Trials for Implementing Cost-effective Fanconi Anemia (FA) Molecular Diagnosis

Original Article

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ABSTRACT

Background: Fanconi anemia (FA) is an inherited rare disorder (1 in 100,000 to 250,000 births) where cells cannot correctly repair interstrand crosslinks (ICLs) resulting in genomic instability that can lead to bone marrow failure, and/or solid tumors. FA is associated with known mutations in at least 22 FA identified genes which occur more frequently among communities with consanguineous marriages as Egypt. We aimed to introduce immunodetection & Western-blotting to identify FA genetic subtypes and then target the pathogenic mutations on the molecular level among the studied patients.

Patients and Methods: Five patients (4 females and one male) clinically diagnosed as FA were referred from the Hereditary Blood Disorders Clinic (HBD) for confirmation by chromosomal breakage analysis using Diepoxybutane (DEB). Using western blotting, FANCA protein was detected in skin fibroblasts derived from FA and controls. Molecular confirmation using Multiplex ligation-dependent probe amplification (MLPA) to screen deletion mutations in the *FANCA* gene followed by a targeted panel includes three *FANC* genes.

Results: Immunodetection of FANCA protein in four patients showed different patterns of FANCG and FA associated peptide 24 (FAAP24). Two patients with the same mutation showed a similar profile of FA core complex detected by FANCA antibody. Two patients had a normal profile. Confirmation by MLPA detection of intragenic homozygous deletions of *FANCA* gene. NGS targeted panel characterized two *FANCA* and one *FANCL* variants.

Key Words: FANCA, FANCL, fanconi anemia, genetic subtypes, immunodetection, western blotting.

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INTRODUCTION

Fanconi anemia (FA) is the most frequent inherited bone marrow failure (IBMF) disorder associated with chromosomal instability, thus cells cannot repair the inter-strand crosslinks (ICLs) correctly causing DNA damage followed by genomic instability and the predisposition to cancer (**Olson, 2020; Gueiderikh *et al*., 2017; Ceccaldi *et al*., 2016; Bogliolo and Surralles, 2015**;). FA frequency varies from 1/350,000 to 1/89 in consanguineous populations as Africans (**Siddiqui and Shamsi, 2019**).

Fanconi anemia is characterized by various clinical manifestations including; short stature, radial ray affection, vertebral anomalies, café au lait spots, microphthalmia, microcephaly and cardiac defects (**Nalepa *and Clapp,* 2018**). However, hematological abnormalities are the hallmark of FA. Signs of progressive BMF as bleeding, recurrent infections and severe anemia are evident at a median age of 7 years (**Quentin *et al*, 2011**), usually associated with poor survival. 20% of FA patients experience acute myeloid leukemia by the age of 20 years, others presented with leukemia & solid tumors as Wilms tumor & medulloblastoma in early onset (2-3 years) (**Siddiqui and Shamsi, 2019; Neveling *et al*., 2009; Alter *et al*., 2007**). Diagnosis of FA is confirmed by hypersensitivity to the clastogenic DNA cross-linking agents as Mitomycin C and Diepoxybutane (DEB) (**Auerbach, 2015**). Fanconi anemia (FA) disorder is caused by known mutations in at least 22 FA identified genes (**Jung *et al*., 2020**), including 18 well-known genes (FANC-A, B, C, D1, D2, E, F, G, I, J, L, N, P, Q, T, U, V, W). The most commonly mutated genes in FA are *FANCA, FANCC*, and *FANCG*; these involved up to 80-90% of all FA cases. FANCA mutations account for nearly 60% of cases worldwide (**Bogliolo *et al*., 2020; Olson, 2020**). Functionally the proteins encoded by these genes is to repair the inter-strand crosslinks (ICL) that typically form during DNA replication and transcription (**Olson, 2020**) through pathway known as the Fanconi pathway (**Pilonetto *et al*., 2017; Solanki *et al*., 2016; Esmail, *et al*. 2016; Amouri *et al*., 2014; Park *et al*., 2013; Castella *et al*., 2011**). A large nuclear FA pathway Core complex (CC) is formed of eight of the upstream complementation

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genes (*FANCA/B/C/E/F/G/L/M*). Mutations in CC cause the complex to be nonfunctional and disrupt the entire FA pathway (**Nicholas *et al*., 2017**).

