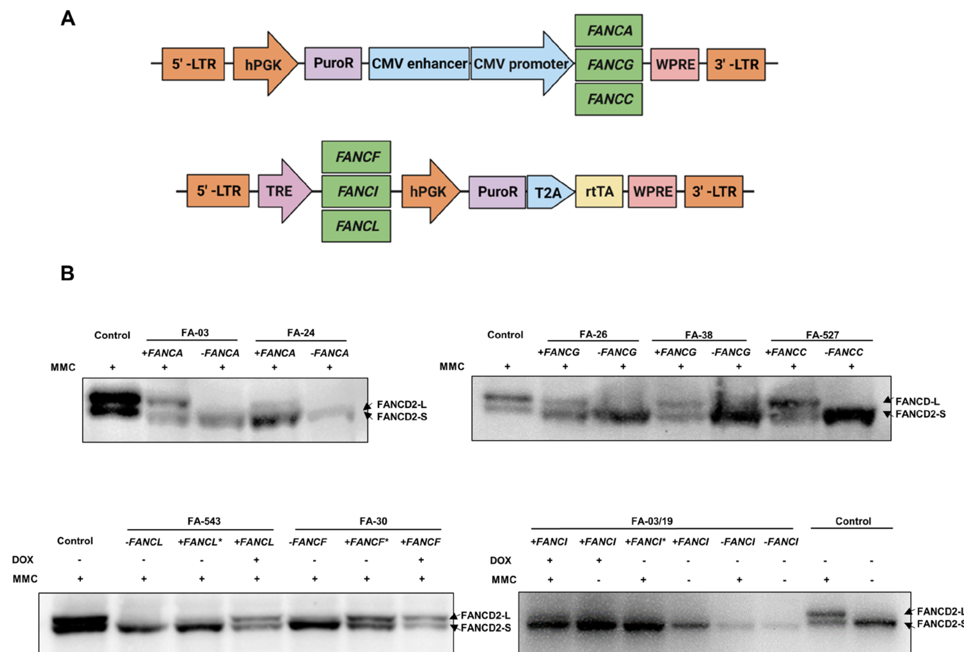

Figure 3 Lentiviral complementation analysis. (A) Lentiviral constitutive expression vectors for complementation analysis of FANCA, FANCG and FANCC and doxycycline-inducible expression vectors for FANCF, FANCI and FANCL. (B) FANCD2 western blot results after complementation of FANCA, FANCG, FANCC, FANCF, FANCI and FANCL genes in the fibroblasts with mutations in these genes. *Leaky expression vector that exhibits transgene expression in the absence of doxycycline (DOX). FA-03, FA-24, FA-26, FA-38, FA-527, FA-543, FA-30, FA-03/19 are patient IDs. CMV, cytomegalovirus; hPGK, human polyglycerate kinase promoter; LTR, long terminal repeat; PuroR, puromycin resistance gene; rtTA, reverse tetracycline-controlled transactivator; TRE, tetracycline response element; T2A, self-cleaving 2A peptide; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element.

of which 32 were novel. Homozygous pathogenic variants were observed in 68 patients, while 14 had compound heterozygous mutations. We found that 14.7% of the *FANCA* pathogenic variants were deletions. There were 14 different deletions in 20 patients. As reported in a previous Indian population study,[22] we also observed a high frequency of *FANCA* exon 27 deletion (3.6%) in our patients (online supplemental figure S3B, table 1). *FANCC*, the second frequently mutated gene with a frequency of 10%–15% in other populations,[20 22] was rare (1.5%) in our patients. The frequency of *FANCG* pathogenic variants was comparable with other populations (11.7% in this study vs 9%–12% in other populations).[2 36] Pathogenic variants in rare FA genes include those in *FANCT/UBE2T* in three patients, *FANCI* in one patient, *FANCJ/BRIP1* in two patients, *FANCF* in two patients and *FANCD1/BRCA2* in one patient (online supplemental table S3, figure 2).

Although pathogenic variants in *FANCL* are rare, WES revealed 27 (19.8%) patients with *FANCL* pathogenic variants in our patients (online supplemental table S3). A synonymous splicing variant c.1092G>A;p.K364=in the *FANCL* gene was found in a homozygous state in 26 (19.1%) patients. Sanger sequence analysis of the PCR-amplified *FANCL* cDNA from a patient with this variant confirmed skipping of exon 13, as reported previously[21](online supplemental figure S5A–C). Lentiviral transduction of wild type *FANCL* cDNA restored FANCD2-Ub in the fibroblasts of a patient with this pathogenic variant (online supplemental figure S5D). All the patients with this pathogenic variant were from South Indian states (12 from Andhra Pradesh, 9 from Kerala, 5 from Tamil Nadu and 1 from Karnataka) (online supplemental figure S1). Although this pathogenic variant was reported previously in 12 Indian patients with FA,[21] our study, with representative samples from all over the country, revealed its frequency among the Indian patients with FA with better accuracy. We identified another *FANCL* pathogenic variant: a nonsense variant c.997C>T; p.Gln333* found in the compound heterozygous state with *FANCL* c.1092G>A;p.K364=in another patient (online supplemental table S3). Other highly frequent pathogenic variants included c.2786A>C (n=5), c.1761–2A>C (n=5) in *FANCG* and c.3066+1G>T (n=4), c.319delG (n=4) and c.826+2T>C (n=4) in *FANCA* (online supplemental table S3).

Determination of pathogenicity of missense variants

We identified 24 missense variants in the 142 patients with FA that we genotyped. The pathogenic effect of these variants was assessed using ACMG guidelines,[37] ClinVar database[37 38] and VarSome variant discovery tool,[39] which use several pathogenicity prediction methods to classify the variants as pathogenic, likely pathogenic or VUS. We identified seven pathogenic variants by ACMG guidelines, eight by ClinVar and six by Varsome (table 2). We also analysed the missense variants using the evolutionary model of variant effect (EVE)[40] tool (https://evemodel.org/) for the pathogenicity prediction, which showed that out of the 24 missense variants in our patients, 20 were pathogenic and 2 were VUS and 2 of them did not have any EVE scores (table 2).

Complementation analysis by lentiviral-mediated gene transfer of wild type cDNA into FA cells and correction of the cellular phenotypes is a feasible method for confirming the pathogenicity of the variants.[15] After antibiotic selection of the fibroblasts transduced with lentiviral vectors encoding wild type cDNAs, the cells were treated with MMC and were analysed for their FANCD2-Ub status. We first validated complementation analysis in the fibroblasts of 13 patients with pathogenic null variants in the FA upstream pathway genes (online supplemental table S7) and observed restoration of FANCD2-Ub in all of

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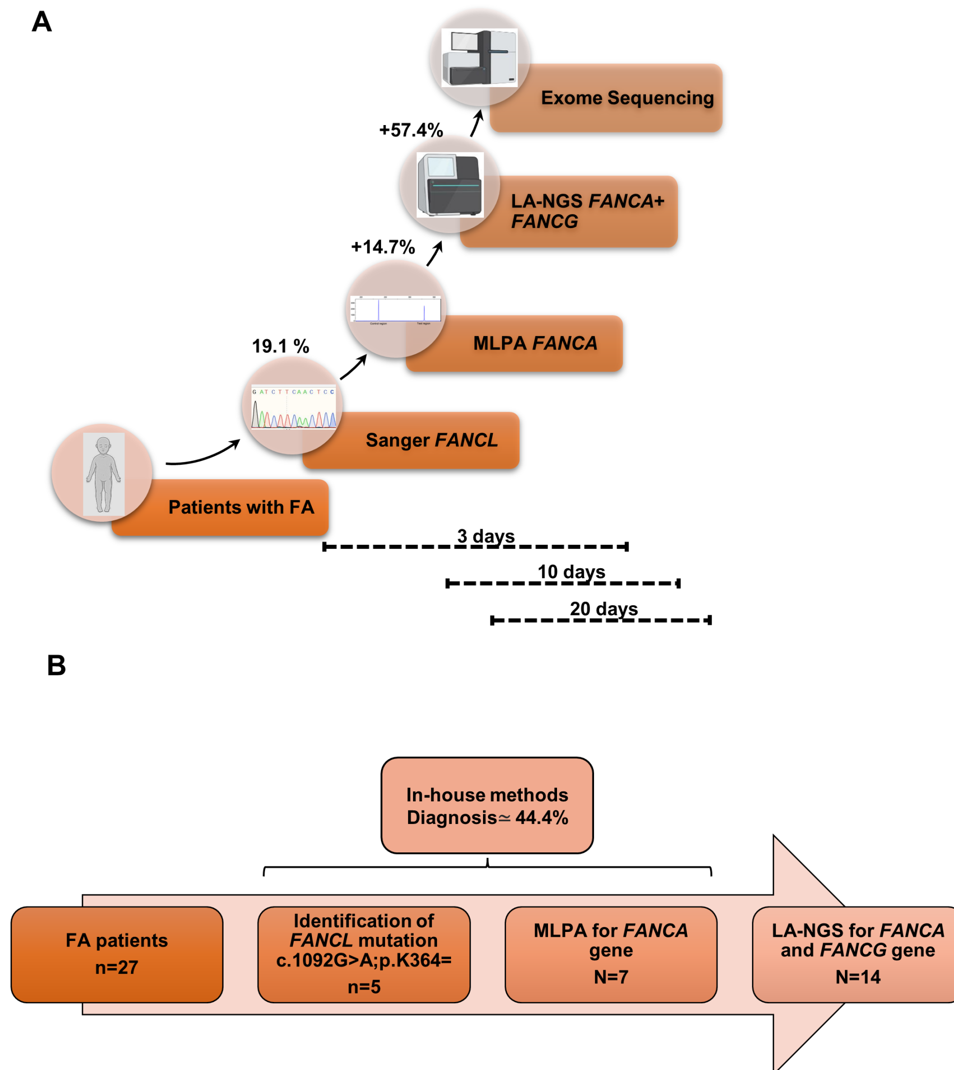

Figure 4 Methodologies for the molecular diagnosis of Fanconi anaemia (FA) in the Indian population. (A) Algorithm for the molecular diagnosis of FA. (B) The new algorithm tested in 27 patients with FA. MLPA, multiplex ligation-dependent probe amplification; LA-NGS, long-amplicon next-generation sequencing.

them (figure 3, online supplemental table S7). Subsequently, we performed complementation analysis in seven patients with VUS and likely pathogenic variants as determined by the ACMG classification in homozygous or compound heterozygous states for whom fibroblasts were available. These included four *FANCA*, 1 *FANCG*, 1 *FANCC* and 1 *FANCF* variants (online supplemental table S7). All these patients showed restoration of FANCD2-Ub after complementation.

A robust molecular diagnosis strategy designed for FA

Our study showed that 57.4% of the patients had SNVs in *FANCA* and *FANCG* genes. Therefore, we developed a LA-NGS method to detect pathogenic variants in these genes. We amplified the *FANCA* gene as six LAs and *FANCG* as one LA by LA-PCR (online supplemental Figure S6A,B), and the PCR products were pooled in a single tube, and NGS and subsequent bioinformatics analysis were performed. The robustness of this method for detecting SNVs was confirmed using DNA samples from 24 patients with known SNVs in *FANCA* and *FANCG* (online supplemental table S5). This method is cost-effective and faster than the current molecular diagnostic strategies and involves less bioinformatics analysis than exome sequencing.

As 19.1% of the patients with FA have *FANCL* c.1092G>A;p.K364=pathogenic variant, Sanger sequencing to detect this variant can be performed as the first test for genotyping the Indian patients with FA. MLPA can detect *FANCA* deletions, which constitute 14.7% of the overall pathogenic variants. The results from these two tests can be obtained in 48 hours. For those who are negative for the pathogenic variants by these two methods, LA-NGS can detect SNVs in the *FANCA* and *FANCG* genes, which constitute ~57% of the FA pathogenic variants. Thus, this algorithm can help in the molecular diagnosis of ~90% of the patients with FA in the Indian population (figure 4A). This diagnostic algorithm was tested in 27 new patients with FA with a median CBA score of 66.8 (0–115) and confirmed that it provides a faster and more cost-effective molecular diagnosis of FA in the Indian population (figure 4B).

DISCUSSION

An accurate laboratory diagnosis of FA is mandatory for the clinical management of this disease. Although CBA is considered a 'gold standard' test for FA, this test has several issues, including laborious standardisation and user variability in the scores. The comparison of CBA and FANCD2-Ub analysis performed in a

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large number of patients with FA in this study confirmed that FANCD2-Ub analysis, which is currently not being used for diagnosis, is also suitable for FA diagnosis (online supplemental table S8). We found increased sensitivity of CBA scores in FA diagnosis when a cut-off of 15 (arrived at using the receiver operating characteristic curve (ROC) curve and Youden's Index) was used. However, a randomised comparative analysis is required to confirm this cut-off. Among 16 patients with low CBA scores, 12 were analysed for FANCD2-Ub analysis. Seven of these 12 patients showed defective FANCD2-Ub (58.3%), and subsequent pathogenic variant analysis confirmed them to be FA cases. Defects in the downstream FA pathway genes, which do not affect FANCD2-Ub, are very rare (2%–6 %)[4 20] in patients with FA, and we also found the downstream pathogenic variants in only ~2.2% of the Indian patients. Therefore, FANCD2-Ub analysis may be used as a reliable test for the diagnosis of FA. Spontaneous reversal of pathogenic variants occurs in the haematopoietic cells of 15%–25% of the patients with FA.[41] FANCD2-Ub analysis performed in both T cells and fibroblasts in 55 patients in this study identified only 3 (5.4%) mosaics, with FANCD2-Ub+ in T cells and FANCD2-Ub− in fibroblasts. The reduced incidence of mosaicism (<15%) observed in our cohort may be because the patients were referred from a haematology clinic after evaluating pancytopenia and other haematological and physical abnormalities.

Detection of defective genes and pathogenic variants is important for genetic counselling and the development of targeted prenatal genetic testing. Early molecular diagnosis is also essential for participation in gene therapy for FA.[42] Although targeted gene panels have been developed for FA,[20 43 44] WES allows the discovery of new genes associated with the diseases. There were very few reports on WES analysis of a limited number of patients with FA, with 15–25 patients.[45] Recently, a comprehensive WES study in 68 European patients with FA identified pathogenic variants in 93.3% of patients.[2] We performed a WES analysis of the largest number of patients with FA and identified pathogenic variants with 95.7% genotyping efficiency. In the six patients for whom only heterozygous variants were identified, gene expression and protein analysis may identify the probably missed pathogenic variants.

NGS has limitations in detecting CNVs. Therefore, robust bioinformatics methods are required to detect deletions. A recent study has applied a bioinformatics tool using custom scripts to identify the deletions in FA genes efficiently.[2] We used ExomeDepth[34] for CNV analysis and applied filters to discard the false positives to obtain 100% accuracy in detecting deletions in our patients. Our results confirmed that the improved bioinformatics could efficiently detect CNVs. As reported earlier in other populations,[46–48] we also found that *FANCA* deletions are very common (14.7%) in Indian patients with FA. The combined analysis of SNVs and CNVs identified the disease-associated genotypes in ~95% of the patients. Such a high pathogenic variant detection rate in FA was reported previously by Bogliolo *et al*,[2] which also analysed both SNVs and CNVs.

