



FANCA Gene in Pakistani Fanconi Anemia Patients: Screening of Mutations in Exon 28 and Exon 29

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ABSTRACT

Objective: This kind of study was conducted first time in Pakistan. Its objective was to ascertain the associated clinical features and analyze the FANCA exon 28 and exon 29 mutations in Pakistani Fanconi anemia (FA) patients. **Methods:** A total of 38 patients with Fanconi anemia were recruited presenting in the Armed forces institute of pathology (AFIP) Rawalpindi Pakistan. They were enrolled in this study on the basis of comprehensive clinical evaluation and positive Diepoxybutane (DEB)/Mitomycin C Chromosomal breakage test. Genomic DNA was extracted from peripheral blood of patients and age and gender-matched controls. Mutation analysis of FANCA gene was done by conventional Polymerase chain reaction (PCR) and DNA sequencing. Various online tools and software were used for analysis of the obtained data and identification of the sequence alterations in FANCA gene in exon 28 and exon 29 of FA patients. **Results and Discussion:** The current study on screening of FANCA mutational analysis in exon 28 and exon 29 revealed four novel mutations. These include three missense variants (p.F876L, p.L883H, and p.K921I) in exon 28 and a novel homozygous frameshift variant (p.S947Ffs.X950) in exon 29. In addition two new intronic variants were also found in this set of patients. **Conclusion:** The sequence variants identified in this study in 10 (26.31%) FA patients in two out of forty-three FANCA gene exons (i.e., exon 28 and exon 29) strongly emphasize the importance of large-scale molecular studies on FANCA gene in Pakistani population.

Keywords: Fanconi anemia, Pakistan, FANCA gene, Mutational analysis, Diepoxybutane/Mitomycin C chromosomal breakage test

INTRODUCTION

Fanconi anemia (FA) is a rare inherited disease characterized by congenital abnormalities, progressive bone marrow failure and increased risk of hematopoietic and epithelial malignancies. The mode of inheritance is autosomal recessive whereas rarely it is inherited in an X-linked manner (FANCB) and as autosomal dominant disease (FANCR). Twenty-one FA genes have yet been identified that take part in maintenance of the genomic stability by FA pathway [1]. Genetically and clinically it is a pleomorphic disease. Cells of the FA patients show hypersensitivity to inter-strand crosslinking agents (ICLs) like mitomycin C (MMC), diepoxybutane (DEB) and cisplatin. The spontaneous chromosomal instability amplified by DNA crosslinking agents is a hallmark feature of Fanconi anemia [2].

Fanconi anemia is the most common cause of childhood inherited aplastic anemia that has been reported in many races

and ethnic groups. It has a slight male predominance with 1.2:1 male to female ratio. The estimated carrier frequency in Americans is 1/181. Relative prevalence widely varies among different ethical backgrounds and due to founder effects, the expected prevalence and carrier rate is much higher in Ashkenazi Jews, Roma population of Spain and black South Afrikaners [3].

Fanconi anemia was first described by Dr. Guido Fanconi who proposed the involvement of multiple genes causing the bone marrow failure in a patient with FA symptoms. Among all Fa genes FANCC was the first gene identified 65 years after the first case of FA was reported [4]. FANCA was the second FA gene and was first identified by Lo Ten Foe and his colleagues in 1996 [5]. Its chromosomal location is 16q24.3 and molecular location is 89, 737, 550 to 89, 816, 657 base pairs. Its open reading frame is distributed among 43 exons ranging in size from 34 to 188 base pairs. FANCA encoded protein weighing 163 kDa, contains 1455 amino acids and is located both in nucleus as well as cytoplasm. It contains a nuclear localization signal (NLS), five functional leucine-rich Nuclear export sequences (NESs) and a partial leucine zipper motif [6]. The broad expression of FANCA in 27 tissues includes lymph node, bone marrow, pancreas, kidney, liver, and skeletal muscles.

Physically the common congenital abnormalities associated with FA are radial ray deformities, Café au lait spots, Short stature, skeletal, central nervous system, cardiovascular and gastrointestinal malformations. The median age for hematological manifestations is about 7 years. Thrombocytopenia, macrocytosis and raised fetal hemoglobin usually appear before patients develop the more severe hematological anomalies [7]. Fanconi anemia pathway is a very complex DNA repair mechanism induced by interstrand crosslinks (ICLs) associated DNA damage during S phase of the cell cycle. Fanconi anemia proteins take part in the repair along with some other proteins such as FAAP24, MHF1. A core complex is formed comprising of FANCA, B, C, E, F, G, M and L that is needed for ubiquitination of FANCD2. Monoubiquitination of FANCD2 remains the mainstay and critical step of FA pathway so that an ID complex comprising of FANCD2 and FANCL is formed that localizes to DNA lesion. It is required for the downstream activation of other proteins such as FANCP and FANCO that unhook the ICLs and repair by FANCD1, J, N, O, R, S and U [8].

