Original Article

Profiling Fanconi Anemia Gene Mutations among Iranian Patients

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Abstract

Background: Fanconi anemia (FA) is a rare genetic syndrome characterized by developmental defects, bone marrow failure, and a high cancer risk. FA is usually inherited as an autosomal recessive condition. This disease is genetically heterogeneous and mutations in 16 different genes have been identified in FA patients to date. An accurate diagnosis needs detection of pathogenic variations in the FA genes along with positive results from chromosome breakage test.

Methods: In this study, 48 families with at least 2 affected FA patients and positive chromosome breakage test were enrolled from the Iranian population. Molecular analysis of FA genes was performed using Next Generation Sequencing (NGS) method and Multiple Ligation Dependent Probe Amplification (MLPA).

Results: Causal mutations for 30 (63%) patients were identified in homozygous or compound heterozygous forms. *FANCA* had the highest mutation frequency rate (83%) followed by *FANCG* (10%), *FANCD2* (3%) and *FANCL* (3%). A significant proportion (44%) of *FANCA* mutations were large rearrangements.

Conclusion: Genetic testing for FA patients improves the accuracy of diagnosis and also will be essential for genetic counselling and prenatal diagnosis for future pregnancies in the family. Availability of NGS technology has made the screening of all known FA genes at once more practical and affordable.

Keywords: Fanconi Anemia, *FANCA*, *FANCG*, gene panel sequencing, Iran

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Introduction

Fanconi anemia (FA) is an autosomal recessive disease with an incidence of one in 350,000 births with a higher frequency in Ashkenazi and South African blacks.[1] This abnormality is characterized by progressive pancytopenia, multiple congenital abnormalities and increased susceptibility to acute myelogenous leukemia, epithelial cancers, particularly in the head and neck, urinary and reproductive systems. Other signs or symptoms of this genetic disorder are mental retardation, short stature, radial aplasia, urinary tract abnormalities, hyper pigmentation and developmental delay.[2] A unique characteristic that distinguishes FA from other chromosome breakage syndromes is the cellular hypersensitivity to DNA cross-linking agents causing chromosome breakage.[3] The clinical features of FA are highly variable, even among individuals within the same family or among patients within the same complementation group,[4] and for this reason, it is helpful to identify the causal genetic mutations in patients with abnormal chromosome breakage test for confirming the diagnosis in these patients.

Currently, mutations with recessive mode of inheritance in 16 different genes are known to cause FA with a total of 307 exons[5,6] (Table 1). All these genes belong to homologous recombination pathway which is involved in repair of DNA double strand breaks.[7] All FA genes are located on autosomal chromosomes except *FANCB* which is on chromosome X.[8]

FANCA gene is the most commonly mutated gene among FA patients and accounts for 60%–70% of cases. *FANCC* and *FANCG* are the next commonly mutated genes which are responsible for 20% of FA patients. Mutations in other FA genes are rare and each gene mutation accounts for only 1%–3% of cases.[2]

Since FA is a chronic life threatening disorder with an increased risk for cancer, determining the causal mutations will be helpful in more accurate diagnosis, prenatal diagnosis and also carrier detection in individuals with a family history of FA. Genetic diagnosis is also important in cases with false negative or inconclusive chromosome breakage test results.[9] The conventional Sanger sequencing-based mutation screening approach for FA is time-consuming, costly, and impractical. Currently, with the use of next generation sequencing (NGS) technology, more comprehensive genetic diagnostic tests could be offered to FA patients.

To the best of our knowledge, there is no previous report of molecular analysis in FA patients in the Iranian population. We report here the molecular genetics results of 48 non-relative FA patients enrolled into the Kariminejad-Najmabadi Pathology and Genetics Center, Tehran, Iran. We screened all the 16 known FA genes in germ-line DNA of these patients using targeted NGS. Additionally, Multiple Ligation Dependent Probe Amplification (MLPA) screening was performed for detecting large rearrangements in *FNACA* gene that could not be identified by sequencing.

