Original research

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

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► Additional supplemental material is published online only. To view, please visit the journal online (http://dx.doi.org/10.1136/jmg-2022-108714).

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Received 18 May 2022
Accepted 3 December 2022
Published Online First 9 March 2023

To cite: Joshi G, Arthur NBJ, Geetha TS, *et al*. *J Med Genet* 2023;**60**:801–809.

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 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

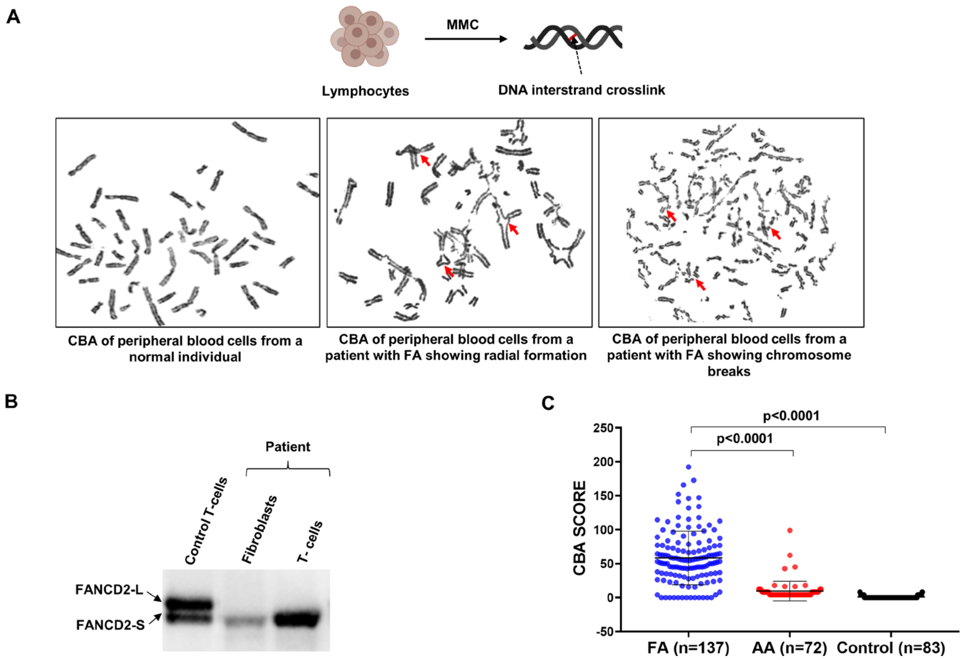
pLX301-*FANCA*, pLX301-*FANCG* and pLX301-*FANCC* plasmids were generated by Gateway cloning, and pCW-*FANCL* and pCW-*FANCF* plasmids were generated by cloning the cDNAs in pCW lentiviral plasmid.

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



CBA of peripheral blood cells from a normal individual

CBA of peripheral blood cells from a patient with FA showing radial formation

CBA of peripheral blood cells from a patient with FA showing chromosome breaks

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).

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

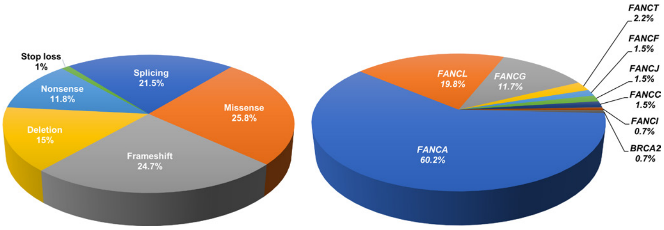


Figure 2 Genotyping of 142 patients with Fanconi anaemia (FA). Left: The percentages of different types of mutations identified. Right: The frequencies of the defective genes in homozygous and compound heterozygous states.

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 (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

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.

