Screening for mutations in two exons of *FANCG* gene in Pakistani population

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Background. Fanconi anemia is a rare autosomal recessive disorder of genetic instability. It is both molecularly and clinically, a heterogeneous disorder. Its incidence is 1 in 129,000 births and relatively high in some ethnic groups. Sixteen genes have been identified among them mutations in *FANCG* gene are most common after *FANCA and FANCC* gene mutations.
Objective. To study mutations in exon 3 and 4 of FANCG gene in Pakistani population.
Methods. Thirty five patients with positive Diepoxybutane test were included in the study. DNA was extracted and amplified for exons 3 and 4. Thereafter Sequencing was done and analyzed for the presence of mutations.
Results. No mutation was detected in exon 3 whereas a carrier of known mutation c.307+1 G>T was found in exon 4 of the *FANCG* gene.
Conclusion. Absence of any mutation in exon 3 and only one heterozygous mutation in exon 4 of *FANCG* gene points to a different spectrum of FA gene pool in Pakistan that needs extensive research in this area.

Key words: Fanconi anemia, *FANCG* gene, screening for mutation, diepoxybutane test

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INRODUCTION AND LITERATURE REVIEW

Fanconi anemia is a rare autosomal recessive disorder. Genomic instability is the characterstic feature of this disease and is manifested both at cellular level and clinically[1]. Cellular manifestations of genetic instability include chromosomal breakage, cell cycle disturbance and increased rate of somatic mutations while phenotypic manifestations include growth retardation, congenital malformations, bone marrow failure, high risk of neoplasia and premature aging[2]. Sixteen FA genes have been identified to date. Each of these corresponds to a different complementation group named as FANCA, -B, -C, -D1, -D2, -E, -F, -G, -I, -J, -L, -M, -N, -O, -P and -Q. These gene take part in the maintenance of genomic stability through Fanconi anemia pathway. A defect in the FA pathway arises due to mutations in any one of the sixteen genes[3]. FA is the most common inherited form of aplastic anemia[4]. The carrier frequency of Fanconi anemia is recently reported to be 1 in 181 that is 30% higher than the previously reported frequency of 1 in 300 in the American population. The incidence in heterozygotes is 1 in 129,000 births[5].

Clinically, Fanconi anemia is characterized by the presence of congenital physical anomalies, bone marrow failure and a predisposition to develop myeloid neoplasms and solid tumors[6]. The mean age for the onset of disease is seven years 1:1 male and female ratio[7]. Congenital physical anomalies include skin pigmentation, radial ray defects and other skeletal malformations, malformations involving the eyes, gastrointestinal tract, genitourinary tract, heart, oral cavity and central nervous system[8]. Approximately 30% patients present with no physical anomaly[9]. Hematologic abnormalities are the most common features of the Fanconi anemia present in 98% of patients. Severity of hematologic abnormality varies from single cytopenia to pancytopenia. The first encountered abnormality after birth is often macrocytosis followed by thrombocytopenia. Patients show features of stressed hematopoiesis such as raised MCV and HbF (ref.[10]). FA patients have a greater predisposition to develop squamous cell cancer (SCC) of head & neck, esophagus, female genital tract and certain liver tumors. It has been observed that there is 4.4 fold higher risk of developing SCC in patients who have undergone bone marrow transplantation[11].

The Fanconi Anemia pathway is a highly complex DNA repair pathway, activated in response to DNA damage to repair interstrand crosslinks (ICLs) during S-phase of the cell cycle[12]. The central event in the pathway is the monoubiquitination of FANCD2, one of the FA proteins. Ubiquitination is the process where a small protein ubiquitin covalently bonds to the target protein. More than half of FA proteins (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCM and FANCL) are required for the monoubiquitination of FANCD2. All these proteins together form FA core complex[13]. Ubiquitinated FANCD2-FANCI complex (ID-complex) localizes to the nuclear foci at the site of DNA damage. Nuclear foci are

structures that form at the site of DNA damage and are directly involved in the DNA repair mechanism. ID-complex recruits endonucleases such as FANCQ and ERCC1 to the ICLs for the DNA repair[14]. FANCD1, FANCJ and FANCN take part in repair downstream the ID complex[15]. In normal circumstances, FA pathway is set off by default, but it is rapidly activated when required. After completion of DNA repair, it is inactivated again. FA pathway is often restricted to S-phase of the cell cycle[16].