FANCA gene mutations show the highest frequency (nearly 65%) of all FA cases and a wide range of heterogeneity (about 200 different mutations) among all FA genes. Almost all types of mutations associated with FANCA have been reported i.e., point mutations including missense and nonsense variants, splicing mutations, microdeletions, micro insertions, duplications, and large intragenic deletions. Large intragenic deletions are the most common among all mutations (<http://www.rockefeller.edu/fanconi/>, accessed on June 9, 2019). Previously three studies on FA patients from Netherland, Italy, Spain, and France reported several mutations many of which were in exon 28 and 29 of FANCA gene [9-11]. The current study describes mutational analysis of these two exons in Pakistani FA patients.

Mutations in FANCA gene are most common among all identified FANCA genes. Fanconi anemia is primarily an autosomal recessive disorder and consanguinity has a significant role in the occurrence of disease. This study is important as it is the first of its kind in Pakistan and was aimed to identify mutations in FANCA gene (exon 28 and 29) in consanguineous Pakistani population.

MATERIALS AND METHODS

Patients

For this study, 50 patients were selected initially on the basis of clinical diagnosis of FA presenting in Armed forces institute of Pathology, Rawalpindi. After informed written consent Blood samples were obtained and 38 out of 50 patients were enrolled after confirmation of Fanconi anemia by Diepoxybutane (DEB)/Mitomycin C (MMC) Chromosomal breakage test. The patients belonged to different ethnic groups of Pakistan, such as Pathans, Pothohari, Punjabis, and Kashmiris. Approval for the study was obtained from the Institutional review board (IRB) University of Health Sciences, Lahore and all procedures performed in this study were in accordance with ethical standards of the institution and Helsinki protocol.

Chromosomal Breakage Test

The test was performed on peripheral blood lymphocytes stimulated by Phytohemagglutinin. The cultures were kept

at 37°C for 72 hours after they were induced with Mitomycin C in a final concentration of 0.01mg/ml. A replicate tube was paired for each culture to serve as untreated control. Cells were arrested at the metaphase stage of cell cycle using Colchicine and then treated with 10% Potassium chloride solution. Methanol: glacial acetic acid (3:1) solution was used for fixation. Cultures from Negative control for each patient were also set for comparison. Chromosomal breakage analysis was performed on 20-25 Giemsa stained metaphases on at least 4 slides for each patient and control and aberrations were scored.

DNA Isolation and Molecular Analysis

Genomic DNA was extracted from peripheral blood using the Chelex method (Chelex® 100, Bio-Rad, California, USA) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was done for exon 28 and 29 with specific primer pairs designed using primer 3 software. PCR products were purified using ethanol precipitation method. Exon 28 and 29 (including their intron/exon boundaries) were sequenced using the Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA) on ABI PRISM 3730 Genetic analyzer following the manufacturer's instructions. SPSS-20 (IBM Corp., USA) was used for statistical analysis of clinical data and Chromas Lite version 2.01 for sequencing data analysis. BLAST 2 (Basic Alignment Search Tool 2) was used to observe the sequence alterations in all patients and controls on NCBI.

RESULTS

This study included 38 unrelated FA patients (18M:20F) with mean age, weight, and height of 11.43 ± 4.21 years, 28.73 ± 12.97 kg and 48.15 ± 6.99 inches respectively whereas the mean age at the time of presentation was 8.39 ± 4.23 years. consanguinity rate was 63.2% and 15 (39.5%) patients had history of affected siblings. Pallor, bruises, and epistaxis were the most common presenting complaints and all the patients had history of recurrent infections. Twenty-eight (73.7%) patients had short stature and 22 (57.9%) had low weight for their age and gender. Thumb abnormalities included hypoplastic thumb in 12 (31.6%) and bifid thumb in 5 (13.2%) patients. Cafe au lait spots were observed in 11 (28.9%) patients. One patient found to have cardiac abnormalities whereas genitourinary abnormalities included one absent kidney in 4 (10.5%) patients and hypospadias in one patient.

