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Analysis of *FANCC* gene mutations (IVS4+4A>T, del322G, and R548X) in patients with Fanconi anemia in Pakistan

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Background/aim: Fanconi anemia (FA) is an autosomal recessive disease determined by mutations in at least 16 genes, with distinct distributions in different populations. To the best of our knowledge, there are no reports regarding the molecular basis of the disease in FA patients in Pakistan. The current study aimed to determine the frequency of *FANCC* gene mutations, i.e. IVS4+4A>T, del322G, and R548X, in FA patients.

Materials and methods: Genomic DNA was obtained from 36 FA patients. All samples were analyzed by polymerase chain reaction and restriction fragment length polymorphism techniques.

Results: Mutation IVS4+4A>T was identified in 26 (72.2%) patients. It was homozygous in 6 and heterozygous in 20 patients. Del322G and R548X were found with the following prevalences: del322G, 5.6%, and R548X, 5.6%. Patients with these two mutations were compound heterozygotes having concomitant IVS4+4A>T mutation.

Conclusion: These results suggest that mutation IVS4+4A>T is the most prevalent mutation in our group of patients. This analysis of Pakistani patients also suggests that there is no significant difference between IVS4+4A>T homozygotes and the rest of the patients with regard to severity of clinical phenotype.

Key words: Fanconi anemia, IVS4+4A>T, Del322G, R548X, *FANCC*

1. Introduction

Fanconi anemia (FA) is a rare autosomal recessive or X-linked genomic instability disorder characterized by hypersensitivity to DNA cross-linking reagents, chromosomal breakage, progressive bone marrow failure, pancytopenia, developmental delay, diverse congenital abnormalities, and a predisposition to solid tumors and leukemias (1). It is the most common type of inherited bone marrow failure syndrome (2). The genetic basis of FA is heterogeneous and many genes are involved in its pathogenesis. Mutations in these genes that code for their respective proteins have been identified in patients with FA (3). The birth incidence of FA is around three per one million. The disease is slightly more common in males than females; the ratio is 1.3:1 due to the *FANCB* gene, which is located on the X chromosome. The estimated heterozygote frequency is one in 300 in Europe and the United States. Its frequency is higher in Afrikaners, Spanish Romani, and Ashkenazi Jews at one in 100. FA is present in all ethnic groups (4). Between 1927 and 2001, 1300 cases of FA were reported in the literature with varying clinical presentations (5).

It is now known that mutations in at least 16 distinct genes with a total of 307 exons are responsible for FA onset, i.e. *FANCA, FANCB*, *FANCC*, *FANCD1/BRCA2*, *FANCD2*, *FANCE*, *FANCF*, *FANCG*, *FANCI*, *FANCJ/BRIP1*, *FANCL*, *FANCM*, *FANCN/PALB2*, *FANCO/RAD51C*, *FANCP/SLX4*, and *FANCQ/ERCC4*. The products of these 16 genes

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are thought to function in a common DNA repair pathway, the FA pathway. These proteins, along with other DNA repair proteins, remove interstrand crosslinks (6). All the genes are quite different from each other and show little or no homology. With the exception of *FANCC* and *FANCG*, which are both located on chromosome 9, and *FANCA* and *FANCN*, which are both located on chromosome 16, the other FA genes are spread over different chromosomes (7). Most FA patients belong to complementation group A, C, or G and their approximate percentage according to the International Fanconi Anemia Registry (IFAR) is as follows: FA-A = 65%, FA-C = 15%, and FA-G = 10%. However, group C is most frequent in Ashkenazi Jews. Its mutations lead to absent or reduced protein production that results in a build-up of DNA errors. Affected individuals develop congenital abnormalities and pancytopenia (8).

Mutation IVS4+4A>T is a splice site mutation and results in deletion of the entire exon 4. Cells with this mutation express a truncated protein of 55 kDa. The truncated protein is functionally inactive (9). The exon 1 mutation, del322G, is a frame-shift mutation and results in a premature stop codon. It involves deletion of a single G at base 322 and consequently a truncated protein of 44 amino acids is produced (10). Similarly, a truncated protein is also produced when thymine is inserted at 1897 bp instead of cytosine in exon 14. This shorter protein is nonfunctional and results from premature termination of protein synthesis, due to mutation R548X in exon 14 (11).