FANCA has high genetic heterogeneity and is the most often mutated FA gene, with frequencies ranging from 60% to 80% in different populations.[2 20 36] However, we found that *FANCA* (60.2%), *FANCL* (19.8%) and *FANCG* (11.7%) are the most common mutated genes in our cohort of patients with FA. Even though we identified a large number of patients (~20%) with homozygous *FANCL* c.1092G>A;p.K364=, they presented diverse phenotypes. More than 83.2% of the patients were homozygous for pathogenic variants in the FA genes due to this population's high consanguinity rate. A large number of patients, 65 (45%), were born from consanguineous marriages. We found only 93 variants in the 142 cases analysed by NGS due to high homozygosity and a few recurrent pathogenic variants. There were 19 recurrent variants found in more than one patient; their frequencies ranged from 1.3% to 17.5%.

We found pathogenic variants in only nine FA genes, *FANCA*, *FANCG*, *FANCC*, *FANCL*, *FANCF*, *FANCT*, *FANCI*, *FANCD1* and *FANCJ*. These genes could be prioritised for designing the FA genotyping panel and the bioinformatics analysis of Indian patients. We found that Sanger sequencing to detect the *FANCL* pathogenic variant and MLPA to detect *FANCA* deletions could diagnose 33.8% of FA cases. We also developed a faster and cost-effective LA-NGS strategy to detect point pathogenic variants in *FANCA* and *FANCG* genes, constituting 57.4% of the genotypes in Indian patients with FA. The presence of a *FANCL* founder variant and the high frequency of *FANCA* and *FANCG* pathogenic variants helped establish a new, faster, cost-effective molecular diagnosis strategy for Indian patients with FA that could diagnose ~90% of the patients with FA. Altogether, the algorithm established would expedite the FA diagnosis and be a cost-effective alternative compared with WES for FA diagnosis.

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Contributors GJ performed research, data analysis and wrote the manuscript. NBJA performed research. TSG performed data analysis. PVRD collected data and wrote the manuscript. KM, DR, ADC, PS and SP performed standardisation of methods and provided the laboratory data. FNA analysed the clinical data. VR performed whole exome sequencing for a part of the patients and critically reviewed the manuscript. AA provided clinical data. VMS provided the chromosome breakage scores for a part of the samples. AS provided clinical data and critical inputs for the manuscript. UPK performed clinical data analysis and provided inputs for the manuscript. BG performed clinical data analysis and supervised the clinical part of the study. SRV designed the research, analysed and interpreted the data, supervised the study and revised and finalised the manuscript. SRV is guarantor for this study and accepts full responsibility for the work and/or the conduct of the study, had access to the data, and controlled the decision to publish.

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Competing interests None declared.

Patient consent for publication Not applicable.

Ethics approval This study involves human participants and was approved by the Institutional Research and Ethics Committee, Christian Medical College, Vellore (R.C.Min.No.6561). Participants gave informed consent to participate in the study before taking part.

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Data availability statement Data are available upon reasonable request.

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Supplementary Methods

Chromosome breakage analysis (CBA)

CBA was performed by culturing T-cells from 0.5mL whole blood in 4.5 mL of RPMI 1640 containing 10% fetal bovine serum (FBS) and 60 µg/mL phytohemagglutinin (Thermo Fisher Scientific) at 37°C/5% CO_2. After 24 h, MMC was added at 50 and 100 ng/mL in the culture, and after 72 h, the cells were arrested at the metaphase by treatment with 0.02 µg/mL colcemid for 1 h. A total of 40 well-spread metaphases of the cells with normal ploidy were analysed. Ambiguous structures were excluded from the analysis. A conservative linear discriminant function that provided 100% sensitivity and 100% specificity was used to calculate the CBA scores.[71]

Isolation and culture of human dermal fibroblasts

Human dermal fibroblasts were isolated and cultured using a previously described protocol.[72] In brief, 1 mm^3 skin biopsies obtained from the patients were washed with phosphate-buffered saline (PBS; HyClone) and incubated overnight at 37°C/5% CO_2 in a 15 mL conical flask with 1 mL of a skin biopsy digestion medium containing DMEM with 20% FBS, 0.25% collagenase type I (ThermoFisher Scientific, 0.05% DNAse I (Merck, and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin). The tube was briefly vortexed the next day, and 7 mL of DMEM containing 20% FBS was added. The dissociated cells were then plated in a T25 culture flask and incubated for 3 days at 37°C (5% CO_2). The medium was replaced on the fourth day with DMEM containing 10% FBS and antibiotics. The cells were cultured with a media change on alternate days until they reached approximately 80% confluency. Cells were then passaged at 1:4 ratio using 0.05% trypsin–EDTA (Thermo Fisher Scientific). After two passages, the cells were cryopreserved.

FANCD2-Ub analysis in the PB cells and dermal fibroblasts

PBMNCs isolated from 5 mL of the blood sample by density gradient separation using Ficoll–Hypaque solution (Hyclone) were cultured in 5 mL of RPMI medium containing 10% FBS and 60 µg/mL phytohemagglutinin. After 4 days of culture, whole-cell lysates were prepared for western blot analysis. Fibroblasts were isolated from patients' skin biopsies for FANCD2 ubiquitination analysis (refer to supplementary methods). 2×10^6 fibroblasts were treated with 0.25 µg/µL MMC for 16 h, and the cells harvested by trypsin–EDTA treatment were used for preparing the cell lysates. Protein separation was performed in a 7% tris–glycine SDS-PAGE gel and transferred on a polyvinylidene difluoride membrane. Non-ubiquitinated and ubiquitinated forms of FANCD2 were probed using an anti-FANCD2 antibody (sc-20022, Santa Cruz Biotechnology) and horseradish peroxidase-conjugated anti-mouse secondary antibody (Cell Signaling) and detection was performed using an enhanced luminol-based chemiluminescence substrate (Westar Supernova, Cyanagen).

Next-generation sequencing

Exon capture for 35 samples was performed using SureSelect Human All Exon V5 (Agilent), 35 samples with xGen Exome Research Panel v1 (IDT), 42 samples with SureSelect Human All Exon V5+UTR (Agilent), 25 samples with SureSelect Human All Exon V6+UTR (Agilent) and 5 samples with a focused exome sequencing panel. All of these panels have 100% coverage for the coding sequence of FA genes. Sequencing was performed on an Illumina HiSeq X system to generate 2×150 bp sequence reads with 8–10 GB of data per sample.

Bioinformatics of exome sequencing data and variant prioritisation

Fastq-mcf (ea-utils-1.1.2-806) was utilised for trimming adapter sequences from the raw reads, and Bedtools-2.17 was used for coverage metrics. A GATK Best Practices bioinformatics analysis using Sentieon (v201808.01) was used to identify germline variants in the samples. The sequences obtained were quality-checked and aligned to the human reference genome (GRCh37/hg19) using the Sentieon aligner. The alignment files were processed using Sentieon to remove duplicates, recalibrate, and re-align indels. Gene annotation of the variants was performed using the Ensembl Variant Effect Predictor against the human gene model (release 91). Single-nucleotide variants (SNVs) with >3% overall population allele frequency in 1000Genome Phase 3[74], gnomAD (v2.1), EVS, dbSNP (v151), as well as MedGenome's Indian population database[76] and deep intronic and intergenic variants were excluded for further analysis. The variants were sequentially prioritised based on their reported status in disease databases (HGMD, ClinVar)and multiple in-silico prediction toools, such as PolyPhen-2, SIFT, MutationTaster2, LRT, EVE.[73] Copy number variants (CNVs) were detected from targeted

sequence data using ExomeDepth (v1.1.10). This algorithm detects CNVs based on comparing the read depths of the test data with the matched aggregate reference dataset. For all the samples, copy number variants (CNVs) were evaluated for identifying deletions and duplications involving one or more exons. Bayes factors of more than 15 were retained. CNVs with low statistical confidence and possibility of being false positive due to the presence of pseudogene in the genome were filtered out. The prioritised variants were classified based on ACMG guidelines. Online tools of Varsome and EVE, a recent computational method, were used together for the classification. Additionally, VarSeq 2.2.0 (Golden Helix Inc., Bozeman, MT, United States), a clinical genomics interpretation and reporting platform, was also used to analyse SNVs and CNVs in the 6 samples in which our pipeline identified heterozygous mutations and therefore could not establish the disease-causing genotypes.

Detection of deletions using multiplex ligation-dependent probe amplification (MLPA) and gene dosage analysis

Deletions in *FANCA* were identified by MLPA using SALSA MLPA P031 and P032 Probe Mixes and SALSA MLPA EK1 reagent kit (MRC Holland) and Coffalyser software (MRC Holland) as per the manufacturer's protocol. To confirm the deletion identified by CNV analysis in the *UBE2T* gene, a gene dosage quantitative multiplex fluorescent-PCR was performed as we described previously[78]. Briefly, a 327 bp fragment from exon 7 of *UBE2T* was amplified along with a short fragment of the human albumin gene (Mayuranathan et al., 2012) by multiplex PCR using fluorescently labeled forward primers **(Table S7)**, and the products were analysed by capillary electrophoresis. For accurate gene dosage analysis, 200ng of DNA and 20 cycles were used for the PCR with 0.4 µM labeled primers. The amplified products were separated by capillary electrophoresis in an ABI-3130 Genetic Analyser (Applied Biosystems), and the results were analysed by GeneMapper software version 4.0 (Applied Biosystems). The peak heights of the *UBE2T* amplicon were intranormalized by dividing them by the peak heights of the albumin gene. Subsequently, internormalization was performed by dividing the intranormalized peak height of *UBE2T* of the patient sample with that of the control sample.

Long-amplicon next-generation sequencing

The *FANCA* gene was amplified as 6 fragments, ranging from 7.15 to 15.25 kb, and *FANCG* as a single fragment of 6.73kb from the patients' DNA samples using GoTaq Long PCR Master Mix (Promega), according to the manufacturer's protocol. Primers were designed using Primer3 (v. 0.4.0) software (http://bioinfo.ut.ee/primer3-0.4.0/) to encompass all the exons and their splice sites **(Table S7)**. The amplified DNA fragments (amplicons) were analysed by 1% agarose gel electrophoresis. Based on the ethidium bromide staining intensities of the PCR amplicons, equal amounts of amplicons were pooled and sheared, and the libraries were prepared using TruSeq Nano DNA Library Preparation Kit (Illumina). The resulting libraries were sequenced on an Illumina NextSeq-500 sequencer to generate 2×150 bp reads at approximately 100× sequencing depth. The generated sequence data were analysed for quality, and a minimum of 75% of the sequenced bases (Q30) were processed to generate FastQ files. The NGS data (FastQ) were analysed by a pipeline developed in Galaxy (galaxyproject.org), as briefly outlined below. The browser extensible data (BED) file of the target region was created using BED Tools. The adapters were trimmed from the raw reads and aligned against the BED file using BWA (Galaxy version 0.7.17.1), alignment post-processing was performed by removing PCR duplicates[79], variant calling was done using FreeBayes, and then the variants were normalised using VCF ALLELIC PRIMITIVES[80]. The VCF files were further annotated using SnpSift (ref seq-hg19). The variants were also annotated using wANNOVAR to determine the mutations' functional consequences.

Plasmids

pLX301-*FANCA*, pLX301-*FANCG* and pLX301-*FANCC* were generated by Gateway cloning using the entry vectors pENTR223.1-*FANCA* (TransOMIC Technologies), pDONR221-*FANCG* (DNASU), and pDONR221-*FANCC* (DNASU), and the destination vector pLX301 (a kind gift from David Root, Addgene ID: 25895). Gateway cloning was carried out using the LR Clonase Enzyme mix (Thermo Scientific). *FANCL* and *FANCF* cDNAs were generated from a normal individual's blood cells using PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa) and gene specific primers (**Table S7**) and amplified using PrimeSTAR GXL PCR master mix (TaKaRa) with primer sequences suitable for Gibson cloning (**Table S7**). The amplified cDNAs were cloned into pCW-Cas9 (a kind gift from Eric Lander and David Sabatini, Addgene ID: 50661) by Gibson assembly using NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs) after removing the Cas9 gene with BamHI and NheI enzymes.

Lentivirus preparation and transduction of fibroblasts

Lentiviruses were prepared using a second-generation packaging system using the envelope plasmid pMD2.G and the packaging plasmid psPAX2 (gifts from Didier Trono, Addgene IDs: 12256 and 12260, respectively). The lentiviral expression, envelope and packaging plasmids were mixed in a 2:1:1 ratio and transfected into HEK 293T cells using the TransIT-293 transfection reagent (Mirus Bio), according to the manufacturer's protocol. The supernatants containing the lentiviral particles were collected at 48, 60, and 72 h post-transfection and were concentrated using Lenti-X Concentrator (Clontech Laboratories), according to the manufacturer's protocol. After reaching 70% confluency, fibroblasts were transduced with an appropriate volume of lentiviruses in 2 mL of medium containing 6 μg/mL polybrene (Sigma–Aldrich). The medium was changed 24 h post-transduction. After 72 h, antibiotics for selecting the transduced cells were added, and the cells were cultured in this antibiotics-containing medium until the selection was complete.

Supplementary Figure legends

Figure S1. Geographical distribution of the FA patients recruited in this study.

Figure S2. Pipeline for the bioinformatics analysis of WES reads. *ClinVar, OMIM, HGMD, SwissVar, 1000 Genome Phase3, gnomAD, EVS, dbSNP, 1000 Japanese Genome and internal Indian population databases were used for variant annotations. $ Nonsynonymous mutations analysed by EVE algorithm.

Figure S3. Detection of large deletions in the FA pathway genes. (A) Representative results of two FANCA deletions detected by bioinformatics analysis in two different patients and confirmation of these deletions by MLPA. FA-24 is heterozygous for a deletion from exon 21 to exon 30, and FA-11 is homozygous for a deletion from exon 32 to exon 36. Probe mixes 1 and 2 cover all the exons of *FANCA*. (B) Position of the different *FANCA* deletions identified in this study (C) Detection of *FANCT(UBE2T)* deletion by bioinformatics analysis. (D-E) Genomic quantitative-PCR for gene dosage analysis to calculate the copy number of exon 7 of the *FANCT* gene. The peak heights of the amplified product of the exon 7 of the *UBE2T* gene and the control albumin gene are shown. Arrow indicates the absence of exome 7 peak in the patient. (E) Graph showing the gene dosage of *FANCT*.

Figure S4. MLPA analysis to detect *FANCA* gene deletions detected in 19 patients.

Figure S5. Molecular analysis of *FANCL* (c.1092G>A;p.K364=) identified in our cohort of FA patients. (A) Diagrammatic representation of the location of the mutation in the *FANCL* gene, exon 13 skipping in *FANCL* mRNA, the position of the primers used for amplification of the cDNA and Sanger sequencing are shown. (B) The PCR amplification of *FANCL* cDNAs from a normal control and an FA patient with the c.1092G>A;p.K364= mutation showing the difference in molecular weights of the amplified products. (C) Sanger sequencing results showing skipping of exon 13 of *FANCL* in the patient. (D) Western blot analysis showing the restoration of FANCD2 Ub after lentiviral complementation of a 'patient's fibroblasts with wild-type *FANCL* cDNA. The results were compared with a normal control and untransduced fibroblasts from a patient with *FANCL* (c.1092G>A;p.K364=) mutation. The vector used for complementation analysis was pCW-*FANCL*, which has doxycycline-inducible *FANCL* expression.