Mutation Analysis for Exon 28 and 29

The analysis of obtained data revealed a total of four mutations of which three were identified in exon 28 and one mutation in exon 29. We also found two intronic sequence variations in intron 27 and intron 28. As per literature review and best of our knowledge all these sequence variations have not been reported previously. We also deposited the new mutations of our study in exon 28 and exon 29 in ClinVar database of NCBI. All the sequence variants detected in this study, along with their respective ClinVar accession numbers, are summarized in Table 1 and their electropherograms are presented in Figure 1.

Table 1 Sequence alterations identified in FANCA gene in Pakistani Fanconi anemia Patients

Sample ID	Exon/Intron	Sequence Alteration	Codon Change	Amino Acid Change	ClinVar Accession No.
FA 05,06,07,12 and 14	Exon 28	c.2729C>A	TTC>TTA	p.876F>L	SCV000239244
FA 08,09 and 10	Exon 28	c.2648T>A	CTT>CAT	p.883L>H	SCV000239243
FA 15	Exon 28	c.2762A>T	AAA>ATA	p.921K>I	SCV000239242
FA 02	Intron 27	c.2602_36C>A	-	-	-
FA 13, 21, 27, 28, 34, 35 and 38	Intron 28	c.2779_56T>A	-	-	-
FA 11	Exon 29	c.2839dupT	TCA>TTC	p.S947FfsX950	SCV000239241

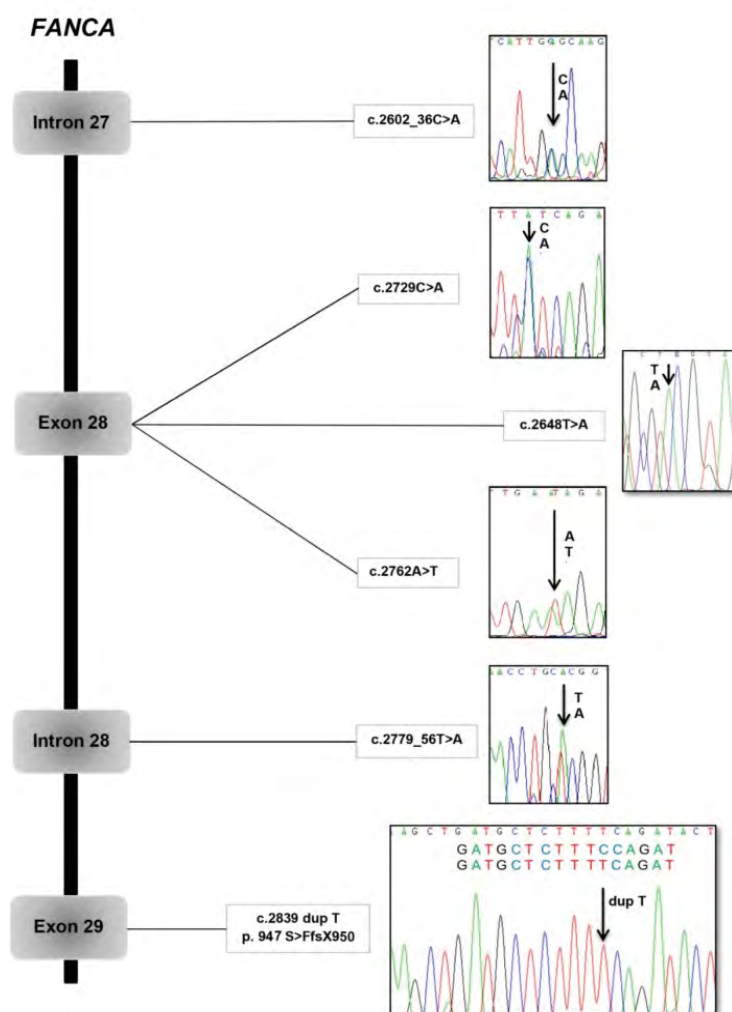


Figure 1 Schematic diagram of FANCA gene and electropherograms of the sequence variants identified in the study. Exons 28 and 29 with intervening introns are displayed in FANCA gene diagram. In each case the specific position of the mutant nucleotide along with base change is indicated by the black arrow

DISCUSSION

Fanconi anemia is a rare disease inherited in an autosomal recessive, X-linked and autosomal dominant fashion. This pleiotropic disorder shows hypersensitivity to the clastogenic effect of ICLs and increased spontaneous chromosomal fragility provides a unique cellular marker for diagnosis of Fanconi anemia. FA genes take part in DNA ICL repair mechanism i.e., FA pathway. FANCA is by far the most frequently affected gene and accounts for about 65% of all diagnosed cases of Fanconi anemia also it harbors an entire spectrum of genetic alterations [12].

