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Characterization of two novel *FANCG* mutations in Indian Fanconi anemia patients

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ABSTRACT

FA is a rare recessive genetic disorder with autosomal or X-linked mode of inheritance and is associated with 19 different FA complementation groups. We have studied three patients clinically diagnosed as FA. All three patients showed a high frequency chromosomal breakage in MMC induced blood cultures and FANCD2 non-monoubiquitination by western blotting. The molecular analysis using direct sequencing revealed two novel mutations in *FANCG*; 2 novel mutations c.1143 + 5G > C and c.883dupG, and a reported mutation c.1471_1473delAAAinsG. We have for the first time modeled FANCG protein with fold based template search using pGenthreader which revealed sequence fold identical to super helical TPR domain of O linked GLCNAC transferase and have studied the impact of mutations on the function and structure of FANCG. All three mutations are potential pathogenic molecular changes which can affect FANCG interactions required for FA pathway, homologous recombination repairs and unhooking step of the ICL repair process.

1. Introduction

FA is a rare recessive genetic disorder with autosomal or X-linked mode of inheritance [1,2]. FA occurs due to defect in DNA damage repair pathway, is characterized by numerous congenital anomalies, cellular hypersensitivity to DNA inter-strand crosslinking (ICL) agents such as Mitomycin C (MMC) and Diepoxybutane (DEB) and increased predisposition to both haematological and solid tumors [1,2]. So far, 19 different FA causing genes (*FANCA-T*) are known to be involved in FA pathway [3]. Among all FA-A, FA-C, and FA-G complementation groups are represented by 60%, 15%, and 10% of FA families respectively [4].

FANCG (OMIM# 602956) is identical to *XRCC9* on chromosome 9p13 and covers around 6 kb with 14 exons, has 2.5 kb mRNA encoding FANCG protein of 622 amino acids of 68 kDa molecular weight [5]. FANCG protein shows no homology to known proteins and its function is thus unclear. FANCG is an integral component of FA nuclear core complex and is also required for formation of another protein complex D1-D2-G-X3 (a complex consisting of FANCD1, FANCD2, FANCG, and XRCC3) [6]. Seven Tetratricopeptide repeat motifs located all over across FANCG are required for mediating protein-protein interactions in these two complexes [6]. FANCG interacts with FANCA through N-terminus, 400–428 amino acids in the C-terminus is required for functional complementation to MMC hypersensitivity and carboxy terminus of FANCG (amino acid 585-622) is required for FANCC binding and optimum FANCA binding in the complex [7]. It has been demonstrated that the FANCG physically interacts with the mitochondrial peroxidase peroxiredoxin-3 (PRDX3) [8]. We report three *FANCG* mutations including two novel mutations in three FA and characterization of their plausible impact using bioinformatics tools.

2. Methods and materials

2.1. Patients

The study was carried out on 3 patients with classical clinical presentations of FA like skin pigmentation abnormalities and congenital malformations, apart from bone marrow failure. The age of

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Table 1
Chromosomal breakage investigation in the three FA cases.

Patient ID	Chromosomal breaks (scored as breaks/cell)[a]			
	MMC (40 ng/ml) induced breakage in patient	Spontaneous breakage in patient	MMC (40 ng/ml) induced breakage in control	Spontaneous breakage in control
Case1	3.2	0.2	0.1	0.02
Case2	4.8	0.14	0.1	nil
Case3	3.9	0.16	0.08	nil

[a] 50 metaphases scored.

these patients, parental history of consanguinity, and clinical presentations are described in Table 1. Institutional Ethics Committee of National Institute of Immunohematology (ICMR) for research on Human subjects had approved the study protocols. Parents had given their written informed consent for their children. The peripheral blood samples were collected in sodium heparin (7cc) and ethylenediaminetetraacetic acid (EDTA) (4cc) vacutainer from the subjects. The clinical details including age, sex, parental consanguinity, parental age, reproductive history and haematological profile were recorded in our proforma.

2.2. Chromosomal breakage test

Chromosome breakage study was done using Phytohemagglutinin (PHA) stimulated lymphocyte cultures as described previously [9,10].

2.3. FANCD2 monoubiquitination detection by western blot

Peripheral blood mononuclear cells were isolated using 5 mL of heparinized blood and cell lysate preparation and western blot of FANCD2 monoubiquitination was performed as described previously [10].