FANCG (OMIM # 602956) previously known as XRCC9, was first identified in Chinese hamster ovary (CHO) mutant UV40 cell line[17]. Its cytogenetic location is 9p13.3 and consists of 6,182 bases and 14 exons. It encodes a protein of mol. wt. 70kDa and 622 amino acids. FANCG is a constituent of the FA core complex. Structurally, it consists of seven tetratricopeptide repeat motifs (TPRs). The TPRs are important for assembly of core complex proteins[18]. FANCG is a phosphoprotein and phosphorylation occurs at serines 7, 383 and 387. Phosphorylation is thought to be important for cellular resistance to DNA crosslinks[19]. It interacts with FANCD2, BRCA2 and XRCC3 in another pathway known as Homologous - recombination pathway. This pathway is involved in resolving the double stranded breaks[20]. It causes disease by both homozygous and compound heterozygous mutations. FANCG is the third most common type of FA accounting for at least 10% of patients. Pathogenic variants in this gene are highly variable. Although, some alleles have been found more commonly in certain populations, for instance, c.307+1G>C is more frequently present in Japanese & Korean population[21], c.925-2A>Gin Brazilian population, c.1480+1G>C in French Canadian population[22,23] and c.637-643delTACCGCC in South African blacks[24]. Mutations in all 14 exons of FANCG have been reported. At least 35 patients have been reported with mutations in exon 3 and 10 patients with mutations in exon 4 (International Fanconi Anemia Mutation Database, accessed on 29-10-14). Seven mutations have been described in exon 3 including c.179delT (ref.[24]), c.212 T>C (ref.[22]), c.219_220insT, c.244dupG (ref.[25]), c.246delA (ref.[24]), c.271_272del (ref.[26]), c.307+1G>C (ref.[25]). Only 2 pathogenic variants have been described in exon 4 including c.313G>T (ref.[25]) and c.346_347del (ref.[22]).

METHODOLOGY

Study subjects

Genomic DNA samples were taken from individuals diagnosed with FA, following written informed consent. These studies were approved by the Institutional Review Board of the University of Health Sciences, Lahore. Genomic DNA was isolated from peripheral blood. Phenol/chloroform extraction and ethanol precipitation was used for the preparation of DNA. A total of 50 patients on the basis of clinical suspicion were initially recruited. The chromosomal breakage analysis came positive in 35 of them. Both male and female patients of all ages were included in the study. Patient biodata, family history, history of disease, general physical examination and laboratory findings were recorded in an intricately designed proforma.

Chromosomal breakage test

Chromosome breakage study was done using Phytohemagglutinin (PHA) stimulated lymphocyte cultures induced with Mitomycin C (MMC) with final concentration of 0.01mg/mL and incubated at 37 °C for 72 h. Cells were arrested with colchicine at metaphase stage, followed by hypotonic solution treatment with 10% potassium chloride, and cells were fixed with fixative (3:1 methanol: glacial acetic acid). At least 4 slides per culture were prepared by dropping suspension of cells in a tube on frosted slides and stained with Giemsa stain. A total of 20-25 metaphases per slide were scored under bright field microscope and chromosomal breakages and radial forms were recorded and compared with the negative control (or non-FA) sample each time.

Mutation screening of *FANCG* gene

Genomic DNA was extracted from peripheral blood using standard phenol chloroform method[27].

PCR was done using 1 µL DNA template, 10X standard Taq Reaction Buffer (2.5 µL), 10 mM dNTPs (1 µL), 10 µM forward primer (1 µL), 10 µM reverse primer (1 µL), Taq DNA Polymerase (0.3 µL), $MgCl_2$ (2.5 µL) and Nuclease free water (15.7 µL) at annealing temperature of 62 °C for exon 3 and 58 °C for exon 4.