Materials and Methods

Study Subjects

Among families who were referred to Kariminejad-Najmabadi

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Pathology and Genetics Centre in 2005–2011 and were suspected to have FA, 48 families were confirmed to have chromosome breakage by cytogenetic approaches. All the subjects or their legal guardians signed consent form and genomic DNA was extracted from blood cells of the family members following the standard salting out procedure. Our families were from different parts of Iran and had at least two affected children. Affected individuals showed short stature, abnormalities of the skin, arms, head, eyes, kidneys, and ears, and developmental disabilities.

The study was approved by the regional ethics committee of University of Social Welfare and Rehabilitation Sciences for Medical Research, Tehran, Iran.

Targeted sequencing

The entire 290 coding exons of all 16 known FA genes (Table 1) plus 20 bp of their flanking regions at both 3' and 5' sides were sequenced. We used AmpliSeq custom designed PCR-based method for target enrichment. A set of 588 primer pairs was designed to amplify the 290 coding exons with a mean amplicon size of 142 bp (range 67–182 bp). All 588 PCRs for each DNA sample were done in two separate pools of multiplex PCR. Each enriched DNA library was tagged with a unique oligonucleotide barcode and each 12 barcoded DNA libraries were pooled and sequenced by Ion Torrent sequencer on a 316 chip. The mean depth of coverage for the targeted region ranged between 106× and 497× with an average of 282× for all 48 samples. The coverage of the targeted region at 20× was between 92% and 97% with an average of 94% for all tested samples. Variants were called in the targeted regions with at least 20× depth of coverage. Variants with alternative allele ratio of less than 0.25, genotype quality of less than 20 or Phred scaled Fisher strand bias of greater than 10 were filtered out. The variant calling was done using Torrent Suite Ver3.4 (ThermoFisher Scientific Inc., Waltham, MA, USA). The SNP & Variation Suite (GoldenHelix Inc., Bozeman, MT, USA) was used for annotating called variants. We removed all the common variants (Minor Allele Frequency, MAF > 1%) reported in public databases and kept our focus on rare variants only (MAF ≤ 1). For this purpose, we used the dbSNP 141 database (http://www.ncbi.nlm.nih.gov /projects/SNP/), Genome 1000 Project (http://www.1000genomes .org/) and NHLBI (National Heart, Lung, and Blood Institute) Exome Sequencing Project (ESP6500, http://evs.gs.washington.e du/EVS/). C scores were calculated by Combined Annotation Dependent Depletion (CADD) algorithm for predicting the functional effect of missense mutations.[10] Missense mutations with a C score higher than 15 were considered to be potentially pathogenic.[10] All the identified pathogenic mutations were confirmed by Sanger sequencing. Sanger sequencing reactions were performed using a BigDye Terminator v3.1 Cycle Sequencing Kit (ThermoFisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's protocol. Sequencing products were analyzed on the ABI prism 3500XL Genetic Analyzer (ThermoFisher Scientific Inc., Waltham, MA, USA).

Multiplex ligation-dependent probe amplification

Since *FANCA* large rearrangements are responsible for about 15%–20% of FA cases,[11] we also performed MLPA for *FANCA* gene for unresolved cases. MLPA was performed using the Salsa MLPA Kit with the probe mix P031 and P032 for *FANCA* gene, using the manufacturer's protocol (MRC Holland Inc., Amsterdam, The Netherlands). The separation and quantification of the MLPA products were performed using ABI Genetic Analyzer 3500XL. The MLPA data were analyzed using Gene Marker V1.97.

Results

Genetic testing of the 16 known FA genes (Table 1) in 48 non-relative FA patients allowed us to identify causal mutations for 30 (63%) of the studied patients and their families. Of those, 25 (83%) had *FANCA* mutations, 3 (10%) had *FANCG* mutations and one patient had mutations in each of the *FANCL* and *FANCD2* genes (Tables 2 and 3). Of all the mutations identified, 20 were novel and 13 were listed in the FA mutation database (http://www.rockefeller.edu/fanconi/mutate/). Of the total 30 patients with identified causal mutations in known FA genes, 27 (90%) were homozygous and just 3 (10%) were compound heterozygous (Tables 2 and 3).