Figure S6. Long amplicon next-generation sequencing to identify mutations in *FANCA* and *FANCG* gene (A) Diagrammatic representation of the regions of *FANCA* and *FANCG* amplified for LA-PCR-NGS. (B) Agarose gel images showing the amplified *FANCA* and *FANCG* gene fragments. *FANCA*: fragment 1- 7126 bp; fragment 2- 8595 bp; fragment 3- 9726 bp; fragment 4- 13739 bp; fragment 5- 13483 bp; fragment 6- 15253 bp; and *FANCG*: fragment 7- 6760 bp.

Figure S7. Overall schematic representation of our study. Comparison of CBA scores and FANCD2-Ub results in the 142 FA patients recruited in this study. The interpretation and primary diagnosis based on the results of CBAs and FANCD2-Ub analysis before performing exome sequencing are shown on the right-hand side. *mosaic patients; # only heterozygous variants identified by NGS; NA: not analysed.

Figure S1

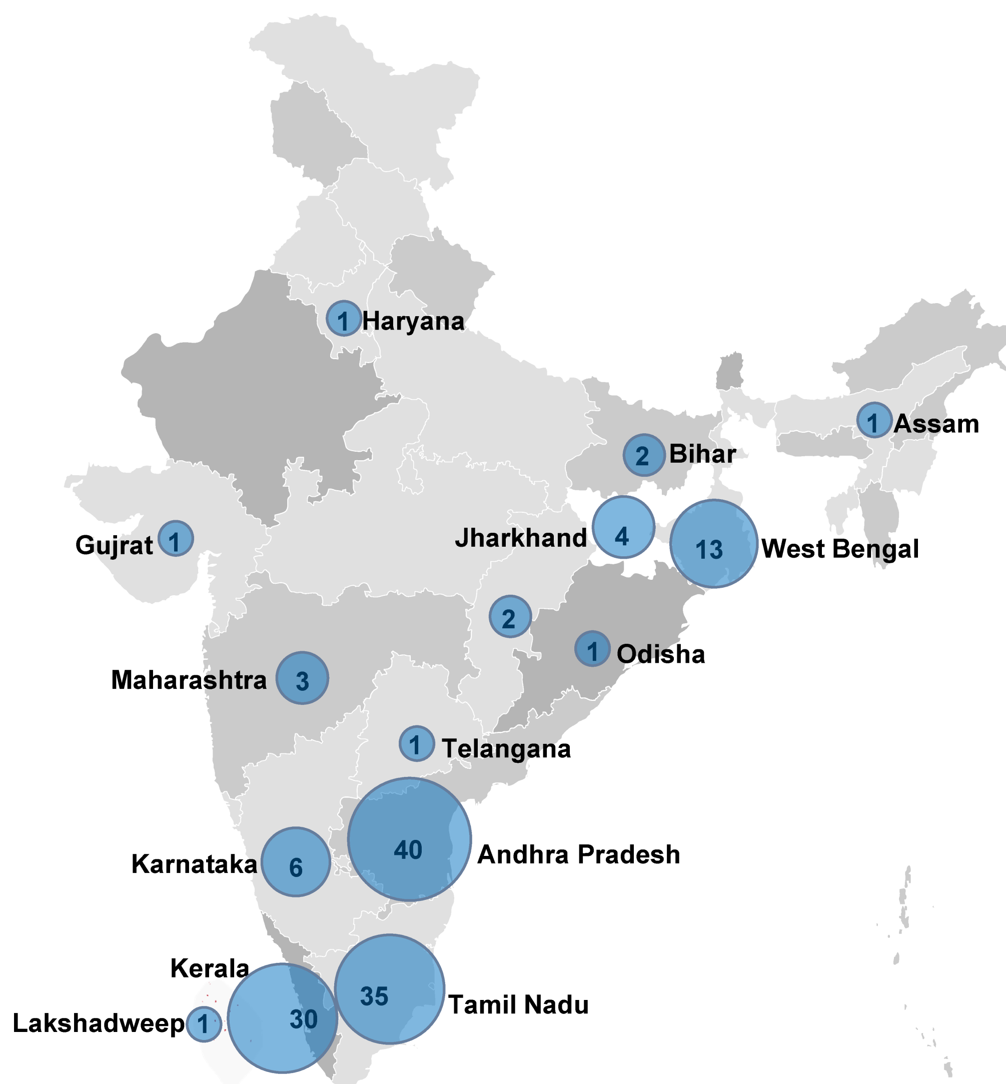

Figure S2

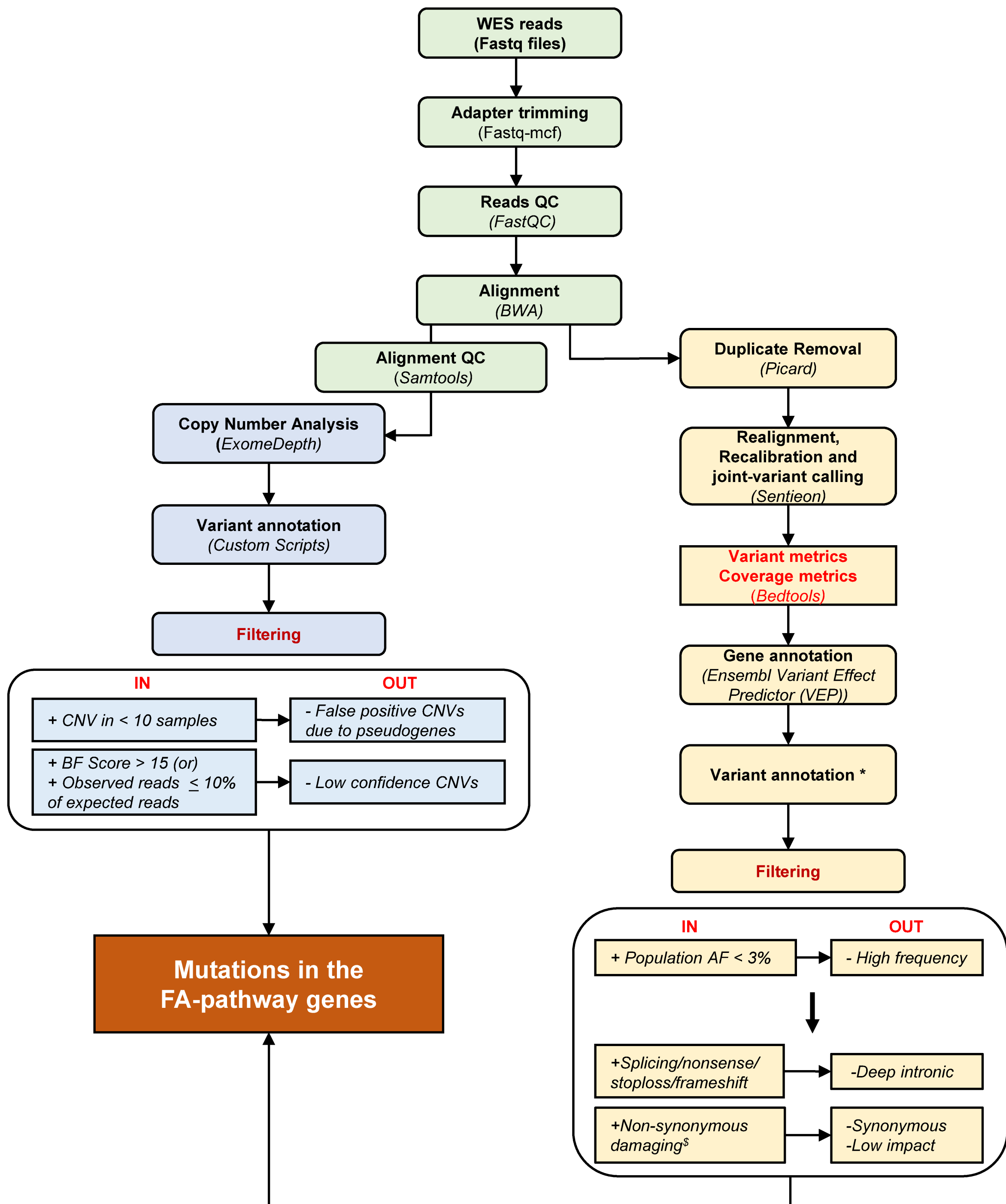

Figure S3

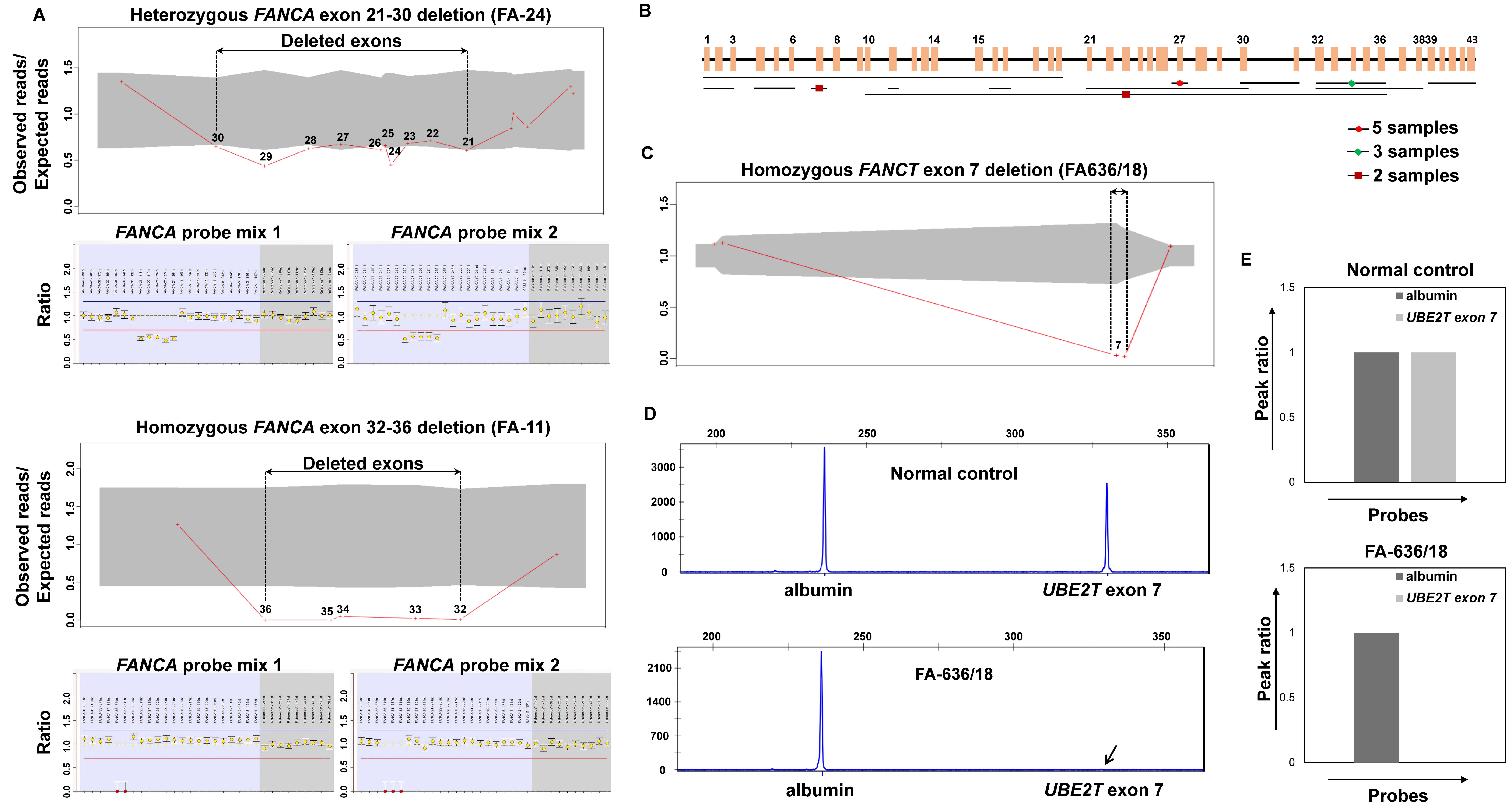

Figure S4

FA-04
FANCA PB 1
FANCA PB 2

FA-11
FANCA PB1
FANCA PB2

FA-16
FANCA PB 1
FANCA PB 2

FA-21
FANCA PB1
FANCA PB2

FA-24
FANCA PB 1
FANCA PB 2

FA-513
FANCA PB 1
FANCA PB2

FA-529
FANCA PB 1
FANCA PB 2

FA-561
FANCA PB 1
FANCA PB2

FA-631/18
FANCA PB 1
FANCA PB 2

FA-672/18
FANCA PB1
FANCA PB2

FA-554
FANCA PB 1
FANCA PB 2

FA-674/18
FANCA PB1
FANCA PB2

FA-622
FANCA PB 1
FANCA PB 2

FA-614
FANCA PB1
FANCA PB2

FA-592

FANCA PB 1 | FANCA PB 2

FA-17/19

FANCA PB1 | FANCA PB2

FA-18/19

FANCA PB 1 | FANCA PB 2

O-117

FANCA PB1 | FANCA PB2

O-126

FANCA PB 1 | FANCA PB 2

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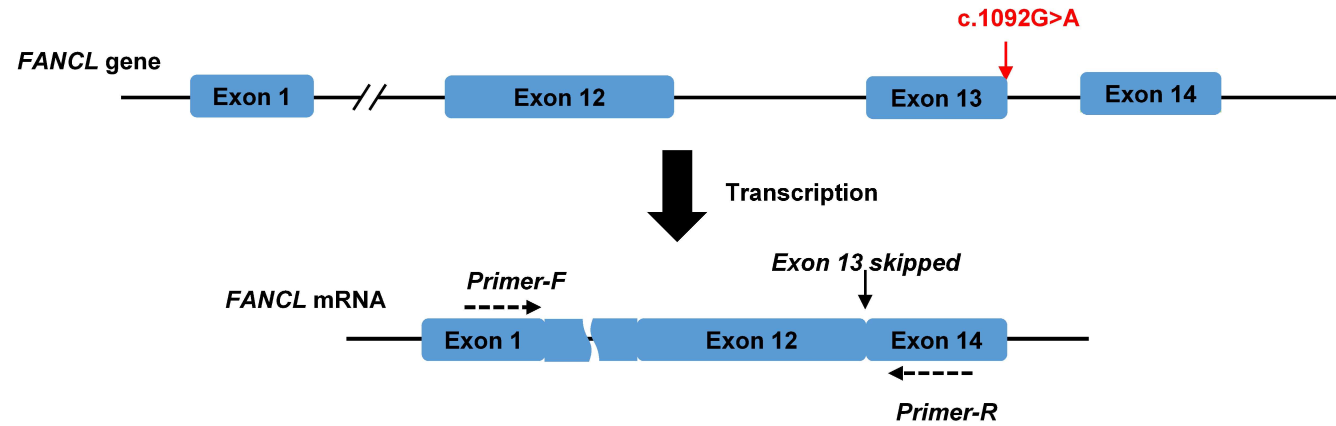
Figure S5

A



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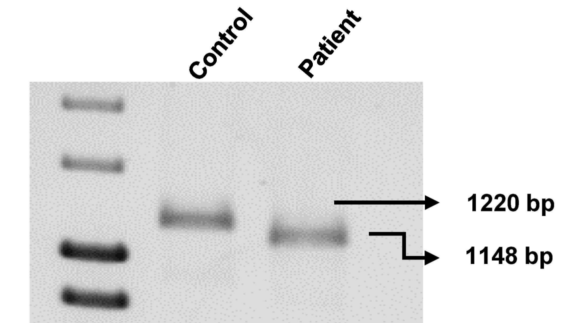