Primers described elsewhere were used for the purpose of amplification as well as sequencing. Amplification products were analyzed using agarose gel electrophoresis and subjected to fluorescence based chain terminator sequencing method using Dye Terminator Cycle sequencing quick start kit (EN 608120) and were analyzed by CEQ ™ 8000 Genome Lab TM series Genetic Analysis System (Beckman Coulter). Sequences were evaluated using ensemble genome browser ENSG00000221829 and aligned to the human reference genome build hg 18 (NCBI36.1) using BLAST program (www.genome.ucsc.edu).

RESULTS

The study included 35 (17M: 18F) patients ranging in age from 4 years to 20 years. History of consanguinity was present in 69% of the patients. Low birth weight was observed in 21 (60%) patients. Short stature was observed in 26 (74.3%) patients. Skin abnormalities were present in 11 (31.4%) patients, all having café-au-lait spots as skin manifestation. Skeletal abnormalities were observed in 17 (48.6%) patients, 9 of which had hypoplastic thumb

Table 1. Baseline laboratory findings of patients.

Laboratory parameter	Median (range)
Hb (g/dL)	8.26 (3.80-13.80)
WBCs (x 10^9/L)	3.19 (0.90-9.1)
Platelets (x 10^9/L)	72.34 (150.0-320.0)

and 8 had bifid thumb. Microcephaly was observed in 19 (54.3%) patients. Microphthalmia was observed in 20 (57.1%) patients. Renal abnormalities were present in 4 (11.4%) patients. Of these, 3 had absent one kidney and 1 patient had bifid ureter.

As far as hematological manifestations are concerned, recurrent infections were the most common finding present in all the patients, followed by pallor in 91.4% of patients and then epistaxis in 54.3% of the patients. Baseline laboratory hematological findings are given in table 1.

Molecular findings

Sequencing of exon 3 was carried out to screen for the mutation c.307+1G >T, which is the most prevalent mutation in Japanese and Korean population and also found in 2 patients of Iranian ethnicity recently[28,29]. Sequencing analysis of exon 3 revealed no sequence alteration in our cohort of patients.

Sequencing of exon 4 was carried out to screen the mutation c.313G>T, which is the founder mutation in the German population. One of our patients was found to be heterozygous for this mutation. This patient was a female patient of 12 years of age diagnosed with FA at the age of eight years. She did not have any congenital physical abnormality and did not belong to a consanguineous pedigree, but she had low birth weight and short stature.

DISCUSSION

Fanconi anemia is the most common cause of congenital aplastic anemia. Due to its genetic and clinical heterogeneity, it has been a disease of great interest for researchers throughout the world[30]. Before the molecular era began, all the research regarding Fanconi anemia was directed towards its phenotypic appearance, but after 1990, most of the research has been focused on the genetics of Fanconi anemia. Sixteen different genes have been discovered to be involved in the pathogenesis of this disease. *FANCG* is the third most common gene involved in the pathogenesis of FA, first two being *FANCA* and *FANCC* respectively[31].

After a thorough literature review and to the best of our knowledge, this gene has never been probed before in Pakistan. The prevalence of this gene has been found to be variable in different populations. For example, it is around 10%, according to the IFAR (FA registry) developed in America[8] and 9% in a study done in Italy[31].