Therefore, this study was designed to determine the frequency of *FANCC* gene mutations, i.e. IVS4+4A>T, del322G, and R548X, in patients with FA in Pakistan.

2. Materials and methods

2.1. Patients

All unrelated patients with confirmed FA who attended the medical, genetic, and hematological services of the Combined Military Hospital and Armed Forces Institute of Pathology were recruited for the present study. Peripheral blood samples were collected from 50 individuals after their informed consent was obtained. Diagnosis of the disease was based on clinical and laboratory data, including hematological analysis, bone marrow aspiration, and biopsy, and was confirmed by DEB test. The study protocol was approved by the institutional ethics committee.

2.2. DEB/MMC test

The DEB test was performed according to conventional methods (12). It was performed on 50 patients and 36 of these turned out to be DEB-positive. They were included in the study. The DEB/MMC test was done on peripheral blood lymphocytes stimulated with phytohemagglutinin in 72-h cultures. Cultures were paired with a replicate set of cultures to serve as untreated controls. MMC at a final concentration of 0.1 μg/mL was also added to the treated cultures. In addition, negative controls from phenotypically normal subjects were also set up for each patient. Analysis was performed on 50 Giemsa-stained metaphases. Chromosomal aberrations were scored.

2.3. Molecular analysis of *FANCC* gene mutations

Genomic DNA was obtained from peripheral blood of patients enrolled in the study using the Chelex method according to the manufacturer's instructions. *FANCC* gene mutations were analyzed by polymerase chain reaction (PCR) followed by restriction site assays.

Primer sequences for the 3 loci (IVS4+4A>T, del322G, R548X) were the same as previously reported in the literature (13). Two of these primers for mutations IVS4+4A>T and del322G introduced artificial restriction sites into PCR products using primer-directed restriction site modification. PCR was carried out in reactions of 20 μL with 150 ng of genomic DNA, 5 pM of each primer, 1X Master Mix (Fermentas Inc., USA), and distilled water. After an initial denaturation at 94 °C for 5 min, samples were amplified for 35 cycles of denaturation at 94 °C for 1 min, annealing for 1 min (50 °C for IVS4+4A>T, 56 °C for del322G, 60 °C for R548X), and extension at 72 °C for 1 min followed by a final extension for 10 min at 72 °C.

Restriction assays were performed by using ScaI, Bsp1286I, and AvaI restriction enzymes (Thermo Scientific Inc., USA) according to the manufacturer's instructions for studying the three mutations of FANCC, i.e. IVS4+4A>T, del322G, and R548X, respectively. The reaction was prepared in a volume of 10 μL containing 3.5 μL of PCR product, 5 U of each enzyme, 0.6 μL of buffer, and 5.5 μL water. The mixture was incubated for 30 min at 37 °C (3 h for ScaI) to let the enzyme digest the PCR product completely. After incubation, the enzyme was deactivated by heating the tubes at 70 °C for 30 min. The PCR digest was electrophoresed on 3% agarose gel. Image Lab software was used to analyze the gel bands of normal (digested into 2 fragments) and mutant (undigested, single band) PCR products. The investigated gene mutations, the sizes of their PCR products, their restriction sites, and the sizes of the normal and mutant fragments obtained after digestion are shown in Table 1.

SPSS 20 (IBM Corp., USA) was used for statistical analysis. Continuous variables such as age, height, weight, duration of illness, and laboratory parameters were expressed as mean ± SD, whereas categorical variables such as sex, clinical features, and mutations were given in the form of frequency and percentage.

3. Results

A total of 36 unrelated FA patients (17 males and 19 females), ranging in age from 5 to 20 years, were included in the study. Mean height and weight of patients was 123.06 ± 17.73 cm and 29.33 ± 13.07 kg respectively,

Table 1. Size of PCR products, their respective restriction enzymes, and fragments obtained after digestion.

Mutation	PCR product (bp)	Restriction enzyme	Restriction site	Size of product (bp) after digestion
IVS4+4A>T	131	ScaI	A	N = 108+23 M = 131
Del322G	151	Bsp1286I	A	N = 129+22 M = 151
R548X	364	AvaI	Nat	N = 231+133 M = 364

ScaI- *Streptomyces caespitosus*; Bsp1286I- *Bacillus sphaericus*; AvaI- *Anabaena variabilis*;
A- artificial; Nat- natural; N- normal; M- mutated.

whereas duration of illness was 37.89 ± 31.15 months. Thirty patients presented with congenital abnormalities such as short stature, microcephaly, microphthalmia, thumb abnormalities, café au lait spots, and congenital absence of one kidney (Table 2). All patients had normal serum electrolytes.