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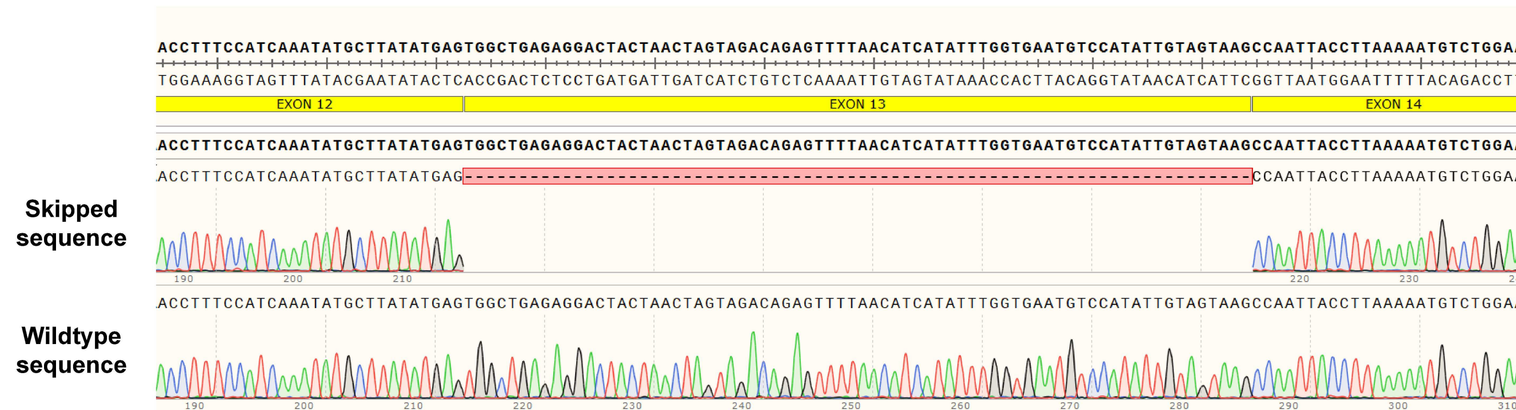

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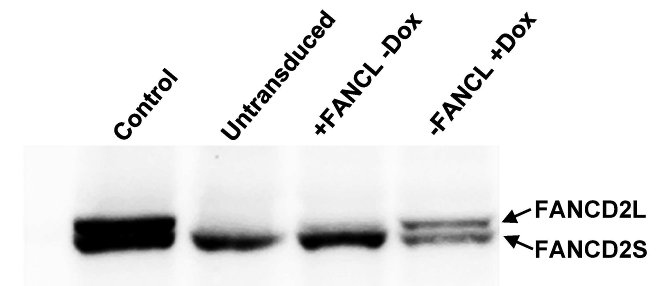

Figure S6

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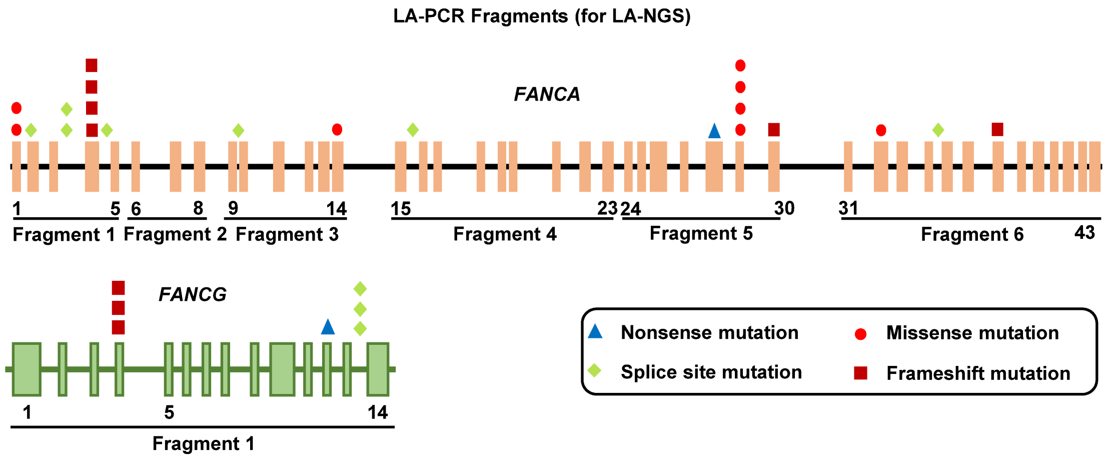

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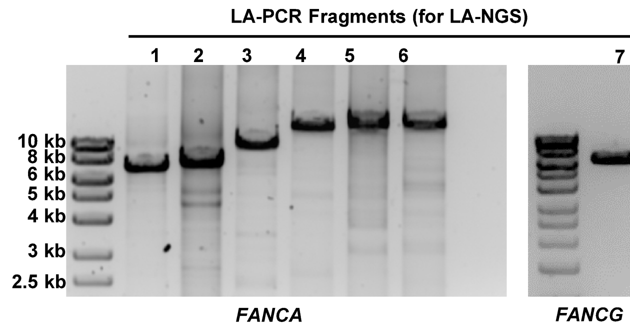

Figure S7

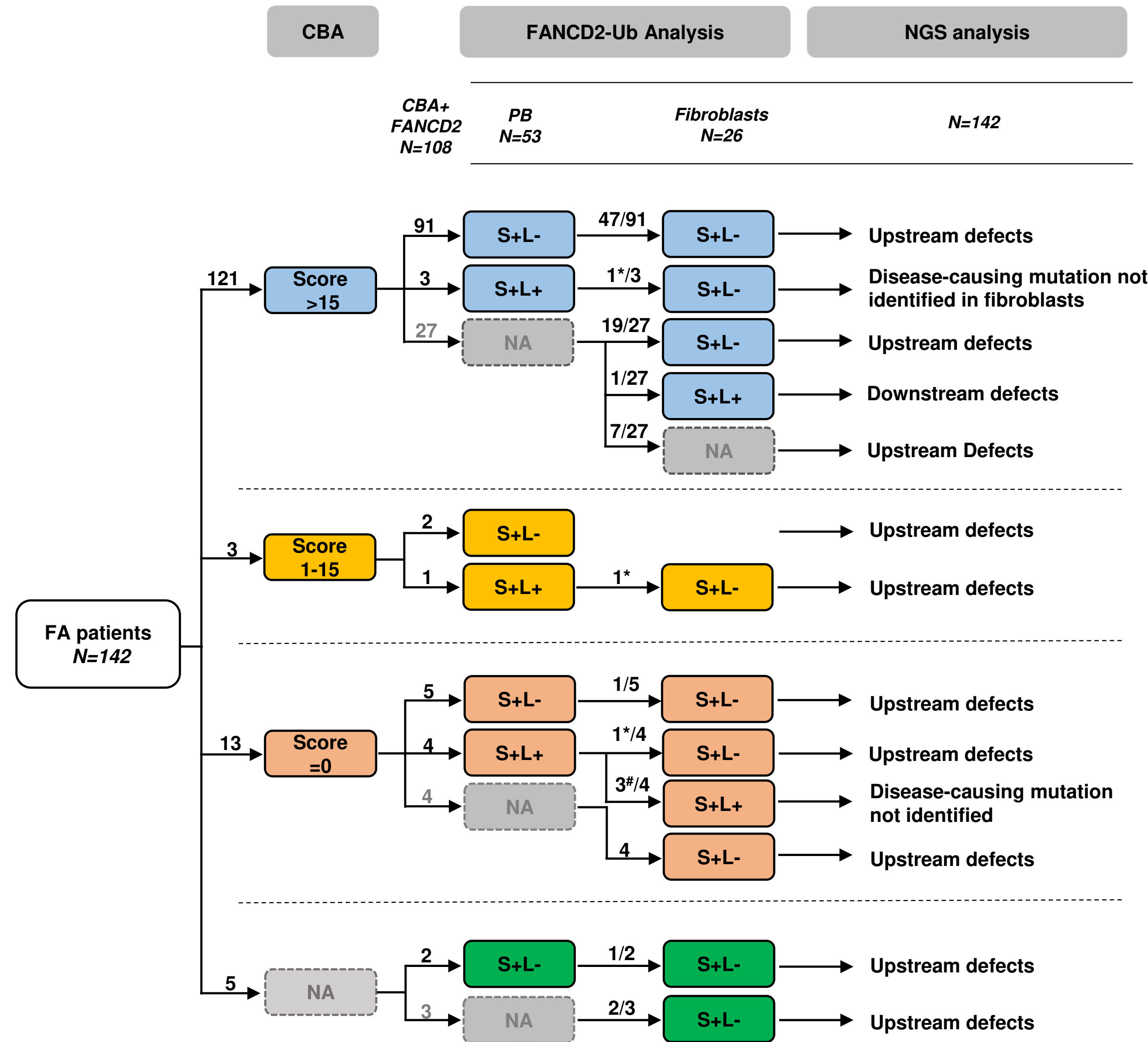

Table S2: FANCD2-Ub status, genotypes and clinical characteristics of the patients with very low CBA scores (0-10)

ID	CBA Score[#]	FANCD2 Ub		DNA change	Gene	Zygosity	Skeletal abnormalities	Facial dysmorphism	Skin changes	Other abnormalities
		PB	Fib							
FA-07	0	FANCD2-Ub-	NA	NC_000002.11:g.58387243C>T	*FANCL*	Homozygous	NA	MCH, frontal blossing	HP, HPOP	NA
FA-29	0	FANCD2-Ub+	FANCD2-Ub+	NC_000003.11:g.10076177A>C	*FANCD2*	Heterozygous	NA	NA	NA	HSK, kidney defects
FA-39	0	FANCD2-Ub+	FANCD2-Ub+	NC_000009.11:g.97873815G>A	*FANCC*	Heterozygous	Thenar hypoplasia	NA	HP	NA
FA-523*	0	FANCD2-Ub+	FANCD2-Ub-	NC_000002.11:g.58387243C>T	*FANCL*	Homozygous	NA	MCH	CAL, HP	NA
FA-525	0	NA	FANCD2-Ub-	NC_000002.11:g.58387243C>T	*FANCL*	Homozygous	Syndactyly, Thumb placed distally	NA	HP, HPOP	NA
FA-528	0	FANCD2-Ub+	FANCD2-Ub+	NC_000017.10:g.79516335G>T	*C17orf70*	Heterozygous	SS	MCH	NA	NA
FA-532	0	NA	FANCD2-Ub-	NC_000002.11:g.58387243C>T	*FANCL*	Homozygous	NA	DF	HP, HPOP	NA
FA-535	0	FANCD2-Ub-	NA	NC_000002.11:g.58387243C>T	*FANCL*	Homozygous	SS	MGH	HP	NA
FA-538	0	FANCD2-Ub-	NA	NC_000002.11:g.58387243C>T	*FANCL*	Homozygous	NA	MCH	CAL	NA
FA-541	0	NA	FANCD2-Ub-	NC_000006.11:g.35423588G>A	*FANCE*	Heterozygous	NA	NA	NA	NA
FA-561	0	FANCD2-Ub-	NA	NC_000016.9:g.89833551_89833647del	*FANCA*	Homozygous	NA	Hypertelorism	HP	NA
FA-554	4.1	FANCD2-Ub-	NA	NC_000016.9:g.89857810C>G NC_000016.9:g.89803957_89806507del	*FANCA*	Compound heterozygous	Bilateral clinodactyly	NA	CAL, HP, Pallor	NA
FA-650/18	4.1	FANCD2-Ub-	NA	NC_000009.11:g.35079204dup	*FANCG*	Homozygous	SS	NA	HP	NA
FA01/19*	8.2	FANCD2-Ub+	FANCD2-Ub-	NC_000002.11:g.58387243C>T	*FANCL*	Homozygous	NA	NA	CAL, HP	NA

SS: short stature; DF: dysmorphic face; HP: hyperpigmentation; MCH: microcephaly; HPOP: hypopigmentation; MGH: micrognathia; CAL: café au lait; HSK: horse shoe kidney.

* mosaicism detected by comparing the FANCD2-Ub analysis in the PB cells and fibroblasts.

[#] CBA score: chromosome breakage analysis score

Table S4: Heterozygous mutations in 6 FA patients

FA ID	Gene	Mutation	VarSome results	EVE results	Type of mutations	CBA score	FANCD2 ubiquitination		Physical anomalies
							T-cells	Fibroblasts	
FA-29	*FANCD2*	NC_000003.11:g.10076177A>C	Likely benign	Pathogenic	Missense	0	FANCD2-Ub+	FANCD2-Ub+	HSK, kidney defects
FA-518	*FANCC*	NC_000009.11:g.97873815G>A	Likely benign	Benign	Missense	0	FANCD2-Ub+	FANCD2-Ub+	Thenar hypoplasia, HP
FA-519	*FANCD2*	NC_000003.11:g.10083368C>T	Pathogenic	NA (nonsense variant)	Nonsense	62.7	FANCD2-Ub-	FANCD2-Ub-	SS, Absent simian crease, TH, TF, HP
FA-528	*C17orf70*	NC_000017.10:g.79516335G>T	Likely benign	NA	Missense	0	FANCD2-Ub+	FANCD2-Ub+	SS, MCH
FA-541	*FANCE*	NC_000006.11:g.35423588G>A	Likely benign	VUS	Missense	0	NA	FANCD2-Ub-	NA
FA-32/19	*FANCA*	NC_000016.9:g.89869731A>T	VUS	Pathogenic	Missense	22.1	FANCD2-Ub-	NA	Musculoskeletal abnormalities, Hypertelorism, MCH, HAP, CAL

HSK: horse shoe kidney; AE: abnormal eyes; MCH: microcephaly; HP: hyperpigmentation; SS: short stature; TH: thumb hypoplasia ;TF: triangular facies