Table 1. List of 16 known Fanconi anemia genes which were screened among 48 Iranian FA patients.

Gene	Cytogenetic Location	Complementation Group	OMIM ID
FANCA	16q24.3	A	607139
FANCB	Xp22.31	B	300515
FANCC	9q22.3	C	613899
BRCA2	13q12.3	D1	600185
FANCD2	3p25.3	D2	613984
FANCE	6p21.3	E	613976
FANCF	11p15	F	613897
FANCG	9p13	G	602956
FANCI	15q26.1	I	611360
BRIP1	17q22	J	605882
FANCL	2p16.1	L	608111
FANCM	14q21.3	M	609644
PALB2	16p12.1	N	610355
RAD51C	17q25.1	O	602774
SLX4	16p13.3	P	613278
ERCC4	16p13.12	Q	133520

Table 2. FA gene mutations identified by next generation sequencing among 48 Iranian FA patients.

Patient ID	Consanguine Parents	Gene	Exon	DNA Change	Protein Change	Mutation Type	Genotype	Reported in FA Mutation Database*
D43067	*Yes*	*FANCA*	38	c.3791_3793delCCT	p.Ser1264del	Inframe deletion	Compound heterozygous	Not reported
			23	c.2057delC	p.Ala686Valfs* 38	Frameshift deletion	Compound heterozygous	Not reported
D42672	*Yes*	*FANCG*	6	c.647-2_649delAGGTC	Not Known	Splice site deletion	Homozygous	Not reported
D45021	*Yes*	*FANCA*	40	c.3993delG	p.Pro1332Leufs*31	Frameshift deletion	Homozygous	Not reported
D45335	*Yes*	*FANCA*	38	c. 3781_3785delTTCTT	p.Phe1261Leufs*15	Frameshift deletion	Homozygous	Not reported
D46069	*Yes*	*FANCA*	30	c.2915G>T	p.Gly972Val+	Missense substitution	Homozygous	Not reported
D47072	*Yes*	*FANCA*	40	c.4006T>G	p.Tyr1336Asp†	Missense substitution	Homozygous	Not reported
D47178	*No*	*FANCA*	3	c.190-2A>G	Not Known	Splice site mutation	Homozygous	Not reported
D48027	*Yes*	*FANCA*	33	c.3348+1G>T	Not Known	Splice site mutation	Homozygous	Not reported
D49068	*Yes*	*FANCG*	2	c.85-2A>T	Not Known	Splice site mutation	Compound heterozygous	Not reported
			4	c.510+1G>T	Not Known	Splice site mutation	Compound heterozygous	Not reported
D49151	*Yes*	*FANCG*	14	c.1772delT	p.Leu591Argfs*3	Frameshift deletion	Homozygous	Not reported
D49237	*Yes*	*FANCL*	4	c.217-2A>G	Not Known	Splice site mutation	Homozygous	Not reported
D42930	*Yes*	*FANCA*	41	c.4124_4125delCA	p.Thr1375Serfs*49	Frameshift deletion	Homozygous	Reported[2]
D43539	*Yes*	*FANCA*	38	c.3788_3790delTCT	p.Phe1263del	Inframe deletion	Homozygous	Reported[2,16,17]
D44329	*Yes*	*FANCA*	11	c.987-990delTCAC	p.His330Alafs*4	Frameshift deletion	Homozygous	Reported[17]
D44709	*No*	*FANCA*	33	c.3348+1G>A	Skipping of exon33	Splice site mutation	Homozygous	Reported[18,19]
D44948	*Yes*	*FANCA*	42	c.4261-2A>C	Skipping of exon42	Splice site mutation	Homozygous	Reported[18]
D47537	*Yes*	*FANCA*	33	c.3286C>T	p.Gln1096*	Premature stop gain	Homozygous	Reported[17]
D49003	*No*	*FANCA*	37	c.3696delT	p.Phe1232Leufs*15	Frameshift deletion	Homozygous	Reported [16]
D44627	*Yes*	*FANCD2*	26	c.2444G>A	p.Arg815Gln	Missense substitution	Homozygous	Reported[20]