A variable degree of pancytopenia was present in all patients. Onset of hematological abnormalities was defined as the time at which a decrease in hemoglobin, white blood cells, or platelets occurred. Mean hemoglobin of patients was found to be 8.24 ± 2.57 g/dL while white blood cell and platelet counts were (2.91 ± 1.42) × 10^9/L and (62.78 ± 33.98) × 10^9/L, respectively. Complete blood counts were done on a Sysmex (XT-1800i). All the patients had bone marrow hypoplasia.

After amplification, restriction site assays were developed for all three known pathogenic mutations. The digested products were analyzed on 3% agarose gel. RFLP results of the three mutations (IVS4+4A>T, del322G, and R548X) are shown in Figures 1a–1c.

IVS4+4A>T was the most prevalent mutation in our patients. It was detected in 26 (72.2%) patients; 20 (55.6%)

Table 2. Clinical characteristics of patients (n = 36).

Variable		Frequency	Percentage (%)
Sex	Male	17	47.2
	Female	19	52.8
Consanguinity		24	66.7
Short stature		27	75
Low weight		20	55.6
Pallor		33	88.9
Epistaxis		19	52.8
Bruises		25	69.4
Microcephaly		23	63.9
Thumb abnormalities	Hypoplastic	11	30.6
	Bifid	3	8.3
Microphthalmia		23	63.9
Café au lait spots		11	30.6
Absent kidney		3	8.3
Elevated liver enzymes		3	8.3