Bone marrow failure and associated complications are the major cause of morbidity and mortality in patients with Fanconi anemia [13]. During the first ten years of life about 75%-90% patients develop bone marrow failure having minor to severe symptoms depending upon the extent and severity of the disease. The exact cause of these defects is not clear but oxidative stress is thought to be a reason for depletion of hematopoietic reserve causing DNA damage. It impairs the maturation and differentiation of hematopoietic cells [14,15].

This study was designed to identify mutations of FANCA gene at exon 28 and exon 29 in the Pakistani population. 10 (26.31%) out of 38 FA patients demonstrated a total of four mutations in FANCA gene in these two exons. Of which nine patients found to have mutations in exon 28 (c.2729C>A in patients FA 05,06,07,12 and 14, c.2648T>A in FA 08,09 and 10 and c.2762A>T in FA 15) and one patient in exon 29 (c.2839dupT in FA 11). Previously a study on 67

Spanish FA patients revealed a broad spectrum of FANCA mutations. Among them 6 mutations (8.95%) were located in exon 28 and 29 in 8 (11.94%) patients [16]. Another study on Italian FA patients showed 4 (30%) mutations located in exon 28 and 29 [17].

As we know that frameshift mutation and deletions are considered pathogenic because of their severity [10], as it is the case with our study individual FA 11. His molecular analysis disclosed a homozygous c.2839dupT mutation at position 947 located in exon 29 that led to truncated FANCA protein. The propositi had bifid right-hand thumb since birth and subsequent medical evaluation showed the congenital absence of left kidney. He suffered from minor nose bleeding and bruises in early years of life that turned into severe form at the age of seven years when he suffered from severe recurrent infections especially respiratory tract infection and profuse epistaxis as well as traumatic and spontaneous bruises. Our patient with this mutation had severe form of disease with transfusion dependency also frequent hospitalization and died of brain hemorrhage. Previously Callen and colleagues reported truncated FANCA mutation (295C>T) in Gypsy Spanish families [18].

Interestingly patient FA 11 with identified frameshift mutation had bifid thumb whereas other patients with identified missense mutation and SNPs have a hypoplastic thumb. This finding may have an association with major pathogenic and moderate to non-pathogenic mutation and severity of skeletal system abnormalities. Previous studies show that null mutations in FANCA gene lead to earlier onset of hematological abnormalities and also patients had shorter survival after diagnosis. The rate of developing AML and MDS was also higher as compared to the patients with altered protein function resulting from heterozygous mutation [19].

This study also revealed one heterozygous missense mutation c.2762A>T in exon 28 (FA-15). The 6 years old boy presented with symptoms of bone marrow failure i.e., pallor, bruises and recurrent infections, low weight short stature and Blood transfusion dependency for his survival. Pathogenicity of missense mutations is often uncertain [10], so is the case with heterogeneous missense mutations identified in our study group with heterozygous missense mutations i.e., c.2729 C>A in exon 28 (FA 05,06,07,12 and 14) and c.2648T>A (FA 08, 09 and 10). These patients presented with different clinical symptoms of varying severity. As explained by Castella and colleagues in 2011 [16], missense mutations make about one-fourth of total mutations found in FANCA gene and any kind of missense or other non-truncating FANCA mutation leads to nonfunctional FANCA protein that is unable to relocate from cytoplasm to nuclei and disrupts the pathway. This may explain the lack of concordance between FANCA mutation and clinical severity in regard to age of onset of hematological abnormalities and number of malformations in patients. Interestingly pleomorphic phenotype and transfusion dependency in these patients also divert the attention towards possible involvement of other FA genes.

As described by previous studies FANCA intron mutations and SNPs are not uncommon. In 2013 a study conducted on the Canadian population revealed numerous FANCA intron mutations including c.2779 T/C in intron 28 and c.2852+137 T/C and c.2852+314 A/T in intron 29 [20]. Two heterozygous intronic variants (c.2602_36C>A and c.2779_56T>A) in intron 27 and 28 respectively were also identified in our study in seven FA patients. Though these variants are not listed in data base SNP list of SNPs, however, significance of these intronic changes remains to be elucidated.