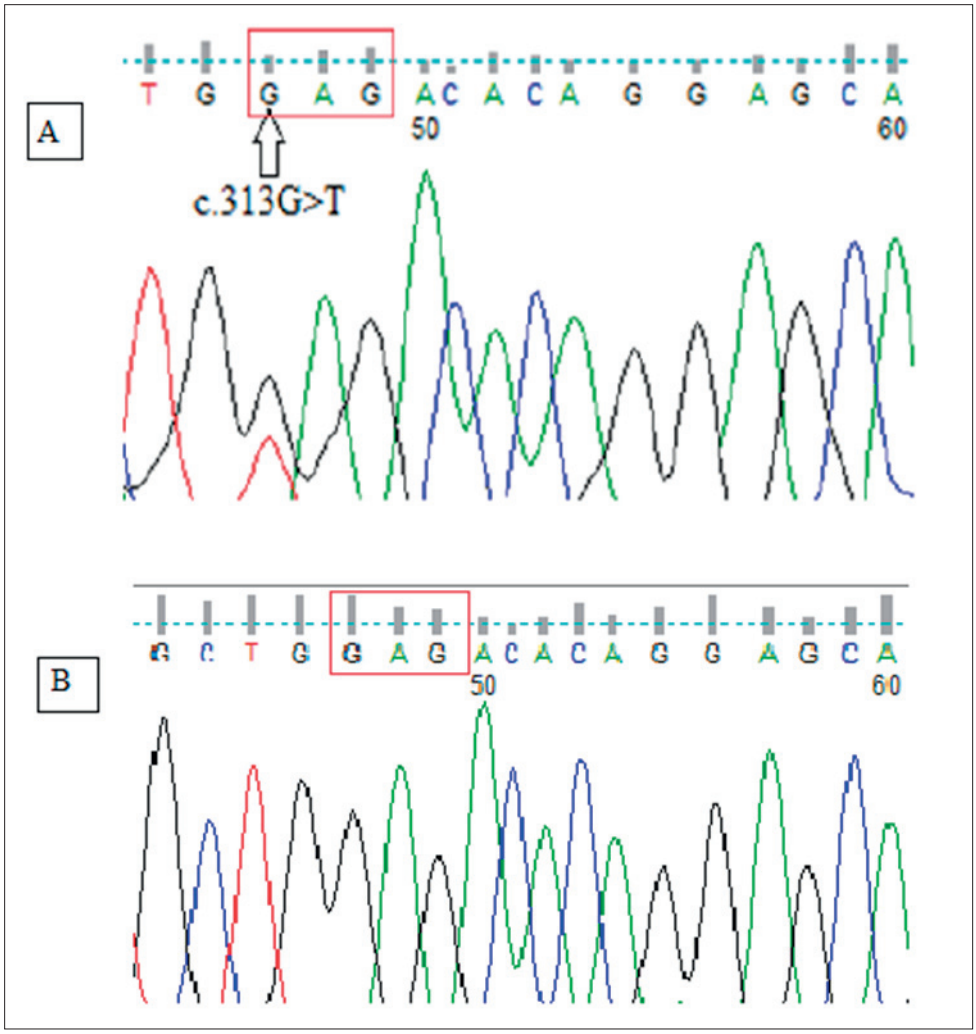

Fig. 1. Electropherogram showing sequencing of exon 4, A shows heterozygous mutation in patient FA-17 by arrow head, B shows the normal sequence in a control

However, it has been found to be much higher in some populations due to founder effects of certain mutations. For instance, the incidence of *FANCG* has been reported to be 77.5-82% due to the common founder mutation of c.637-643delTACCGCC in South African blacks[24]. This trend of variance in the prevalence of FA genes exists for all FA genes. For example, Ashkenazi Jews have the *FANCC* as the most prevalent genetic mutation because of the founder effect of IVS4 +4 A>T mutation[32]. A study done recently in Egypt on four exons of *FANCA gene* has revealed no mutation found to the researchers' surprise as FANCA is the most prevalent gene throughout the world so far[33]. FANCA has also been the most prevalent mutation found in our neighboring country India whereas FANCG has not been reported in any study done yet[34-37].