* Fanconi Anemia Mutation Database (http://www.rockefeller.edu/fanconi/mutate/)

+ The FANCA p.Gly972Val has not been reported in any of NHLBI Exome sequencing project, G1000 project and dbSNP141 databases. The C score for this variant based on CADD algorithm[10] for predicting functional effect of missense mutations is 15.35.

† The FANCA p.Tyr1336Asp has not been reported in any of NHLBI Exome sequencing project, G1000 project and dbSNP141 databases. The C score for this variant based on CADD algorithm[10] for predicting functional effect of missense mutations is 17.00.

Table 3. FANCA large rearrangements identified in 48 Iranian FA patients

Family Number	Large rearrangement	Genotype	Reported in FA Mutation Database*
D43306	*FANCA* Ex 1-22 del	Homozygous	Not reported
D43420	*FANCA* Ex30 del	Homozygous	Reported[12]
D43567	*FANCA* Ex1-44 del	Homozygous	Reported[16]
D44144	*FANCA* Ex30 del	Homozygous	Reported[12]
D44004	*FANCA* Ex1-26 del	Homozygous	Not reported
D44550	*FANCA* Ex1-22 del, *FANCA* Ex18-20 del	Compound Heterozygous	Not reported, Reported[2]
D45531	*FANCA* Ex7 del	Homozygous	Not reported
D46114	*FANCA* Ex20-22 del	Homozygous	Not reported
D46088	*FANCA* Ex20-28 del	Homozygous	Not reported
D48898	*FANCA* Ex1-22 del	Homozygous	Not reported
D49107	*FANCA* Ex16-22 del	Homozygous	Reported[17]

* Fanconi Anemia Mutation Database (http://www.rockefeller.edu/fanconi/mutate/)

FANCA mutations

From a total of 48 studied families, 25 (52%) had mutations in *FANCA* gene that were identified by combination of MLPA and targeted NGS sequencing; of those, 23 patients had homozygous mutations and the other 2 were compound heterozygous. Of the total 27 *FANCA* mutations identified among 25 patients, 15 (56%) were novel and not previously reported in the FA Mutation Database.

Small Nucleotide changes: In 8 families, we identified nine novel variants in *FANCA* gene, seven homozygous and two-compound heterozygous. Of those, one was missense substitution (c.4006T>G), one was nonsense variant (c.2915G>T), three were frame-shift deletions (c.2057delC, c.3993delG and c.3781_3785delTTCTT), three were splice site mutations (c.190-2A>G, c.3348+1G>T and c.4261-2A>C) and the last mutation was an in-frame deletion (c.3791_3793delCCT) (Table 2). We also identified six known *FANCA* mutations in six families, all homozygous. Of those, three were frame-shift deletions (c.4124_4125delCA, c.987_990delTCAC and c.3696delT), one was splice site mutation (c.3348+1G>A), one was an in-frame deletion (c.3788_3790delTCT) and the last one was a premature stop gain mutation (c.3286C>T) (Table 2).

***Large Rearrangements*:** Using MLPA method, we found 12 different large rearrangements in *FANCA* gene among 11 families: 10 homozygous and two-compound heterozygous. We found deletions ranging from a single exon loss, such as exons 7 and 30 to deletion of the entire *FANCA* gene (Table 3).

FANCG mutations

Four different *FANCG* mutations were found among three FA patients, two homozygous and two-compound heterozygous and all were novel (Table 2). These mutations included a frame-shift deletion (c.1772delT) and three splice site mutations (c.647-2_649delAGGTC, c.85-2A>T and c.510+1G>T).