A continuously updated FA mutation database enlists more than 30 mutations in exon 28 and 29 of FANCA gene (<http://www.rockefeller.edu/fanconi/genes>, accessed on June 9, 2019). Of these, five mutations (p.R880, p.H913R, p.D924Y, c.2778+1G>A and c.2778+83C>G) in exon 28 and four mutations (c.2840C>G, c.2852G>A, c.2812_2830 dup and c.2851C>T) in exon 29 were reported in an Italian cohort [11]. All of these mutations were localized in the BRCA1 interacting domain of the FANCA protein which is crucial for the interaction of core complex and FA/BRCA pathway for DNA repair. Other studies have also reported a diverse spectrum of mutations in exon 28 and 29 including a splicing variant c.2778+1G>C in intron 28 [21], a 33 bp deletion of exon 28, an aberrant mutation c.2807A>G (p.E936G) in exon 29 [9,10] and other such variants identified in Spanish, Japanese and Italian cohorts [16,17]. A schematic representation of mutational diversity in FANCA is presented in Figure 2. The results of these studies on diverse populations including Pakistani patients highlight the importance of the FANCA protein domain encoded by exon 28 and 29 in the DNA repair.

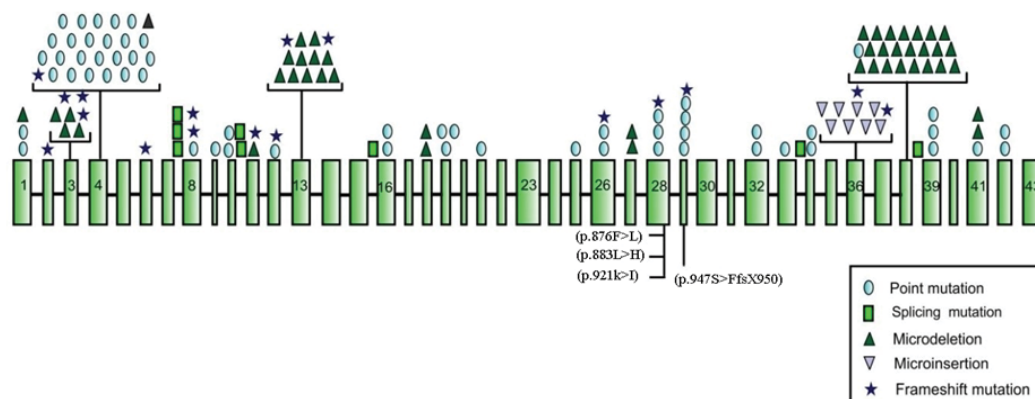


Figure 2 Schematic illustration of FANCA gene and its mutation spectrum. Different mutations (symbols shown in the box) in the respective exons of FANCA gene reported in other populations are shown above. Mutations identified in Pakistani Fanconi anemia patients in exon 28 and exon 29 are shown below. (Adapted from Castella et al., 2011) [16].

CONCLUSION

We present a study that is first of its kind from Pakistan on FANCA gene mutations in exon 28 and 29. It reveals a total of four new mutations out of which in exon 28 we report three heterozygous mutations: c.2729C>A in five patients, c.2648T>A in three and c.2762A>T in one patient and one homozygous mutation c.2839dupT in one patient in exon 29. We report that 10 (26.31%) of our patients from a total of 38 FA patients demonstrate mutations in these two exons. This strongly emphasizes the need for screening for mutation spectrum in all 43 exons of FANCA gene to establish an estimate of FANCA being the most common gene involved or not in causing mutations in Pakistani Fanconi anemia patients. The current study also reveals two heterozygous intronic variants c.2602_36C>A in one patient and c.2779_56T>A in seven patients in intron 27 and 28 respectively. Although a clear genotype-phenotype correlation could not be established in present study, however, to facilitate the prenatal diagnosis of FA and to seek an informative genotype-phenotype correlation, further large-scale studies on FANCA gene and other genes from FA/BRCA complex are recommended.

DECLARATIONS

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Authors' Contributions

SI participated in sample collection, experiment, data analysis, and manuscript writing. IA participated in sample collection and experiment. SB contributed in manuscript proofreading and final version of this article, AA participated in bioinformatic analysis and article writing. NA contributed to sample collection and clinical analysis of the patients. SJ participated in experiments. SK participated in data analysis and manuscript preparation. SM directly participated in the conception, planning, and execution of the study.

Conflicts of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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