Researchers from our institution have already worked on *FANCA* and *FANCC* genes. Their work has shown *FANCA* mutations in 47% of patients and *FANCC* in 72% of patients[38]. So we decided to take up the third most prevalent gene i.e., FANCG. This gene comprises of fourteen exons. Mutations have been reported in all of them. Most frequently found mutation in this gene is c.307+1G>T mutation (Fanconi anemia data base accessed on 27th March 2016 http://www.rockefeller.edu/fanconi/genes/jumpg). This is also the founder mutation in Japanese and Korean populations and is found to have a common ancestor haplotype in East Asia[39]. This mutation is also reported in 2 patients from Iran[29]. Therefore, taking into consideration the prevalence of this mutation in Asian countries and especially in Iran, it was decided to probe into this mutation in our country. However, we did not find this mutation in any of our patients in homozygous or in the heterozygous state. Another common mutation is c.313G>T, which is also a founder mutation in a German population[21]. In our study, we found a heterozygous c.313G>T mutation.

This variance in prevalence of gene mutations can be explained on two bases. Firstly, that we have sequenced only two exons of the *FANCG* gene, whereas others have studied all fourteen exons. Secondly, this heterogeneity can also be due to difference in geographic and ethnic origin as Indians have also not found any *FANCG* mutation to date.

As far as the phenotype of FA is concerned, it is a phenotypically heterogeneous disease. Each patient of FA is presented with a unique set of physical characteristics. Median age at diagnosis in our study is 11 years with a range of 4-20 years similar to the finding of Solomon *et. al*., conducted in India. However, this finding is exceptionally different from studies done in western countries where the median age at diagnosis is 6 years[40]. This difference is indicating the trend of late diagnosis of FA in Southeast Asia. Boost in the knowledge of physicians about the physical findings of FA is needed to enable them to make early diagnosis because most of the patients in our country present when there is bone marrow failure.

Despite clinically well documented congenital and somatic physical abnormalities, most of the patients of FA present with bone marrow failure[41]. The most common presenting complaint in our cohort of patients has been recurrent infections (present in 100% of patients) and bleeding tendencies (present in 82% of patients) with the most frequent site of the bleeding being nose. This finding is consistent with most other publications[32,37]. Pallor is also a very frequent finding in most studies done worldwide[40]. We also observed pallor in more than 50% of patients. Although, bleeding tendency and recurrent infections are manifestations of thrombocytopenia and neutropenia, we found anemia to be more severe in our patients at the time of diagnosis consistent with the finding of Feben et al. Most of our patients came with severe aplastic anemia at first presentation. This finding is similar to the most other publications[37].

Hematologic malignancies and solid tumors are a frequent finding in Fanconi anemia patients and a lot of work has been done on this aspect[6,42]. We did not find any patient with hematologic or solid tumors. Most of the studies done on malignancies found in FA were retrospective or a long term follow-up studies, whereas our study was a descriptive study and purposive sampling was done. Our study did not include any long term follow-up. Moreover, solid tumors tend to develop later in the course of disease. Median age of onset of solid tumors is around 30 years in literature[43]. But in our part of world patients hardly cross the age of 20 years, mainly because of limited availability of bone marrow transplant and other health facilities. It is reported that there is a 13 % chance of developing AML in patients of FA by age 50 with a maximum number of patients between 15 and 35 years[44]. Our patients had a median age of 11 years, too young to show such development.

CONCLUSION

We report the presence of one heterozygous patient carrying c.313G>T mutation in *FANCG* gene.

This is the first report of FANCG gene mutation in Pakistan that is confirmed molecularly. We have also described the phenotypic presentation of FA patients in our country. Current study also revealed that most of the patients in Pakistan seek for medical aid very late and diagnosed as patients of Fanconi anemia when they have developed complications due to bone marrow failure.

Limitations

Financial and time restraints were the major limitation of our research.

Our study includes 35 FA patients, which is a small sample size. However, given the rarity of the disease, this sample size was deemed adequate for the research.

Our study mostly includes patients who presented to the hospital for severe cytopenias, thus they represent the severe spectrum of the disease. So our data do not truly represent the phenotype of the disease.

Author contributions: UA: sample collection, experiment, data analysis and manuscript writing; SI: sample collection, writing and proof reading of manuscript; IA: sample collection and manuscript writing. SK: study planning, experiment and data analysis. NA, NiA: sample collection,

clinical assessment of the patients and experiment; SM: conception, planning and execution of the study.
Conflict of interest statement: None declared.

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