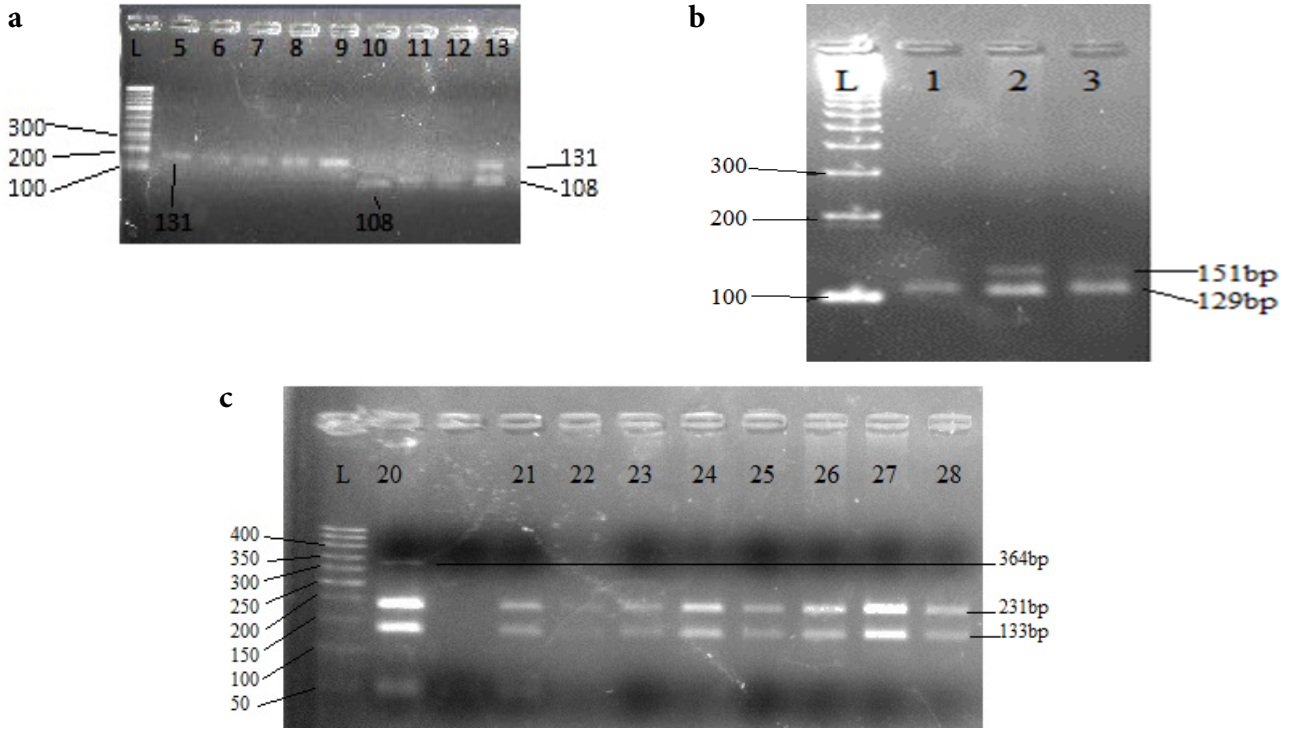

Figure 1. a) RFLP results of IVS4+4A>T mutation. A 100-bp ladder is shown on the extreme left side. Samples 5–13 are shown. Bands of samples 5–9 are of 131 bp and they are homozygous for the mutation. Bands of samples 10–12 are of 108 bp and are normal for this mutation. Sample 13 shows 2 bands of 108 and 131 bp and it is heterozygous for this mutation. b) RFLP results of del322G mutation. A 100-bp ladder is shown on the left side. Samples 1–3 are shown. The band of sample 1 is of 129 bp and it is normal for this mutation. Bands of samples 2 and 3 are of 129 and 151 bp and are heterozygous for this mutation. c) RFLP results of R548X mutation. A 50-bp ladder is shown on the extreme left side. Sample 20 is heterozygous for this mutation, showing 3 bands of 133, 231, and 364 bp. Samples 21–28 are normal for this mutation, showing 2 bands of 133 and 231 bp.

were heterozygous and 6 (16.7%) were homozygous. There were no homozygotes for the other two mutations. The patients who were heterozygous for del322G also had concomitant IVS4+4A>T mutation and they were compound heterozygotes. The other 2 patients who were heterozygous for R548X mutation were also compound heterozygotes. In addition to R548X mutation, they also had IVS4+4A>T mutations; one patient was homozygous for IVS4+4A>T and the other was heterozygous for it, as shown in Table 3.

Previous studies showed that patients homozygous for IVS4+4A>T have a severe phenotype (14). We divided the patients into two groups: homozygous for IVS4+4A>T (group 1) and other patients including those heterozygous for IVS4+4A>T and the rest of the patients (group 2). The patients were compared on the basis of hematological abnormalities and congenital malformations. There was no significant difference in the clinical presentation of patients of the 2 groups. Café au lait spots, congenital absence of one kidney, and elevated liver enzymes were present only in group 2 and microphthalmia and microcephaly were slightly higher in group 1 but the difference was not significant (Figure 2).

4. Discussion

We have screened these three mutations of the *FANCC* gene in 36 unrelated FA patients after confirming their diagnosis by chromosomal breakage test. The mean age of the studied group was 11.54 ± 4.26 years. Congenital abnormalities were frequent and were equally distributed in all patients. Most of the patients presented with severe hematological manifestations including bone marrow hypoplasia.

Previous studies showed that the distribution of FA gene mutations is quite variable in different populations. *FANCC* gene mutations are more common in Ashkenazi Jews and the Japanese population (15,16). In the current study, 72.2% of the patients (n = 26) were found to have *FANCC* gene mutations, both homozygous and heterozygous. Similarly, in another study conducted in Brazil, *FANCC* gene mutations were found in two-thirds of the patients (17).

IVS4+4A>T was the most common mutation found in this study; 16.7% patients (n = 6) were homozygous whereas the remaining 55.6% or 20 patients were heterozygous (carriers) for this mutation. It was observed that most of the patients (61.5%) recruited in this study

Table 3. Molecular features of 36 patients with Fanconi anemia.