2.4. Direct sequencing of FANCA, FANCC, and FANCG

2.4.1. Genomic DNA and total RNA extraction from peripheral blood

QIAamp DNA Blood Mini Kit (Qiagen) and QIAamp RNA Blood Mini Kit (Qiagen) was used for extraction of the total genomic DNA and RNA. The concentrations of DNA and RNA were determined on nanodrop spectrophotometer. The RNA reverse transcribed to first-strand cDNA by using RevertAid H minus First Strand cDNA Synthesis Kit (Thermo Scientific).

2.4.2. PCR amplification of the genomic DNA

Sequence for amplification of *FANCA, FANCC, and FANCG* exon specific primers were designed as published by Gille JJP et al., 2012 [11]. Mutations have been submitted to Leiden Open Source Variation Database (LOVD v.3.0) database (http://databases.lovd.nl/).

2.4.3. PCR amplification of cDNA by RT-PCR and direct sequencing

The entire coding sequence of *FANCG* was screened using three pairs of primers designed based on the *FANCG* sequence (RefSeq NM_004629.1). The three partially overlapping cDNA fragments were amplified using 3 pairs of primers (primer sequence available on request) encompassing 14 exons of *FANCG* gene.

The PCR reaction mixture was prepared and parameters were optimized as described previously [10]. The PCR amplification products were checked on 2% agarose gel electrophoresis and subsequently sequenced using ABI Prism 3130 Automated DNA Sequencer with forward, reverse primers as well as internal forward and reverse primers designed specifically to cover the entire coding region of *FANCG* gene. The sequences were manually compared with *FANCG* gene RefSeq NM_004629.1 using ChromasLite 2.1.1 DNA sequencing software.

2.4.4. In silico *analyses*

2.4.4.1. Splice region mutation analysis. Human Splicing Finder (HSF), a bioinformatics tool was used to predict the effects of mutations on splicing signals (http://www.umd.be/HSF3/) [12].

2.4.4.2. Modeling of FANCG wildtype and mutant proteins. Wild type Fanconi anemia group G protein sequence (FANCG protein) was considered for 3D modeling. To begin with, PDB-BLAST based search [13] was initiated so as to identify potential templates. With no reported 3D coordinates from Protein Data Bank [14], here we opted for fold based search using pGenTHREADER software [15]. In each case potential 3D structure was selected based on their confidence score (based on *p*-value) which are characterized into a) GUESS (<1) b) LOW (<=0.1) c) MEDIUM (<=0.01) d) HIGH (<=0.001) and e) CERT (<=0.0001). In addition to this, structure with better Net score (raw Genthreader score) was considered. In essence, the selected 3D structure with highest confidence and net scores were considered as template for homology modeling using Modeller.15v software [16]. Here the query sequences of FANCG protein (case 2 and case 3) are aligned against the selected template using *align2d.py* command. Followed by this the aligned file was considered for homology modeling using *model-single.py* command. Finally models were selected based on their DOPE score (Discrete Optimized Protein Energy) which is a statistical potential used to assess homology models in protein structure prediction [17]. All the generated models were visualized using Chimera software [18].

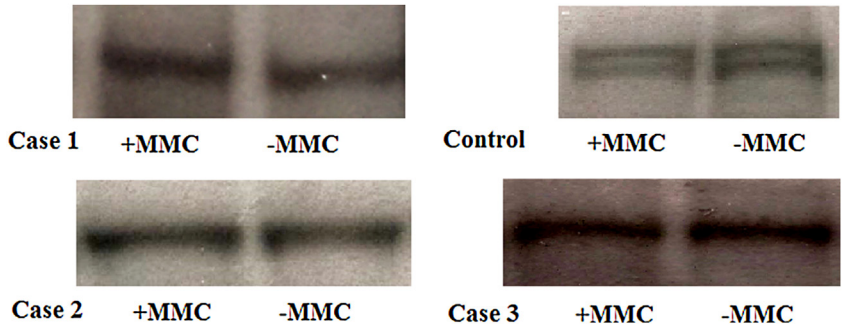

Fig. 1. Western blot for detection of FANCD2 monoubiquitination for the three FA-G cases and non-FA control samples. +MMC indicates monoubiquitination event in response to MMC induced DNA damage and −MMC indicates spontaneously occurring monoubiquitination in response to DNA damage during cellular processes.