Table S5: Mutations identified in *FANCA* and *FANCG* genes by LA-NGS

FA ID	Gene	Mutation	Type of mutations	Site of mutation
FA-31	*FANCA*	NC_000016.9:g.89882944C>G	Splice site	Intron 1
FA-521	*FANCA*	NC_000016.9:g.89877481T>G	Splice site	Intron 3
FA-500/ FA-05	*FANCA*	NC_000016.9:g.89857866C>T	Missense	Exon 14
FA-503/ FA-15	*FANCA*	NC_000016.9:g.89851261C>T	Splice site	Intron 15
FA-505/ FA-09	*FANCA*	NC_000016.9:g.89877340_89877341del	Splice site	Intron 4
		NC_000016.9:g.89877448del	Frameshift deletion	Exon 4
FA-515/ FA-03	*FANCA*	NC_000016.9:g.89813237A>C	Splice site	Intron 34
		NC_000016.9:g.89828423T>G	Missense	Exon 29
		NC_000016.9:g.89882965;C>T	Missense	Exon 1
FA-551/ FA-18	*FANCA*	NC_000016.9:g.89828357C>G	Missense	Exon 29
FA-555/ FA-516	*FANCA*	NC_000016.9:g.89825022del	Frameshift insertion	Exon 30
FA-568	*FANCA*	NC_000016.9:g.89828423T>G	Missense	Exon 29
FA-574/ FA-511	*FANCA*	NC_000016.9:g.89831327G>A	Nonsense	Exon 28
FA-20/19	*FANCA*	NC_000016.9:g.89877481T>G	Splice site	Intron 3
FA-27/19	*FANCA*	NC_000016.9:g.89866011A>G	Splice site	Intron 9
FA-36/19	*FANCG*	NC_000009.11:g.35074215T>G	Splice site	Intron 13
FA-37/19	*FANCG*	NC_000009.11:g.35074215T>G	Splice site	Intron 13
FA-573	*FANCA*	NC_000016.9:g.89877448del	Frameshift Deletion	Exon 4
		NC_000016.9:g.89883022A>T	Missense	Exon 1
FA-580	*FANCG*	NC_000009.11:g.35078222_35078223delinsAGCAGTT	Frameshift insertion deletion	Exon 4
FA-581	*FANCA*	NC_000016.9:g.89809216_89809219del	Frameshift Deletion	Exon 37
FA-584	*FANCA*	NC_000016.9:g.89877327_89877344del	Frameshift Deletion	Exon 4, Intron 4
FA-588	*FANCG*	NC_000009.11:g.35074215T>G	Splice site	Intron 13
FA-591	*FANCA*	NC_000016.9:g.89866011A>G	Splice site	Intron 9
FA-593	*FANCA*	NC_000016.9:g.89828358G>A	Missense	Exon 29
FA-598	*FANCG*	NC_000009.11:g.35075059G>A	Nonsense	Exon 12
FA-599	*FANCA*	NC_000016.9:g.89877327_89877344del	Frameshift Deletion	Exon 4, Intron 4
FA-622	*FANCA*	NC_000016.9:g.89816231_89816232del	Frameshift deletion	Exon 32

Table S6: List of primers used in this study

Purpose	Target Gene	Forward Primer (5'-3')	Reverse primer (5'-3')
Long amplicons for NGS	*FANCA*	TGGTCTCTTCAGGACCAACC	TGCGATAAGCCAAGATAGCA
		GATCTGGATGGAGGCAACAGAGT	CCCAACAAGAGATGACCGGATAC
		GGGCTTTGTTTGAGGAAGTCTGTT	CGAGAAGGTGAGCTTTCTGTACCA
		TACCCCTAAGGATCCCAAAAGGAT	AGAAGGCTCCATGCGTCTAA
		GCCTCGACTGGTCTAGAAGTCC	TGCCCAGGATCTACTAGGCCATTT
		AGGGGCAGGGTAAACAATGTGAGT	CCCTCAAGTACCACATGACCAAAC
	FANCG	GCTCGACAGTGAGCAGAGAAAGGAT	CTTTCACCCTGTACCCACACAGACA
Gene dosage PCR	*UBE2T*	TTGATAATCTACCAGAGGCT	GGCCAGTTTACTCCCAGACA
Primer for cDNA synthesis of *FANCL* gene	*FANCL*	-	TTAAGTTTCCAGCTCTTCAC
Primer for detection of mutation (c.1092G>A;p.K364=) in *FANCL*		CATCCCTTACTCTTGCAAAACA	TCACCTAGGAAATCTAGAAAAGGA
Amplification of *FANCL* cDNA		GTCTAGAGCTTTTCTGTGTT	TTAAGTTTCCAGCTCTTCAC
Primer for cDNA synthesis of *FANCF* gene	*FANCF*	-	ATACTTTGGACACACGAAGG
Gibson cloning of *FANCF* gene into pCW vector	*FANCF*	tggctagcGTAGGGCCTTCGCGCAC	ccggatccCATATATTTGGTGAGAACATTGTAATTTTCATTTTGTAAAC
Gibson cloning *FANCL* gene into pCW vector	*FANCL*	cagatcgcctggagaattggGTTTCTCCGGACTTCGAG	aaggcgcaaccccaaccccgCGAAATGTTGTATTCTTATTTCAGTG

Table S7: The genotypes of the FA patients selected for complementation analysis

ID	Gene	Zygosity	DNA change	Type of mutation	Varsome	EVE
FA-01	*FANCG*	Homozygous	NC_000009.11:g.35074215T>G	Splice site	Pathogenic	NA
FA-03	*FANCA*	Compound heterozygous	NC_000016.9:g.89813237A>C	Splice site	Pathogenic	NA
			NC_000016.9:g.89828423T>G	Missense	VUS	Pathogenic
FA-03/19	*FANCI*	Compound heterozygous	NC_000015.9:g.89804822del	Frameshift deletion	Pathogenic	NA
			NC_000015.9:g.89848828_89848832del	Intronic splice acceptor variant	VUS	NA
FA-04	*FANCA*	Compound heterozygous	NC_000016.9:g.89882396T>C	Splice site	Pathogenic	NA
			NC_000016.9:g.89811367_89865640del	Deletion	Pathogenic	NA
FA-06	*FANCA*	Homozygous	NC_000016.9:g.89828423T>G	Missense	Likely pathogenic	Pathogenic
FA-12	*FANCA*	Compound heterozygous	NC_000016.9:g.89809286_89809290dup	Frameshift insertion	Likely pathogenic	NA
			NC_000016.9:g.89857867G>A	Missense	Likely pathogenic	Pathogenic
FA-18	*FANCA*	Homozygous	NC_000016.9:g.89828357C>G	Missense	Likely pathogenic	Pathogenic
FA-24	*FANCA*	Compound heterozygous	NC_000016.9:g.89809302C>T	Nonsense	Pathogenic	NA
			NC_000016.9:g.89828358_89842224del	Deletion	Pathogenic	NA
FA-26	*FANCG*	Homozygous	NC_000009.11:g.35076427_35076431del	Splice site	VUS	NA
FA-30	*FANCF*	Homozygous	NC_000011.9:g.22646233T>C	Stop loss	VUS	NA
FA-31	*FANCA*	Homozygous	NC_000016.9:g.89882944C>G	Splice site	Pathogenic	NA
FA-36	*FANCG*	Homozygous	NC_000009.11:g.35075650_35075651del	Frameshift deletion	Pathogenic	NA
FA-38	*FANCG*	Compound heterozygous	NC_000009.11:g.35078223A>G	Missense	VUS	Pathogenic
			NC_000009.11:g.35076856_35076857del	Frameshift deletion	VUS	NA
FA-40	*FANCA*	Homozygous	NC_000016.9:g.89877448del	Frameshift deletion	Pathogenic	NA
FA-517	*FANCC*	Homozygous	NC_000009.11:g.97873912C>A	Nonsense	Pathogenic	NA
FA-527	*FANCC*	Homozygous	NC_000009.11:g.97864081T>G	Missense	VUS	Pathogenic
FA-531	*FANCG*	Homozygous	NC_000009.11:g.35076026C>G	Splice site	Pathogenic	NA
FA-533	*FANCA*	Compound heterozygous	NC_000016.9:g.89858399_89858400del	Frameshift deletion	VUS	NA
			NC_000016.9:g.89806402C>T	Missense	VUS	Pathogenic
FA-543	*FANCL*	Homozygous	NC_000002.11:g.58387243C>T	Exonic splice donor variant	Pathogenic	NA
FA-641/18	*FANCF*	Homozygous	NC_000011.9:g.22647316A>C	Missense	VUS	VUS

NA- not applicable
VUS- Variants of uncertain significance

Table S8: Calculation of sensitivity of CBA and FANCD2 Ub analysis with NGS as gold standard

CBA

	Disease			
Test	**Present**	**n**	**Absent**	**n**
Positive	True Positive	119	False positive	2
Negative	False Negative	13	True Negative	3
Sensitivity	91.50%			
Specificity	60%			

FANCD2 Ub analysis

	Disease			
Test	**Present**	**n**	**Absent**	**n**
Positive	True Positive	130	False positive	2
Negative	False Negative	2	True Negative	3
Sensitivity	97.00%			
Specificity	60%			

CBA and FANCD2 Ub analysis is compared to NGS

Table S1: Spectrum of congenital abnormalities in 142 patients

ID	Region	Gene	Gender	Diagnosis	Treatment	Consanguinity	Skeletal abnormalities	Facial dysmorphism	Skin changes	Other abnormalities	Relatives with FA
FA-01/ FA-536	Tamil Nadu	FANCG	M	NA	Danazol	Yes	NA	NA	HPOP,Leukoplakia	NA	NA
FA01/19	Kerala	FANCL	M	MDS	BMT	NA	NA	NA	CAL, HP	NA	NA
FA-02	Tamil Nadu	FANCJ	M	MDS	Danazol	No	NA	MCH	CAL	NA	NA
FA-03/ FA-515	Assam	FANCA	F	NA	Danazol	NA	TH, TA	DF	HP	NA	NA
FA-03/19	West Bengal	FANCI	M	NA	Stanozolol	No	SS	LSE, MOA, MCH, TF	HP, CAL	NA	Sister
FA-04/ FA-509	Kerala	FANCA	M	NA	Stanozolol	No	BT, Absent thumb	LSE	HP, HPOP, CAL	NA	NA
FA-05/ FA-500	Tamil Nadu	FANCA	M	NA	Stanozolol	Yes	NA	NA	HP	NA	NA
FA-06	West Bengal	FANCA	M	MDS	BMT	NA	SS, TH, Thenar hypoplasia	TF	HPOP, HP	NA	NA
FA-07	Andhra Pradesh	FANCL	M	NA	Stanozolol/BMT	NA	NA	MCH, frontal blossing	HP, HPOP	NA	NA
FA-08/ FA-552	Andhra Pradesh	FANCA	M	DKC	Stanozolol/BMT	Yes	NA	NA	HP, leukoplakia	NA	NA
FA-09/ FA-505	Kerala	FANCA	F	NA	Danazol/BMT	No	NA	NA	HPOP	NA	NA
FA-10	Tamil Nadu	FANCA	F	NA	Danazol	Yes	Clinodactyly	MCH, MGH, HAP	HP, POH	NA	NA
FA-11/ FA-524	Kerala	FANCA	M	NA	Stanozolol	No	SS, Arachinodactyly	DF	HP	NA	NA
FA-12	West Bengal	FANCA	F	NA	Danazol	No	NA	NA	HP, POH	NA	NA
FA-12/19	Kerala	FANCA	M	NA	Stanozolol	No	Hypothenar, clinodactyly, partial syndactyly	HAP	CAL	HSK	NA
FA-13	Kerala	FANCG	M	NA	Stanozolol	NA	NA	MCH,TF	HPOP, HP	HSK	NA
FA-14/ FA-534	Andhra Pradesh	FANCL	M	NA	Stanozolol	No	NA	NA	HP	NA	NA
FA-15 FA-503	Andhra Pradesh	FANCA	F	NA	Danazol/BMT	NA	SS	MCH	HP	ARP	Sister
FA-16	Andhra Pradesh	FANCA	F	NA	Danazol	Yes	SS	MGH	HP	NA	NA
FA-17/19	Tamil Nadu	FANCA	M	NA	Stanozolol	No	NA	MCH	CAL	NA	Brother
FA-18/ FA-551	Andhra Pradesh	FANCA	M	AML	Stanozolol	No	SS	LSE	HP	NA	Sister had AA
FA-18/19	Tamil Nadu	FANCA	M	NA	Stanozolol	No	BT, polydactyly	DF, EF, HAP	HP	NA	Brother
FA18/20	Kerala	FANCL	F	NA	Danazol/BMT	No	Nail dystrophy, Scoliosis	NA	HP, oral leukoplakia, CAL	NA	NA
FA-19	Karnataka	FANCL	M	NA	Stanozolol	NA	BT, SS	MOA, MCH	HP	NA	NA
FA-20	Tamil Nadu	FANCG	M	NA	Stanozolol	Yes	BT, Polydactyly	AE,MGH	HP	NA	NA
FA-20/19	Andhra Pradesh	FANCA	M	NA	Stanozolol	Yes	NA	DF	HP	Ectopic kidney	NA
FA-21/ FA-510	Jharkhand	FANCA	M	NA	Stanozolol	Yes	BT	AE	HP	NA	NA
FA-22	Kerala	FANCA	M	NA	Stanozolol/ BMT	Yes	Polydactyly	DF	Petechiae, POH	NA	NA
FA-23	Tamil Nadu	FANCA	F	NA	NA	No	Thumb Abnormalities	MCH	HPOP, HP	NA	NA
FA-24/ FA-502	Andhra Pradesh	FANCA	M	HCC	Stanozolol/ BMT	No	Thenar hypoplasia	NA	CAL, HP	NA	NA
FA-25	Andhra Pradesh	FANCL	M	NA	Stanozolol/ BMT	No	SS	NA	HP	Ectopic kidney	NA
FA-26/ FA-530	Tamil Nadu	FANCG	M	NA	Stanozolol	Yes	SS, Polydactyly, Clubbing	MCH	HPOP, HP	NA	NA
FA-27	Andhra Pradesh	FANCA	F	NA	Danazol	Yes	NA	NA	HP	NA	NA
FA-27/19	Tamil Nadu	FANCA	M	NA	Stanozolol	Yes	Triphalageal thumb	Hypertelorism, HAP	HP	NA	Sister, 1 death at birth
FA-28	Tamil Nadu	FANCA	F	NA	Danazol	Yes	SS	MCH	HP	NA	NA
FA-29	Chattisgarh	FANCD2	F	NA	BMT	Yes	NA	NA	NA	HSK	NA
FA-30/ FA-501	Tamil Nadu	FANCF	M	NA	Stanazolol	NA	Thenar hypolasia	LSE, MCH	HPOP, CAL, HP	NA	NA
FA-30/19	Andhra Pradesh	FANCL	F	NA	Danazol	Yes	NA	MCH	HP, HPOP	NA	NA
FA-31/ FA-514	Andhra Pradesh	FANCA	F	NA	Danazol	No	SS	HAP	HP	NA	NA
FA-32	Jharkhand	FANCA	M	NA	Stanozolol	No	SS	LSE	HP, HPOP	NA	NA
FA-33	Tamil Nadu	FANCA	M	NA	Stanozolol/ BMT	Yes	NA	NA	HP	NA	Sister with AA, brother died
FA-34	Tamil Nadu	FANCA	M	NA	Stanozolol/ BMT	Yes	SS	TF	CAL, HP	NA	NA
FA-35	Kerala	FANCA	M	NA	BMT	NA	TH, SD	Depressed nasal bridge, MCH	CAL	NA	NA
FA-36/ FA-526	Andhra Pradesh	FANCG	M	NA	Stanozolol	Yes	NA	TF, ASE	CAL, HP	NA	NA
FA-36/19	Tamil Nadu	FANCG	M	AML	Stanozolol	No	TH	HAP, small mouth and eyes, ASE	HP, CAL	Small kidneys	Sister
FA-37/19	Tamil Nadu	FANCG	F	MDS	Danazol	No	NA	HAP, small mouth and eyes, ASE	HP, CAL	NA	Brother
FA-38	Maharashtra	FANCG	F	NA	BMT	No	Subtle thumb abnormality	NA	HP	NA	NA
FA-39 /FA-518	Andhra Pradesh	FANCC	M	NA	Stanozolol	Yes	Thenar hypoplasia	NA	HP	NA	NA
FA-49/19	Andhra Pradesh	FANCL	M	DKC	NA	Yes	SS	NA	HP, HPOP, CAL	Hypoplastic kidneys	NA
FA-511	Andhra Pradesh	FANCA	M	NA	Stanozolol/ BMT	Yes	SST	NA	HP, CAL	NA	Sibling died of FA-AML
FA-513	Andhra Pradesh	FANCA	M	NA	Stanozolol	Yes	TH	LSE	HP, CAL	NA	NA
FA-516	West Bengal	FANCA	M	NA	Stanozolol	Yes	NA	LSE, MCH	CAL	NA	NA
FA-517	Kerala	FANCC	M	NA	Danazol/ BMT	Yes	TA, Ectrodactyly, Clinodactyly	HAP, MOA, MCH	CAL	NA	NA
FA-519	West Bengal	FANCD2	M	NA	Stanozolol	No	SS, Absent simian crease, TH	TF	HP	NA	NA
FA-521	Andhra Pradesh	FANCA	M	NA	Stanozolol	Yes	TH	AE	HP	NA	NA
FA-523	Kerala	FANCL	M	NA	Stanozolol	No	NA	MCH	CAL, HP	NA	NA
FA-525	Tamil Nadu	FANCL	M	NA	Stanozolol/ BMT	No	Syndactyly, DPT	NA	HP, HPOP	NA	NA
FA-527	Chattisgarh	FANCC	F	NA	Danazol/ BMT	No	SS, Clinodactyly	NA	HP	NA	NA
FA-528	Haryana	C17orf70	F	NA	BMT	No	SS	MCH	NA	NA	NA
FA-529	Tamil Nadu	FANCA	F	NA	Danazol	No	SS, BT	MCH	HP	NA	NA