Case	Age (years)	Sex	Abnormal gene	Genotype		
				IVS4+4A>T	R548X	Del322G
1	8	F	FANCC	IVS4+4A>T/–	–/–	–/–
2	8	F	FANCC	IVS4+4A>T/–	–/–	Del322G/–
3	6	M	FANCC	IVS4+4A>T/–	–/–	Del322G/–
4	15	M	FANCC	IVS4+4A>T/–	–/–	–/–
5	11	F	FANCC	IVS4+4A>T/IVS4+4A>T	–/–	–/–
6	6.5	M	FANCC	IVS4+4A>T/IVS4+4A>T	–/–	–/–
7	20	F	FANCC	IVS4+4A>T/IVS4+4A>T	–/–	–/–
8	15	M	FANCC	IVS4+4A>T/IVS4+4A>T	–/–	–/–
9	10	M	FANCC	IVS4+4A>T/IVS4+4A>T	–/–	–/–
10	17	F	NA	–/–	–/–	–/–
11	9	M	NA	–/–	–/–	–/–
12	8	F	NA	–/–	–/–	–/–
13	11	M	FANCC	IVS4+4A>T/–	–/–	–/–
14	10	M	FANCC	IVS4+4A>T/IVS4+4A>T	R548X/–	–/–
15	6	M	FANCC	IVS4+4A>T/–	–/–	–/–
16	8	M	NA	–/–	–/–	–/–
17	12	F	NA	–/–	–/–	–/–
18	20	M	NA	–/–	–/–	–/–
19	18	F	FANCC	IVS4+4A>T/–	–/–	–/–
20	14	M	FANCC	IVS4+4A>T/–	R548X/–	–/–
21	12	F	FANCC	IVS4+4A>T/–	–/–	–/–
22	13	M	FANCC	IVS4+4A>T/–	–/–	–/–
23	17	M	FANCC	IVS4+4A>T/–	–/–	–/–
24	19	F	FANCC	IVS4+4A>T/–	–/–	–/–
25	18	F	FANCC	IVS4+4A>T/–	–/–	–/–
26	9	F	FANCC	IVS4+4A>T/–	–/–	–/–
27	7	M	FANCC	IVS4+4A>T/–	–/–	–/–
28	13	M	FANCC	IVS4+4A>T/–	–/–	–/–
29	11	F	NA	–/–	–/–	–/–
30	10	F	FANCC	IVS4+4A>T/–	–/–	–/–
31	5	F	FANCC	IVS4+4A>T/–	–/–	–/–
32	7	F	FANCC	IVS4+4A>T/–	–/–	–/–
33	8	F	FANCC	IVS4+4A>T/–	–/–	–/–
34	10	M	NA	–/–	–/–	–/–
35	11	F	NA	–/–	–/–	–/–
36	13	F	NA	–/–	–/–	–/–

M- Male; F- female; NA- not available; (–/–): absence of both alleles.

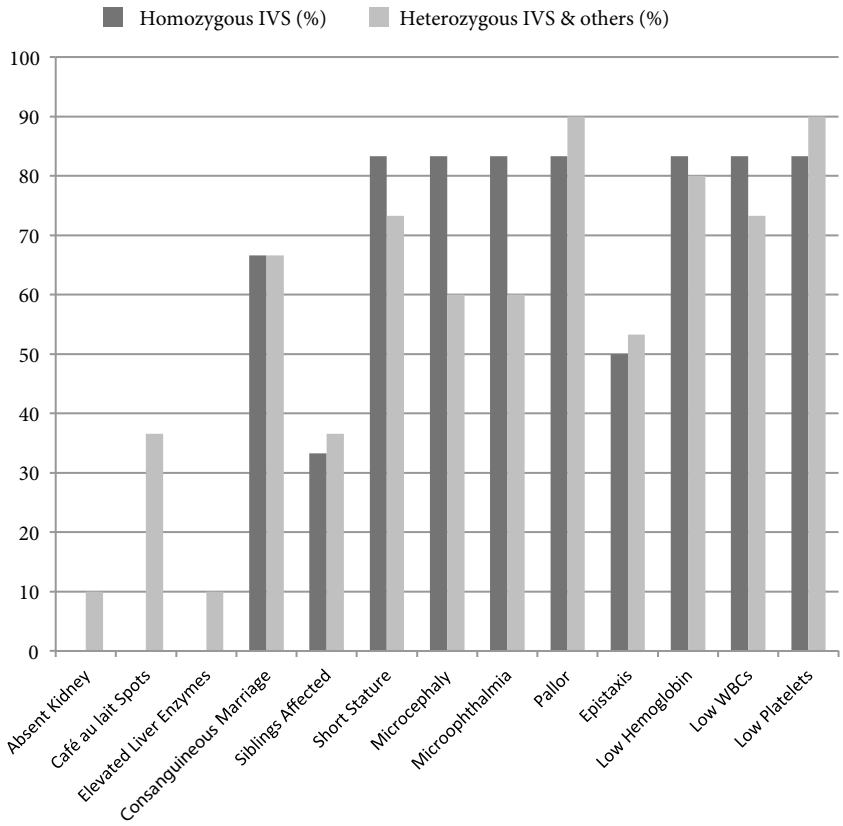

Figure 2. Comparison between groups 1 and 2.