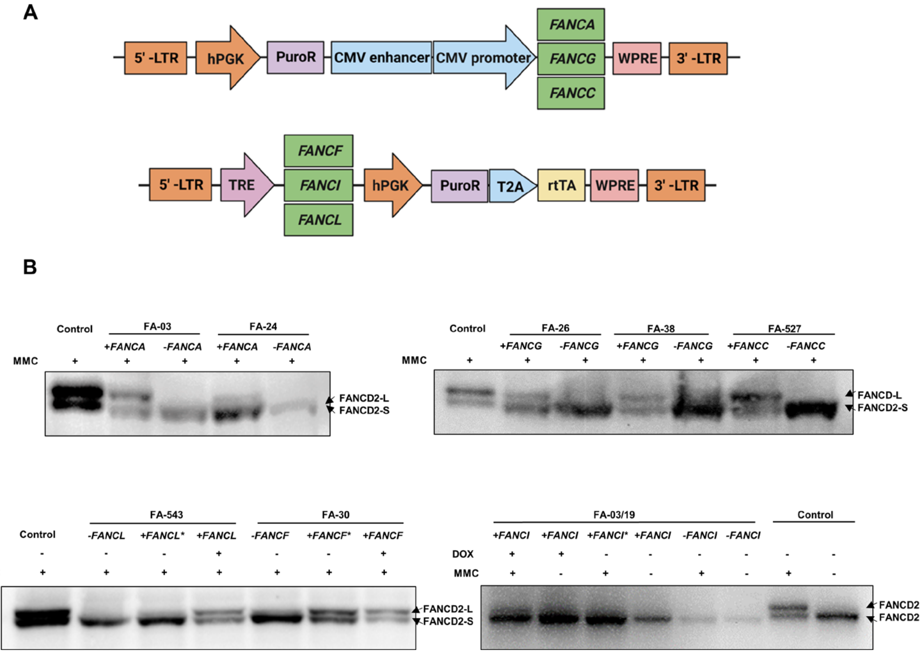


Figure 3 Lentiviral complementation analysis. (A) Lentiviral constitutive expression vectors for complementation analysis of FANCA, FANCG and FANCC and doxycycline-inducible expression vectors for FANCF, FANCI and FANCL. (B) FANCD2 western blot results after complementation of FANCA, FANCG, FANCC, FANCF, 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

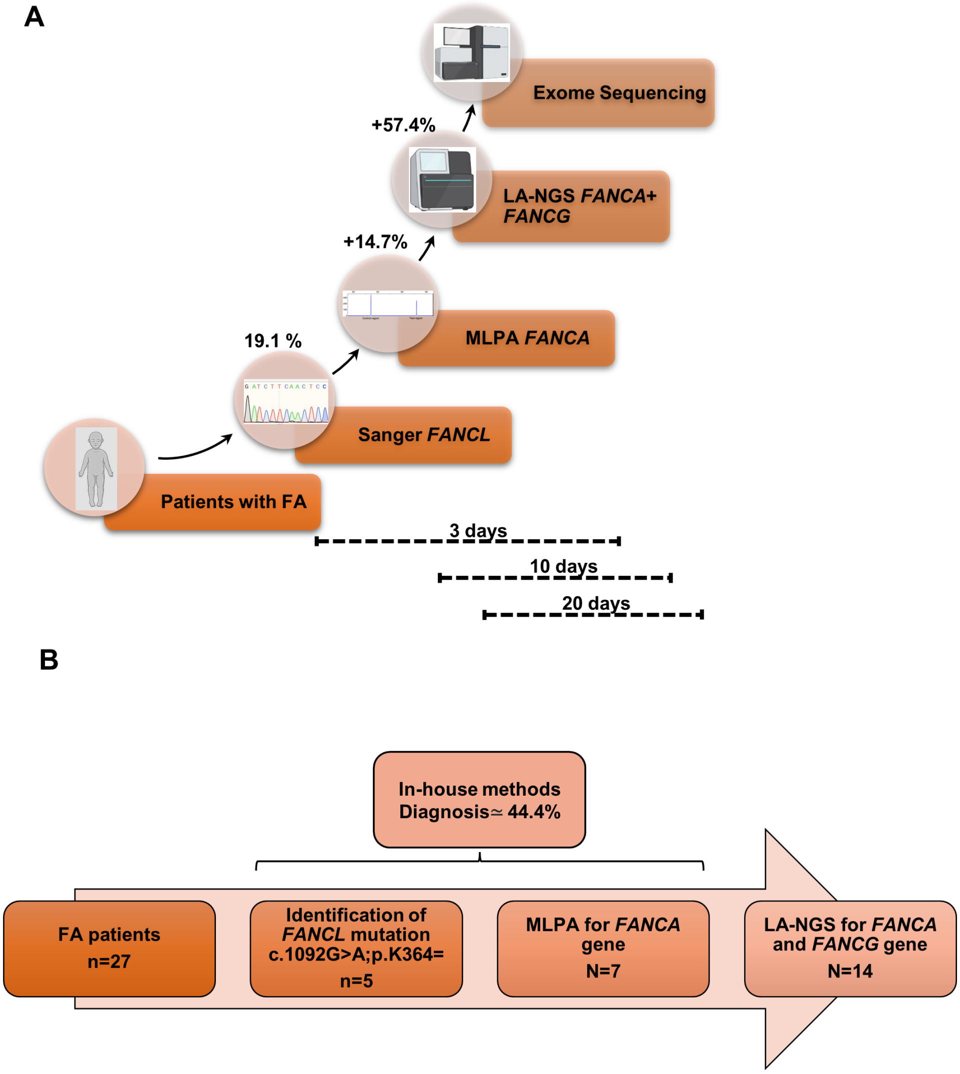


Figure 4 Methodologies for the molecular diagnosis of Fanconi anaemia (FA) in the Indian population. (A) Algorithm for the molecular diagnosis of FA. (B) The new algorithm tested in 27 patients with FA. MLPA, multiplex ligation-dependent probe amplification; LA-NGS, long-amplicon next-generation sequencing.

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 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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Acknowledgements The authors thank MedGenome Labs Ltd, Bengaluru, India, for the exome sequencing service.

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.

Funding The Wellcome Trust DBT India Alliance Senior Research Fellowship to SRV (Grant number IA/S/17/1/503118) and MedGenome Labs Ltd.

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.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available upon reasonable request.

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