FA-531	Karnataka	FANCG	M	NA	BMT	Yes	Clinodactyly	NA	HP, CAL	ARP	NA
FA-532	Andhra Pradesh	FANCL	F	NA	Danazol	Yes	NA	DF	HP, HPOP	NA	NA
FA-533	Maharashtra	FANCA	F	NA	Danazol/ BMT	No	NA	HAP, MGH	HP	NA	NA
FA-535	Andhra Pradesh	FANCL	M	NA	Stanozolol	Yes	SS	MGH	HP	NA	NA
FA-538	Andhra Pradesh	FANCL	M	NA	Stanozolol	NA	SS, TH	MCH, HAP	CAL	NA	NA
FA-543	Andhra Pradesh	FANCL	F	NA	Danazol	Yes	Nail dystrophy	HAP	HPOP	NA	NA
FA-544	Andhra Pradesh	FANCA	M	NA	Stanozolol	NA	Clinodactyly	LSE, HAP	HPOP, CAL, HP	NA	NA
FA-547	Karnataka	FANCA	F	NA	Danazol	Yes	Skeletal deformity	MOA ,DF, MGH	HPOP	NA	NA
FA-548	Kerala	FANCL	M	NA	Stanozolol	No	SS	MCH, DF	HP, HPOP	NA	NA
FA-549	Bihar	FANCA	M	NA	Stanozolol	No	BT, Polydactyly	NA	HP	NA	NA
FA-554	Kerala	FANCA	M	NA	Stanozolol	No	SS, Bilateral clinodactyly	NA	CAL, HP, Pallor	NA	Sister
FA-556	Karnataka	FANCA	M	NA	Stanozolol	No	NA	NA	HP	Kidney abnormalities	NA
FA-557	Kerala	FANCA	M	NA	Stanozolol/ BMT	No	NA	NA	HP, HPOP	NA	NA
FA-561	Tamil Nadu	FANCA	F	MDS	Danazol/ BMT	Yes	SS	Hypertelorism	HP	NA	NA
FA-564	Tamil Nadu	FANCL	M	NA	Stanozolol	Yes	SS	MOA	COP, CAL	NA	NA
FA-565	West Bengal	FANCA	M	MDS	Stanozolol	No	Clinodactyly	MCH, MOA	CAL, Pallor	NA	NA
FA-573	Kerala	FANCA	M	NA	Stanozolol/ Metformin	No	SS	NA	CAL	Hypogonadism	NA
FA-581	Andhra Pradesh	FANCA	M	NA	Stanozolol/ BMT	Yes	SS, TH	MCH, LSE, MOA	POH	NA	NA
FA-588	Kerala	FANCG	M	NA	Stanozolol/ BMT	No	SS, Short neck, thumb abnormalities	MCH, hypertelorism	POH, HP	NA	NA
FA-591	Tamil Nadu	FANCA	F	NA	Danazol	Yes	Thenar and hypothenar atrophy	NA	HP, CAL	NA	yes
FA-592	Andhra Pradesh	FANCA	F	NA	Danazol/ BMT	No	SS	NA	POH, CAL	NA	NA
FA-593	Tamil Nadu	FANCA	M	MDS	Stanozolol	Yes	TH (R), TA (L)	NA	HP	NA	NA
FA-595	Tamil Nadu	FANCL	M	NA	Stanozolol	No	NA	NA	CAL, HPOP	NA	Brother
FA-598	Karnataka	FANCG	M	NA	Stanozolol/ BMT	Yes	SS	NA	CAL	Atrophic left kidney	NA
FA-599	Tamil Nadu	FANCA	F	NA	Danazol	Yes	NA	Small and closely set eyes, HAP	CAL, HP	kidney abnormalities	NA
FA-601	Tamil Nadu	FANCL	M	NA	Danazol/ BMT	No	NA	Oral leukoplakia, MCH	HP, HPOP, POH	NA	Brother
FA-614	Andhra Pradesh	FANCA	M	NA	Stanozolol/ BMT	No	SS, clinodactyly	HAP	HPOP	NA	NA
FA-622	West Bengal	FANCA	F	NA	Danazol	No	NA	NA	HP	NA	NA
FA-629/18	Gujrat	FANCG	M	NA	NA	No	SS, TH	HAP, EF	NA	Absent kidney	NA
FA-631/18	Kerala	FANCA	F	MDS	Danazol	No	NA	DF	NA	NA	Sister died during chemotherapy
FA-636/18	Karnataka	FANCT	F	MDS/AML	NA	No	SS, congenital kyphoscoliosis, SD	MCH, TF	HP	Solitary kidney	NA
FA-637/18	Tamil Nadu	FANCJ	M	MDS	BMT	No	NA	NA	NA	Ectopic kidney	NA
FA-638/18	Andhra Pradesh	FANCL	M	NA	Stanozolol	No	Bilateral clinodactyly, SST	LSE, MOA	COP, HP, CAL	NA	NA
FA-641/18	Kerala	FANCF	M	MDS	BMT	No	Hypoplasia of upper and lower limbs	NA	HP, CAL	NA	NA
FA-650/18	Andhra Pradesh	FANCG	F	NA	Danazol	Yes	SS, TA	NA	HP	NA	NA
FA-652/18	West Bengal	FANCA	F	NA	Danazol	Yes	NA	DF	NA	NA	NA
FA-659/18	Tamil Nadu	FANCA	M	NA	Stanozolol/ BMT	Yes	congenital TH, CTEV	MCH, LSE	POH, CAL	Congenital acyanotic heart disease	NA
FA-660/18	Tamil Nadu	FANCA	F	NA	NA (Pregnant)	Yes	Polydactyly	HAP	HP	NA	NA
FA-665/18	Kerala	FANCL	F	NA	Danazol	Yes	SS, clinodactyly	MCH, LSE, HAP	CAL, COP	NA	NA
FA-672/18	Kerala	FANCA	M	NA	Danazol	No	BT	HAP	CAL	NA	NA
FA-674/18	Kerala	FANCA	M	NA	Stanozolol	No	TH	MCH	Patches over body	kidney abnormalities, ARP	NA
FA-675/18	Kerala	FANCL	F	NA	Stanozolol/ BMT	No	NA	MCH, EF	NA	NA	NA
O-117	Tamil Nadu	FANCA	F	NA	Danazol/ BMT	Yes	SS, emaciated	MCH, MOA, LSE, HAP, EF	POH, HPOP	NA	NA
O-123	Kerala	FANCL	F	NA	Danazol	No	BT	MCH	POH, HPOP, CAL	NA	NA
O-126	Andhra Pradesh	FANCA	M	MDS-AML	Stanozolol/ BMT	Yes	Simian crease in both hands	LSE, hypertelorism	HP	NA	NA
FA-40	Tamil Nadu	FANCA	M	NA	NA	Yes	NA	TF	CAL	kidney abnormalities	NA
FA-522	Kerala	FANCL	M	NA	NA	No	Thenar and hypothenar hypoplasia	NA	CAL, HP	NA	NA
FA-541	Tamil Nadu	FANCE	F	NA	BMT	Yes	NA	NA	NA	NA	NA
FA-542	NA	FANCA	F	NA	NA	NA	NA	NA	NA	NA	NA
FA-568	Andhra Pradesh	FANCA	M	MDS	BMT	No	NA	NA	NA	NA	Sibling died
FA-580	Bihar	FANCG	F	NA	Danazol	No	NA	NA	NA	Ectopic kidney	NA
FA-584	Tamil Nadu	FANCA	F	NA	Danazol	Yes	NA	NA	Pigmented tongue	NA	NA
FA-646/18	Andhra Pradesh	FANCA	F	NA	Danazol/ BMT	Yes	NA	NA	NA	Anorectal malformation	Sister died with congenital anomaly
FP-23-P-11	Andhra Pradesh	FANCA	M	NA	Danazol	No	NA	NA	NA	Anorectal malformation	NA
P-177	West Bengal	FANCA	F	NA	Danazol	No	NA	Epicanthus	NA	NA	NA
P-603	Andhra Pradesh	FANCL	M	NA	Stanozolol	Yes	Clinodactyly	LSE, HAP, depressed nasal bridge, MCH	Ichthyosis, CAL, HP	NA	NA
FA-04/20	Kerala	FANCA	F	NA	Stanozolol/ BMT	No	NA	NA	NA	NA	NA

FA-06/20	Telangana	FANCA	F	NA	Danazol	Yes	Polydactyly	HAP, EF	NA	NA	NA
FA-10/20	Andhra Pradesh	FANCA	F	NA	Danazol	Yes	NA	MCH, HAP	HP, CAL	NA	NA
FA-10/21	Andhra Pradesh	FANCA	F	NA	Danazol	Yes	Thenar and hypothenar hypoplasia	MCH, Small and closely set eyes, HAP	CAL	NA	NA
FA-11/20	West Bengal	FANCA	M	NA	Stanozolol	Yes	SS	MCH, TF, HAP	CAL	NA	NA
FA-15/20	Lakshadweep	FANCG	M	NA	Stanozolol	No	SS	NA	HP	ARP	NA
FA-16/20	Andhra Pradesh	FANCA	F	MDS	Danazol	No	NA	NA	NA	NA	NA
FA-21/20	Andhra Pradesh	FANCA	F	MDS	BMT	Yes	NA	MCH	HP	NA	NA
FA-5/21	West Bengal	FANCT	M	MDS	Danazol	No	NA	NA	NA	NA	NA
FA-01/20	Kerala	FANCG	M	NA	Stanozolol	Yes	Clinodactyly	MCH, MGH	NA	Left ectopic kidney	NA
FA-02/21	West Bengal	FANCA	M	NA	Stanozolol	No	SS	NA	NA	NA	NA
FA-08/19	Andhra Pradesh	FANCA	F	MDS	Danazol	Yes	SS, Polydactyly	NA	HP	ongenital acyanotic heart diseas	NA
FA-13/19	Kerala	FANCA	F	NA	Danazol	No	Polydactyly, Thumb anomalies	MCH, HAP	CAL	NA	NA
FA-18/21	Tamil Nadu	FANCA	M	MDS	Stanozolol	Yes	SS, TH	EF	HP	NA	NA
FA-21/21	Kerala	FANCA	F	NA	Danazol/BMT	No	NA	NA	HP	NA	NA
FA-23/19	Tamil Nadu	FANCA	M	NA	Stanozolol	Yes	SS	EF, HAP	NA	NA	NA
FA-26/21	Jharkhand	FANCA	M	NA	NA	Yes	Hypothenar wasting	NA	HP	Anorectal malformation	NA
FA-30/21	Tamil Nadu	UBE2T/FANCT	F	NA	NA	Yes	Polydactyly	NA	HP	HSK	NA
FA-31/21	Odisha	FANCA	F	NA	Danazol	NA	NA	EF, HAP	NA	NA	NA
FA-32/19	Kerala	FANCA	M	NA	Stanozolol	Yes	Musculoskeletal abnormalities	Hypertelorism, MCH, HAP	CAL	NA	NA
FA-33/19	Maharashtra	FANCL	M	NA	Danazol	No	NA	NA	HP, CAL	Small testes	NA
FA-33/21	Jharkhand	FANCA	M	NA	NA	Yes	NA	Fanconi facies	CAL	NA	NA
FA-34/19	Kerala	FANCL	M	NA	Stanozolol	No	NA	NA	HP	NA	NA
FA-35/21	Andhra Pradesh	FANCA	F	NA	Danazol	Yes	Thenar hypoplasia	NA	NA	NA	NA
FA-649/18	West Bengal	BRCA2	M	AML	NA	No	Thumb Abnormalities	MCH	HP, HPOP	Wilms tumor	Sibling died of FA-AML
FA-12/22	Andhra Pradesh	FANCL	F	NA	Danazol	Yes	Clinodactyly	TF, MCH	HP	Anorectal malformation	NA

Skeletal abnormalities: SS- Short stature; TH- Thumb hypoplasia; BT- Bifid thumb; SST- Short stubby toes; TA- Thumb aplasia; CTEV- Congenital talipes equinovarus; HSK- Horse shoe kidney; DPT- Distally placed thumb; ARP- absent radial pulse; SD- Sprengel's deformity

Facial Dysmorphism: DF- Dysmorphic Face; MCH- Microcephaly; MGH- Micrognathia; AE- Almond shaped eyes; Low set ears- LSE; High arched palate- HAP; Arched palate- AP; Elfin Facies- EF; TF- Triangular facies; Microopthalamia- MOA; ASE- Antemongoloid slant of eyes

Skin changes: HP- Hyperpigmentation; HPOP- Hypopigmentation; CAL- Café au lait; COP- Circum oral pigmentation; POH- Perioral hypopigmentation

Other anomalies: HSK- Horse-shoe kidney

Table S3. Disease associated genotypes identified by exome sequencing in 136 out of 142 FA patients