were born of consanguineous marriages. Earlier it was claimed by Verlander et al. that IVS4+4A>T is the most common mutation in Ashkenazi Jews and is present in >80% of FA patients (14). Later, an analysis of Ashkenazi Jews in another study confirmed that this mutation is responsible for most of the cases of FA among them and its carrier frequency is 1 in 89. It was also suggested that this mutation is extremely rare in other populations, as it was absent in Iraqi Jews. The same results were confirmed by the IFAR (18). In another study, it was reported that IVS4+4A>T was the most prevalent mutation of the *FANCC* gene and it was detected in 15.9% of Brazilian FA patients (17). The same mutation was also present in 31% of Japanese patients. Of these, 27.6% were homozygous. Moreover, these results indicate that IVS4+4A>T mutation is also common in other populations of non-Jewish origin (16).

FA is characterized by a wide variety of clinical phenotypes. It has also been reported that in Ashkenazi Jews, IVS4+4A>T is associated with a severe clinical phenotype, multiple congenital abnormalities, and short survival time in comparison with other *FANCC* gene mutations or non-*FANCC* gene mutations (15). Contrary to this, our patients with IVS4+4A>T mutation did not exhibit phenotypic severity of that degree. However, Futaki et al. studied the same mutation and compared homozygous patients with IVS4+4A>T mutation with the rest of the patients. They concluded that it is not associated with a severe phenotype in Japanese patients as it is seen to be in Ashkenazi Jews (16). Therefore, to check the association of IVS4+4A>T mutation with the severity of disease in our population, patients were divided into 2 groups; group 1 included patients who were homozygous for the mutation and group 2 included heterozygous cases and the rest of the patients. Both groups were compared on the basis of congenital and hematological abnormalities and it was observed that there was no significant difference in clinical presentations of disease between the 2 groups. Congenital absence of one kidney, café au lait spots, and elevated liver enzymes were absent in group 1 and were present in group 2. We could not compare the survival time of patients due to the limited time duration of the study. Taken together, these results suggest that the severity of the clinical phenotype is similar between IVS4+4A>T homozygotes and other FA patients included in our study. Wolf presented the same explanation in his study: the same mutations can result in nonuniform clinical pictures. He reported that phenotypic variations do exist for the same mutations among different ethnic

groups. It was stated that the presence of modifier genes at other loci, epigenetic changes such as methylation, and environmental influences such as exposure to pathogens could be possible explanations. These factors might alter the expression of mutations (19).

Screening of mutations, i.e. del322G and R548X, in our patients revealed that each of these was present in only 5.6% or 2 patients. All the patients were compound heterozygotes. In the Brazilian population, del322G and R548X were found to be 11.45% and 2.3%, respectively. Compound heterozygosity with one mutated allele of both IVS4+4A>T and del322G was also been described in the same population (17). Gillio et al. found that compound heterozygotes might have mild phenotypes (15), but we did not observe any significant difference in the clinical presentation between compound heterozygotes and other patients.

In conclusion, we found that IVS4+4A>T mutation was the only mutation present in a high frequency in our patients and there was no significant difference in the clinical presentation of homozygous cases and the rest of the patients. The high frequency of IVS4+4A>T homozygotes and compound heterozygotes was expected as most of the patients were children of consanguineous parents. Furthermore, heterozygous FA gene mutations may also contribute to the severity and prognosis of the disease because some of the mutations, as described earlier, might have deleterious or protective effects on other mutations. This indicates the importance of molecular analysis for a better understanding of disease pathology and diagnosis. Some concomitant mutations have protective or deleterious effects on phenotype of patients so it is recommended to analyze all the mutations of the *FANCC* gene and correlate their effects with clinical presentations of the patients and their parents to determine their exact genotype for advanced FA research and gene therapy.

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