FANCD2 and FANCL Mutations

Of the 48 FA patients tested, only one patient was found to have a mutation in each of the *FANCD2* and *FANCL* genes and both were homozygous. The *FANCD2* mutation was a missense mutation (c.2444G>A, p.Arg815Gln) which was reported several times in the FA mutation database. The *FANCL* mutation was a novel splicing mutation (c.217-2A>G) seen in a 4-year-old girl with FA.

Discussion

We screened all 16 known FA genes among 48 FA patients having clinical features of the disorder plus positive chromosome breakage test. In these patients, we could find causal mutations for 30 (63%) of them. For the 30 FA patients with confirmed molecular diagnosis, 25 (83%) patients had *FANCA* mutations of which 14 had a small number of nucleotide changes identified by NGS and the other 11 had large rearrangements detected by MLPA. The other 5 (17%) patients with confirmed molecular diagnosis had mutations in three different FA genes including *FANCG*, *FANCD2* and *FANCL*.

In this study, molecular genetic analysis of FA was performed for the first time in Iran. Our results showed that the FA diagnosis could be confirmed genetically in the majority of patients (63%) who have been diagnosed clinically. As expected, the majority of the patients (83%) had *FANCA* mutations which is relatively higher than other reports indicating that *FANCA* mutations account for 60%-70% of FA patients.[2,4] To provide more accurate results and improve diagnosis for FA patients, we also analyzed large deletions in *FANCA* gene in patients who did not have any small nucleotide changes on any of the 16 known FA genes. We found that large rearrangements are responsible for 44% of the FA patients with *FANCA* mutations. This is comparable with what was reported in the Japanese population.[12] In that study, 77% of unrelated Japanese FA families had *FANCA* mutations, of which 30% were large deletions in *FANCA* gene. In a recent study, 202 FA patients were tested and large deletions were found in *FANCA*, *FANCC*, *FANCD2*, and *FANCB* genes among 35% of the tested patients.[13]

Mutations in three FA genes (*FANCA*, *FANCC*, and *FANCG*) were screened among 30 Korean FA patients using direct sequencing and MLPA and almost half of the patients were *FANCA* mutation carriers and the other half were *FANCG* mutation carriers.[14] No *FANCC* mutation carrier was found in that study which is comparable with our findings.

We could not find the causal mutations for 18 of the total 48 tested FA patients. There are several possible explanations for

this. First, some of these patients could have mutations in any of the 16 known FA genes that have been missed and we could not detect them. This could be small nucleotide changes missed by NGS or large rearrangements in genes other than FANCA that have not been tested for large rearrangements in our study. Second, it is possible that some of those patients have a diagnosis other than FA such as other chromosome breakage syndromes including ataxia telangiectasia, xeroderma pigmentosum or Bloom syndrome.[15] Finally, it is possible that there are other unknown FA genes and some of these patients may have mutations on them.

Genetic testing for FA is a valuable tool for families with FA patients from several aspects. First, it confirms the diagnosis of FA among those patients with inconclusive chromosome breakage test or those with clinical features overlapping with other chromosome breakage tests. Second, knowing the family mutations could be used for prenatal screening in future pregnancies and avoiding the birth of another child with FA in the family. Finally, the heterozygous carriers among the extended family of the FA patients could be identified by knowing the exact family mutations to avoid familial marriages which could potentially result in having a child with FA in future. This could be important for consanguineous populations such as Iran in which marriages inside extended families are common.

In conclusion, NGS-based gene panel testing for all FA genes in combination with MLPA for *FANCA* could detect causal mutations for the majority of patients. Considering the benefits of knowing the causal genetic mutations for confirming the diagnosis for the patients and also genetic counselling and prenatal diagnosis for future pregnancies in the families plus the affordability of the genetic testing using NGS technology, molecular genetic testing should be offered to all clinically diagnosed FA patients.

Conflict of interest statement

All authors declare the absence of conflict of interests

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