Sample ID	Gene	DNA change	Type of mutation	cDNA change	Amino acid change	dbSNP ID	Zygosity	Varsome Results	EVE Results
FA-01	*FANCG*	NC_000009.11:g.35074215T>G	3' splice site mutation	NM_004629.2:c.1761-2A>C	-	rs765150956	Homozygous	Pathogenic	NA
FA-02	*FANCJ (BRIP1)*	NC_000017.10:g.59857679T>A	Missense	NM_032043.3:c.1878A>T	p.Glu626Asp	rs1567812484	Homozygous	Pathogenic	Pathogenic
FA-03	*FANCA*	NC_000016.9:g.89813237A>C	5' splice site mutation	NM_000135.4:c.3408+2T>G	-	Novel	Compound heterozygous	VUS	NA
		NC_000016.9:g.89828423T>G	Missense	NM_000135.4:c.2786A>C	p.Tyr929Ser	Novel		VUS	Pathogenic
FA-04	*FANCA*	NC_000016.9:g.89882396T>C	3' splice site mutation	NM_000135.4:c.80-2A>G	-	Novel	Compound heterozygous	VUS	NA
		NC_000016.9:g.89811367_89865640del	Deletion	NM_000135.4:c.827_3626del	-	Novel		Likely pathogenic	NA
FA-05	*FANCA*	NC_000016.9:g.89857866C>T	Missense	NM_000135.4:c.1304G>A	p.Arg435His	rs1060501879	Homozygous	Pathogenic	Pathogenic
FA-06	*FANCA*	NC_000016.9:g.89828423T>G	Missense	NM_000135.4:c.2786A>C	p.Tyr929Ser	Novel	Homozygous	Likely pathogenic	Pathogenic
FA-07	*FANCL*	NC_000002.11:g.58387243C>T	Exonic splice donor variant	NM_001114636.1:c.1107G>A	p.Lys369(=)	rs577063114	Homozygous	Pathogenic	NA
FA-08	*FANCA*	NC_000016.9:g.89805365dup	Frameshift insertion	NM_000135.4:c.4185dupG	p.Ile1396AspfsTer29	Novel	Homozygous	Pathogenic	NA
FA-09	*FANCA*	NC_000016.9:g.89877340_89877341del	5' splice site mutation	NM_000135.4:c.426_426+1delAG	K143Rfs*7/5' splice variant	rs763114336	Compound heterozygous	VUS	NA
		NC_000016.9:g.89877448del	Frameshift deletion	NM_000135.4:c.319delG	p.Val107PhefsTer31	rs1411237340		VUS	NA
FA-10	*FANCA*	NC_000016.9:g.89809218_89809219del	Frameshift deletion	NM_000135.4:c.3761_3762delAG	p.Glu1254GlyfsTer23	rs868273545	Homozygous	Pathogenic	NA
FA-11	*FANCA*	NC_000016.9:g.89811367_89816310del	Deletion	NM_000135.4:c.3067_3626del	-	Novel	Homozygous	Pathogenic	NA
FA-12	*FANCA*	NC_000016.9:g.89809286_89809290dup	Frameshift insertion	NM_000135.4:c.3690_3694dupGCACT	p.Phe1232CysfsTer17	Novel	Compound heterozygous	Likely pathogenic	NA
		NC_000016.9:g.89857867G>A	Missense	NM_000135.4:c.1303C>T	p.Arg435Cys	rs148473140		Likely pathogenic	Pathogenic
FA-13	*FANCG*	NC_000009.11:g.35077267_35077273del	Frameshift deletion	NM_004629.2:c.637_643delTACCGCC	p.Tyr213LysfsTer6	rs587776640	Homozygous	Pathogenic	NA
FA-15	*FANCA*	NC_000016.9:g.89851261C>T	Exonic splice donor variant	NM_000135.4:c.1470+1G>A	p.Lys369Lys	rs1555556175	Homozygous	Pathogenic	NA
FA-16	*FANCA*	NC_000016.9:g.89833551_89833647del	Deletion	NM_000135.4:c.2505_2601del	p.Lys835SerfsTer22	George et al. 2021	Homozygous	Pathogenic	NA
FA-18	*FANCA*	NC_000016.9:g.89828357C>G	Missense	NM_000135.4:c.2852G>C	p.Arg951Pro	Novel	Homozygous	Likely pathogenic	Pathogenic
FA-19	*FANCL*	NC_000002.11:g.58387243C>T	Exonic splice donor variant	NM_001114636.1:c.1107G>A	p.Lys369(=)	rs577063114	Homozygous	Pathogenic	NA
FA-20	*FANCG*	NC_000009.11:g.35074215T>G	3' splice site mutation	NM_004629.2:c.1761-2A>C	-	rs765150956	Homozygous	Pathogenic	NA
FA-21	*FANCA*	NC_000016.9:g.89807212_89816310del	Deletion	NM_000135.4:c.3067_3828del	-	Novel	Homozygous	Pathogenic	NA
FA-22	*FANCA*	NC_000016.9:g.89866011A>G	5' splice site mutation	NM_000135.4:c.826+2T>C	-	Novel	Homozygous	Pathogenic	NA
FA-23	*FANCA*	NC_000016.9:g.89818545C>A	5' splice site mutation	NM_000135.4:c.3066+1G>T	-	rs587783028	Homozygous	Pathogenic	NA
FA-24	*FANCA*	NC_000016.9:g.89809302C>T	Nonsense	NM_000135.4:c.3671G>A	p.Trp1224Ter	Novel	Compound heterozygous	Likely pathogenic	NA
		NC_000016.9:g.89828358_89842224del	Deletion	NM_000135.4:c.1827_2852del	-	Castella et al. 2011		Likely pathogenic	NA
FA-25	*FANCL*	NC_000002.11:g.58387243C>T	Exonic splice donor variant	NM_001114636.1:c.1107G>A	p.Lys369(=)	rs577063114	Homozygous	Pathogenic	NA
FA-26	*FANCG*	NC_000009.11:g.35076427_35076431del	5' intronic splice donor variant (+2)	NM_004629.2:c.1076+3_1076+7delGAGGT	-	rs780410457	Homozygous	VUS	NA
FA-27	*FANCA*	NC_000016.9:g.89818545C>A	5' splice site mutation	NM_000135.4:c.3066+1G>T	-	rs587783028	Homozygous	Pathogenic	NA
FA-28	*FANCA*	NC_000016.9:g.89882944C>G	5' splice site mutation	NM_000135.4:c.79+1G>C	-	rs1483028018	Homozygous	Pathogenic	NA
FA-30	*FANCF*	NC_000011.9:g.22646233T>C	Stop loss	NM_022725.4:c.1124A>G	p.Ter375TrpextTer33	Novel	Homozygous	VUS	NA
FA-31	*FANCA*	NC_000016.9:g.89882944C>G	5' splice site mutation	NM_000135.4:c.79+1G>C	-	rs1483028018	Homozygous	Pathogenic	NA
FA-32	*FANCA*	NC_000016.9:g.89824984C>G	5' splice site mutation	NM_000135.4:c.2981+1G>C	-	Novel	Homozygous	Pathogenic	NA
FA-33	*FANCA*	NC_000016.9:g.89813247dup	Frameshift insertion	NM_000135.4:c.3401dupT	p.Phe1135LeufsTer80	Novel	Homozygous	Pathogenic	NA
FA-34	*FANCA*	NC_000016.9:g.89818545C>A	5' splice site mutation	NM_000135.4:c.3066+1G>T	-	rs587783028	Homozygous	Pathogenic	NA
FA-35	*FANCA*	NC_000016.9:g.89806416del	Frameshift deletion	NM_000135.4:c.3920delA	p.Gln1307ArgfsTer2	rs1228394297	Homozygous	Pathogenic	NA
FA-36	*FANCG*	NC_000009.11:g.35075650_35075651del	Frameshift deletion	NM_004629.2:c.1246_1247delCT	p.Leu416MetfsTer2	Novel	Homozygous	Pathogenic	NA
FA-38	*FANCG*	NC_000009.11:g.35078223A>G	Missense	NM_004629.2:c.425T>C	p.Leu142Pro	Novel	Compound heterozygous	VUS	Pathogenic
		NC_000009.11:g.35076856_35076857del	Frameshift deletion	NM_004629.2:c.792_793delAG	p.Arg264SerfsTer24	Novel		VUS	NA
FA-40	*FANCA*	NC_000016.9:g.89877448del	Frameshift deletion	NM_000135.4:c.319delG	p.Val107PhefsTer31	rs1411237340	Homozygous	Pathogenic	NA
FA-511	*FANCA*	NC_000016.9:g.89831327G>A	Nonsense	NM_000135.4:c.2749C>T	p.Arg917Ter	rs1060501880	Homozygous	Pathogenic	NA
FA-513	*FANCA*	NC_000016.9:g.89880929_89883055del	Deletion	NM_000135.4:c.-31_283del	-	Savoia et al. 1996	Homozygous	Pathogenic	NA
FA-516	*FANCA*	NC_000016.9:g.89825022del	Frameshift deletion	NM_000135.4:c.2944delA	p.Thr982ProfsTer7	Novel	Homozygous	Pathogenic	NA
FA-517	*FANCC*	NC_000009.11:g.97873912C>A	Nonsense	NM_000136.3:c.1162G>T	p.Gly388Ter	rs371897078	Homozygous	Pathogenic	NA
FA-521	*FANCA*	NC_000016.9:g.89877481T>G	3' splice site mutation	NM_000135.4:c.284-2A>C	-	rs756023006	Homozygous	Pathogenic	NA
FA-522	*FANCL*	NC_000002.11:g.58387243C>T	Exonic splice donor variant	NM_001114636.1:c.1107G>A	p.Lys369(=)	rs577063114	Homozygous	Pathogenic	NA
FA-523	*FANCL*	NC_000002.11:g.58387243C>T	Exonic splice donor variant	NM_001114636.1:c.1107G>A	p.Lys369(=)	rs577063114	Homozygous	Pathogenic	NA
FA-525	*FANCL*	NC_000002.11:g.58387243C>T	Exonic splice donor variant	NM_001114636.1:c.1107G>A	p.Lys369(=)	rs577063114	Homozygous	Pathogenic	NA
FA-527	*FANCC*	NC_000009.11:g.97864081T>G	Missense	NM_000136.3:c.1585A>C	p.Thr529Pro	rs587778326	Homozygous	VUS	Pathogenic
FA-529	*FANCA*	NC_000016.9:g.89833551_89833647del	Deletion	NM_000135.4:c.2505_2601del	p.Lys835SerfsTer22	George et al. 2021	Homozygous	Pathogenic	NA
FA-531	*FANCG*	NC_000009.11:g.35076026C>G	3' splice site mutation	NM_004629.2:c.1077-1G>C	-	Novel	Homozygous	Pathogenic	NA
FA-532	*FANCL*	NC_000002.11:g.58387243C>T	Exonic splice donor variant	NM_001114636.1:c.1107G>A	p.Lys369(=)	rs577063114	Homozygous	Pathogenic	NA
FA-533	*FANCA*	NC_000016.9:g.89858399_89858400del	Frameshift deletion	NM_000135.4:c.1164_1165delAG	p.Arg388SerfsTer20	Novel	Compound heterozygous	VUS	NA
		NC_000016.9:g.89806402C>T	Missense	NM_000135.4:c.3934G>A	p.Asp1312Asn	Novel		VUS	Pathogenic
FA-534	*FANCL*	NC_000002.11:g.58387243C>T	Exonic splice donor variant	NM_001114636.1:c.1107G>A	p.Lys369(=)	rs577063114	Homozygous	Pathogenic	NA
FA-535	*FANCL*	NC_000002.11:g.58387243C>T	Exonic splice donor variant	NM_001114636.1:c.1107G>A	p.Lys369(=)	rs577063114	Homozygous	Pathogenic	NA
FA-538	*FANCL*	NC_000002.11:g.58387243C>T	Exonic splice donor variant	NM_001114636.1:c.1107G>A	p.Lys369(=)	rs577063114	Homozygous	Pathogenic	NA
FA-542	*FANCA*	NC_000016.9:g.89866011A>G	5' splice site mutation	NM_000135.4:c.826+2T>C	-	Novel	Homozygous	Pathogenic	NA
FA-543	*FANCL*	NC_000002.11:g.58387243C>T	Exonic splice donor variant	NM_001114636.1:c.1107G>A	p.Lys369(=)	rs577063114	Homozygous	Pathogenic	NA
FA-544	*FANCA*	NC_000016.9:g.89858878C>T	5' splice site mutation	NM_000135.4:c.1083+1G>A	-	Novel	Homozygous	Pathogenic	NA
FA-547	*FANCA*	NC_000016.9:g.89845356G>A	Nonsense	NM_000135.4:c.1771C>T	p.Arg591Ter	rs753980264	Homozygous	Pathogenic	NA
FA-548	*FANCL*	NC_000002.11:g.58387243C>T	Exonic splice donor variant	NM_001114636.1:c.1107G>A	p.Lys369(=)	rs577063114	Homozygous	Pathogenic	NA
FA-549	*FANCA*	NC_000016.9:g.89828423T>G	Missense	NM_000135.4:c.2786A>C	p.Tyr929Ser	Novel	Homozygous	Likely pathogenic	Pathogenic
FA-554	*FANCA*	NC_000016.9:g.89857810C>G	5’ Splice site mutation	NM_000135.4:c.1359+1G>C	-	rs1555561294	Compound heterozygous	Likely pathogenic	NA
		NC_000016.9:g.89803957_89806507del	Deletion	NM_000135.4:c.3829_*1052del	-	Novel		Likely pathogenic	NA
FA-556	*FANCA*	NC_000016.9:g.89806405del	Frameshift deletion	NM_000135.4:c.3931delA	p.Ser1311ValfsTer52	Novel	Homozygous	Pathogenic	NA
FA-557	*FANCA*	NC_000016.9:g.89882943A>G	5' splice site mutation	NM_000135.4:c.79+2T>C	-	rs1319795682	Homozygous	Pathogenic	NA

FA-561	*FANCA*	NC_000016.9:g.89833551_89833647del	Deletion	NM_000135.4:c.2505_2601del	p.Lys835SerfsTer22	George et al. 2021	Homozygous	Pathogenic	NA
FA-564	*FANCL*	NC_000002.11:g.58387243C>T	Exonic splice donor variant	NM_001114636.1:c.1107G>A	p.Lys369(=)	rs577063114	Homozygous	Pathogenic	NA
FA-565	*FANCA*	NC_000016.9:g.89883009C>T	Nonsense	NM_000135.4:c.15G>A	p.Trp5Ter	Novel	Homozygous	Pathogenic	NA
FA-568	*FANCA*	NC_000016.9:g.89828423T>G	Missense	NM_000135.4:c.2786A>C	p.Tyr929Ser	Novel	Homozygous	Likely pathogenic	Pathogenic
FA-629/18	*FANCG*	NC_000009.11:g.35075954C>G	5' intronic splice donor variant (+5)*	NM_004629.2:c.1143+5G>C	-	rs778328620	Homozygous	Pathogenic	NA
FA-631/18	*FANCA*	NC_000016.9:g.89831438G>A NC_000016.9:g.89811367_89816310del	Nonsense Deletion	NM_000135.4:c.2638C>T NM_000135.4:c.3067_3626del	p.Arg880Ter -	rs762804216 Novel	Compound heterozygous	Pathogenic Pathogenic	NA NA
FA-636/18	*UBE2T/FANCT*	NC_000001.10:g.202300964_202301088del	Deletion	NM_014176.4:c.470_594del	p.Ala157GlyfsTer18	Novel	Homozygous	Pathogenic	NA
FA-637/18	*FANCJ (BRIP1)*	NC_000017.10:g.59885995G>A	Missense	NM_032043.3:c.751C>T	p.Arg251Cys	rs752309409	Homozygous	Likely pathogenic	Pathogenic
FA-638/18	*FANCL*	NC_000002.11:g.58387243C>T	Exonic splice donor variant	NM_001114636.1:c.1107G>A	p.Lys369(=)	rs577063114	Homozygous	Pathogenic	NA
FA-641/18	*FANCF*	NC_000011.9:g.22647316A>C	Missense	NM_022725.4:c.41T>G	p.Leu14Arg	Novel	Homozygous	VUS	VUS
FA-646/18	*FANCA*	NC_000016.9:g.89828357C>T	Missense	NM_000135.4:c.2852G>A	p.Arg951Gln	rs755922289	Homozygous	Likely pathogenic	Pathogenic
FA-650/18	*FANCG*	NC_000009.11:g.35079204dup	Frameshift insertion	NM_004629.2:c.119dupA	p.Gln41AlafsTer16	Novel	Homozygous	Pathogenic	NA
FA-652/18	*FANCA*	NC_000016.9:g.89816214G>A	Missense	NM_000135.4:c.3163C>T	p.Arg1055Trp	rs753063086	Homozygous	Likely pathogenic	Pathogenic
FA-659/18	*FANCA*	NC_000016.9:g.89809284A>G	Missense	NM_000135.4:c.3689T>C	p.Leu1230Pro	Novel	Homozygous	VUS	Pathogenic
FA-660/18	*FANCA*	NC_000016.9:g.89865641C>T	3' splice site mutation	NM_000135.4:c.827-1G>A	-	rs753728435	Homozygous	Pathogenic	NA
FA-665/18	*FANCL*	NC_000002.11:g.58387243C>T	Exonic splice donor variant	NM_001114636.1:c.1107G>A	p.Lys369(=)	rs577063114	Homozygous	Pathogenic	NA
FA-672/18	*FANCA*	NC_000016.9:g.89811367_89816310del	Deletion	NM_000135.4:c.3067_3626del	-	Novel	Homozygous	Pathogenic	NA
FA-674/18	*FANCA*	NC_000016.9:g.89816214G>A NC_000016.9:g.89811367_89865640del	Missense Deletion	NM_000135.4:c.3163C>T NM_000135.4:c.827_3626del	p.Arg1055Trp -	rs753063086 Novel	Compound heterozygous	Pathogenic Pathogenic	Pathogenic NA
FA-675/18	*FANCL*	NC_000002.11:g.58387243C>T NC_000002.11:g.58388695G>A	Exonic splice donor variant Nonsense	NM_001114636.1:c.1107G>A NM_001114636.1:c.997C>T	p.Lys369(=) p.Gln333Ter	rs577063114 rs776298788	Compound heterozygous	Pathogenic Pathogenic	NA NA
FA18/20	*FANCL*	NC_000002.11:g.58387243C>T	Exonic splice donor variant	NM_001114636.1:c.1107G>A	p.Lys369(=)	rs577063114	Homozygous	Pathogenic	NA
FA01/19	*FANCL*	NC_000002.11:g.58387243C>T	Exonic splice donor variant	NM_001114636.1:c.1107G>A	p.Lys369(=)	rs577063114	Homozygous	Pathogenic	NA
FP-23-P-11	*FANCA*	NC_000016.9:g.89828423T>G	Missense	NM_000135.4:c.2786A>C	p.Tyr929Ser	Novel	Homozygous	VUS	Pathogenic
FA-17/19	*FANCA*	NC_000016.9:g.89871689_89871801del	Deletion	NM_000135.4:c.597_709del	p.Ser199GlyfsTer24	Esmail nia et al. 2016	Homozygous	Pathogenic	NA
FA-18/19	*FANCA*	NC_000016.9:g.89871689_89871801del	Deletion	NM_000135.4:c.597_709del	p.Ser199GlyfsTer24	Esmail nia et al. 2016	Homozygous	Pathogenic	NA
FA-20/19	*FANCA*	NC_000016.9:g.89877481T>G	3' splice site mutation	NM_000135.4:c.284-2A>C	-	rs756023006	Homozygous	Pathogenic	NA
FA-27/19	*FANCA*	NC_000016.9:g.89866011A>G	5' splice site mutation	NM_000135.4:c.826+2T>C	-	Novel	Homozygous	Pathogenic	NA
FA-30/19	*FANCL*	NC_000002.11:g.58387243C>T	Exonic splice donor variant	NM_001114636.1:c.1107G>A	p.Lys369(=)	rs577063114	Homozygous	Likely pathogenic	NA
FA-36/19	*FANCG*	NC_000009.11:g.35074215T>G	3' splice site mutation	NM_004629.2:c.1761-2A>C	-	rs765150956	Homozygous	Pathogenic	NA
FA-37/19	*FANCG*	NC_000009.11:g.35074215T>G	3' splice site mutation	NM_004629.2:c.1761-2A>C	-	rs765150956	Homozygous	Pathogenic	NA
FA-49/19	*FANCL*	NC_000002.11:g.58387243C>T	Exonic splice donor variant	NM_001114636.1:c.1107G>A	p.Lys369(=)	rs577063114	Homozygous	Pathogenic	NA
FA-573	*FANCA*	NC_000016.9:g.89877448del NC_000016.9:g.89883022A>T	Frameshift deletion Missense	NM_000135.4:c.319delG NM_000135.4:c.2T>A	p.Val107PhefsTer31 p.Met1Lys	rs1411237340 rs769479800	Compound heterozygous	Pathogenic Pathogenic	NA NA
FA-580	*FANCG*	NC_000009.11:g.35078222_35078223delinsAGCAGT	Frameshift insertion	NM_004629.2:c.425_426delTGinsAACTGCT	p.Leu142GlnfsTer12	Novel	Homozygous	Pathogenic	NA
FA-581	*FANCA*	NC_000016.9:g.89809216_89809219del	Frameshift deletion	NM_000135.4:c.3759_3762del	p.Glu1254SerfsTer11	Novel	Homozygous	Pathogenic	NA
FA-584	*FANCA*	NC_000016.9:g.89877327_89877344del	Frameshift deletion	NM_000135.4:c.423_426+14del	-	Novel	Homozygous	Pathogenic	NA
FA-588	*FANCG*	NC_000009.11:g.35074215T>G	3' splice site mutation	NM_004629.2:c.1761-2A>C	-	rs765150956	Homozygous	Pathogenic	NA
FA-591	*FANCA*	NC_000016.9:g.89866011A>G	5' splice site mutation	NM_000135.4:c.826+2T>C	-	Novel	Homozygous	Pathogenic	NA
FA-595	*FANCL*	NC_000002.11:g.58387243C>T	Exonic splice donor variant	NM_001114636.1:c.1107G>A	p.Lys369(=)	rs577063114	Homozygous	Pathogenic	NA
FA-598	*FANCG*	NC_000009.11:g.35075059G>A	Nonsense	NM_004629.2:c.1501C>T	p.Gln501Ter	Novel	Homozygous	Pathogenic	NA
FA-599	*FANCA*	NC_000016.9:g.89877327_89877344del	Frameshift deletion	NM_000135.4:c.423_426+14del	-	Novel	Homozygous	Pathogenic	NA
FA-601	*FANCL*	NC_000002.11:g.58387243C>T	Exonic splice donor variant	NM_001114636.1:c.1107G>A	p.Lys369(=)	rs577063114	Homozygous	Pathogenic	NA
FA-614	*FANCA*	NC_000016.9:g.89849270_89849510del	Deletion	NM_000135.4:c.1472_1624del	-	Neil V morgan et al. 1999	Homozygous	Pathogenic	NA
O-117	*FANCA*	NC_000016.9:g.89874703_89877479del	Deletion	NM_000135.4:c.285_596del	-	Savoia et al. 1996	Homozygous	Pathogenic	NA
O-123	*FANCL*	NC_000002.11:g.58387243C>T	Exonic splice donor variant	NM_001114636.1:c.1107G>A	p.Lys369(=)	rs577063114	Homozygous	Pathogenic	NA
O-126	*FANCA*	NC_000016.9:g.89862316_89862426del	Deletion	NM_000135.4:c.894_1004del	p.Trp298_Lys335delinsGly	Solanki et al. 2016	Homozygous	Pathogenic	NA
P-177	*FANCA*	NC_000016.9:g.89813297C>G	Missense	NM_000135.4:c.3350G>C	p.Arg1117Thr	Novel	Homozygous	Likely pathogenic	Pathogenic
FA-12/19	*FANCA*	NC_000016.9:g.89877448del NC_000016.9:g.89882396T>C	Frameshift deletion 3' splice site mutation	NM_000135.4:c.319delG NM_000135.4:c.80-2A>G	p.Val107PhefsTer31 -	rs1411237340 Novel	Compound heterozygous	Pathogenic Pathogenic	NA NA
FA-622	*FANCA*	NC_000016.9:g.89818552_89825113del NC_000016.9:g.89816231_89816232del	Deletion Frameshift deletion	NM_000135.4:c.2853_3060del NM_000135.4:c.3146_3147delTT	- p.Phe1049Ter	Novel Novel	Compound heterozygous	Pathogenic Pathogenic	NA NA
FA-593	*FANCA*	NC_000016.9:g.89828358G>A	Missense	NM_000135.4:c.2851C>T	p.Arg951Trp	rs755546887	Homozygous	Likely pathogenic	Pathogenic
FA-592	*FANCA*	NC_000016.9:g.89845258_89882945del NC_000016.9:g.89833551_89833647del	Deletion Deletion	NM_000135.4:c.79_1777del NM_000135.4:c.2505_2601del	- p.Lys835SerfsTer22	Castella et al. 2011 George et al. 2021	Compound heterozygous	Pathogenic Pathogenic	NA NA
FA-03/19	*FANCI*	NC_000015.9:g.89804822del NC_000015.9:g.89848828_89848832del	Frameshift deletion Intronic splice acceptor variant	NM_001113378.2:c.295delC NM_001113378.2:c.3256-8_3256-4delTCTTT	p.His99IlefsTer10 -	rs759398314 Novel	Compound heterozygous	Pathogenic VUS	NA NA
P-603	*FANCL*	NC_000002.11:g.58387243C>T	Exonic splice donor variant	NM_001114636.1:c.1107G>A	p.Lys369(=)	rs577063114	Homozygous	Pathogenic	NA
FA-04/20	*FANCA*	NC_000016.9:g.89877448del	Frameshift deletion	NM_000135.4:c.319del	p.Val107PhefsTer31	rs1411237340	Homozygous	Pathogenic	NA
FA-06/20	*FANCA*	NC_000016.9:g.89805352G>A	Missense	NM_000135.4:c.4198C>T	p.Arg1400Cys	rs745882980	Homozygous	Pathogenic	Pathogenic
FA-10/20	*FANCA*	NC_000016.9:g.89805352G>A	Missense	NM_000135.4:c.4198C>T	p.Arg1400Cys	rs745882980	Homozygous	Pathogenic	Pathogenic
FA-10/21	*FANCA*	NC_000016.9:g.89809216_89809219del	Frameshift deletion	NM_000135.4:c.3759_3762del	p.Glu1254SerfsTer11	Novel	Homozygous	Pathogenic	NA
FA-11/20	*FANCA*	NC_000016.9:g.89806411_89806427dup	Frameshift insertion	NM_000135.4:c.3909_3925dup	p.Thr1309ArgfsTer6	Novel	Homozygous	Pathogenic	NA
FA-15/20	*FANCG*	NC_000009.11:g.35077289del	Frameshift deletion	NM_004629.2:c.619del	p.Leu207SerfsTer2	Novel	Homozygous	Pathogenic	NA
FA-16/20	*FANCA*	NC_000016.9:g.89818545C>A	5' splice site mutation	NM_000135.4:c.3066+1G>T	-	rs587783028	Homozygous	Pathogenic	NA
FA-21/20	*FANCA*	NC_000016.9:g.89805660C>A	Nonsense	NM_000135.4:c.4048G>T	p.Glu1350Ter	Novel	Homozygous	Pathogenic	NA
FA-5/21	*UBE2T/FANCT*	NC_000001.10:g.202302631T>G	Missense	NM_014176.4:c.232A>C	p.Asn78His	rs776219033	Homozygous	VUS	Pathogenic
FA-01/20	*FANCG*	NC_000009.11:g.35074476dup	Nonsense	NM_004629.2:c.1652dup	p.Tyr551Ter	Novel	Homozygous	Pathogenic	Pathogenic
FA-02/21	*FANCA*	NC_000016.9:g.89807252A>G NC_000016.9:g.89849441C>T	Missense Missense	NM_000135.4:c.3788T>C NM_000135.4:c.1540G>A	p.Phe1263Ser p.Ala514Thr	George et al. 2021 Novel	Compound heterozygous	Likely pathogenic VUS	VUS Pathogenic

FA-08/19	*FANCA*	NC_000016.9:g.89833551_89833647del	Deletion	NM_000135.4:c.2505_2601del	p.Lys835SerfsTer22	George et al. 2021	Homozygous	Pathogenic	NA
FA-13/19	*FANCA*	NC_000016.9:g.89831446G>C	Nonsense	NM_000135.4:c.2630C>G	p.Ser877Ter	Solanki et al. 2016	Homozygous	Likely pathogenic	NA
FA-18/21	*FANCA*	NC_000016.9:g.89836600G>A	Missense	NM_000135.4:c.2290C>T	p.Arg764Trp	De Rocco et al. 2014	Homozygous	Pathogenic	Pathogenic
FA-21/21	*FANCA*	NC_000016.9:g.89816138C>T	Missense	NM_000135.4:c.3239G>A	p.Arg1080Gln	rs1555538571	Homozygous	Likely pathogenic	Pathogenic
FA-23/19	*FANCA*	NC_000016.9:g.89836973T>G	Exonic splice donor variant	NM_000135.4:c.2221A>C	p.Arg741=	Novel	Homozygous	Likely pathogenic	NA
FA-26/21	*FANCA*	NC_000016.9:g.89809211_89809212insCT	Frameshift insertion	NM_000135.4:c.3761_3762insAG	p.Glu1255GlyfsTer12	rs868273545	Homozygous	Pathogenic	NA
FA-30/21	*UBE2T/FANCT*	NC_000001.10:g.202300964_202301088del	Deletion	NM_014176.4:c.470_594del	p.Ala157GlyfsTer18	Novel	Homozygous	Pathogenic	NA
FA-31/21	*FANCA*	NC_000016.9:g.89851302A>G	Missense	NM_000135.4:c.1430T>C	p.Leu477Ser	Novel	Homozygous	Pathogenic	Pathogenic
FA-33/19	*FANCL*	NC_000002.11:g.58387243C>T	Exonic splice donor variant	NM_001114636.1:c.1107G>A	p.Lys369(=)	rs577063114	Homozygous	Pathogenic	NA
FA-33/21	*FANCA*	NC_000016.9:g.89809211_89809212insCT	Frameshift insertion	NM_000135.4:c.3761_3762insAG	p.Glu1255GlyfsTer12	rs868273545	Homozygous	Pathogenic	NA
FA-34/19	*FANCL*	NC_000002.11:g.58387243C>T	Exonic splice donor variant	NM_001114636.1:c.1107G>A	p.Lys369(=)	rs577063114	Homozygous	Pathogenic	NA
FA-35/21	*FANCA*	NC_000016.9:g.89805365dup	Frameshift insertion	NM_000135.4:c.4185dup	p.Ile1396AspfsTer29	Novel	Homozygous	Pathogenic	NA
FA-649/18	*BRCA2*	NC_000013.10:g.32893238G>C	Missense	NM_000059.4:c.92G>C	p.Trp31Ser	Caleca et al. 2018	Homozygous	Likely pathogenic	NA
FA-12/22	*FANCL*	NC_000002.11:g.58387243C>T	Exonic splice donor variant	NM_001114636.1:c.1107G>A	p.Lys369(=)	rs577063114	Homozygous	Pathogenic	NA

NA: Not applicable as EVE is available only for missense mutations

* likely pathogenic by ACMG guidelines but pathogenic by Varsome.