Molecular subtyping is critically needed for diagnosis and management of FA patients however, it is not an easy task because the disease is caused by hereditary patterns characterized by different point mutations and large genomic deletions in nearly 22 genes. Various molecular screening tools could be used starting from PCR-direct sequencing, Multiplex ligation-dependent probe amplification (MLPA) progressing to NGS through whole-exome sequencing (WES) or targeted exome sequencing (targeted-seq) (**Rio *et al*., 2019**). We aimed at establishment of an economic method for subtyping FA with the cost-effective immunohistochemistry and Western blotting to detect the altered protein expression in patients' skin fibroblasts. In addition to the detection of pathogenic gene variants using different molecular tools.

PATIENTS AND METHODS:

Five patients clinically diagnosed as FA on the basis of clinical features including progressive pancytopenia, characteristic congenital abnormalities such as radial defects, skin pigmentation, and affected growth.

Patients were recruited from the HBD clinic, the Medical Research Excellence Centre of National Research Centre (NRC) of Egypt over a period of more than two years. Written informed consents were obtained from all participant' legal guardians. This study was approved by the institutional Review Board of NRC according to the Helsinki Declaration. In all cases, a diagnosis of FA was confirmed cytogenetically through the demonstration of increased chromosomal breakage following exposure to the clastogenic diepoxybutane (DEB) (**Auerbach, 2015**). In addition, five healthy individuals of matching age and sex were included as control group.

Methods:

Skin fibroblast culture

Skin fibroblasts were cultured from skin biopsies under the standard conditions. Briefly, the cells were cultured in RPMI medium with 10 % FBS and 1 % penicillin/ streptomycin. The cells were grown at 37℃ and 5 % CO2. The cells were harvested at 70-80 % confluence.

Western blot

Fifty micrograms of proteins extracted from fibroblasts were loaded onto 4-12 % SDS-PAGE gel, along with a molecular weight marker. Proteins were transferred on a nitrocellulose membrane. The membrane was incubated overnight with appropriate 1:5,000 dilution of rabbit anti-FANCA. The membrane was incubated with 1:2,500 dilution of anti-rabbit at room temperature for 1 h. The signal was developed by performing a chemiluminescent reaction. Images were acquired using CDD camera for chemiluminescence detection.

Molecular Genotyping of FA Patients:

The molecular genotyping was performed according to the assigned complementation group to allow identification of causative mutations in our FA patients as follow:

DNA extraction from collected blood samples using QIAgene extraction Kit following the manufacturer's instructions. DNA concentration and quality was assessed using Nanodroppe Nanodrop 2000 (Thermo Scientific).

Multiplex Ligation-Dependent Probe Amplification (MLPA) tests for FANCA gene.

Patients subtyped as complementation group FANCA were first studied using Multiplex ligation dependent probe amplification SALSA MLPA kits (P031 & P032 FANCA) for *FANCA* gene for the detection of possible *FANCA* gene deletions. Target DNA was denatured for 5 minutes at 98°C, probe mix was be added, after which the mixture was heated for 1minute at 98°C and incubated at 60°C overnight (16 hr); after addition of ligase, the mixture was incubated at 54°C for 15minutes. Ligase was subsequently inactivated at 98°C for 5 minutes. Next, ligation product was transferred to PCR mix. The PCR reaction was carried out for 35cycles (30 seconds at95°C, 30seconds at 60°C, and 60seconds at 72°C). The fragments were analyzed on an ABI model 3500 capillary sequencer (Applied Biosystems) using gene scan-TAMRA 500-size standard (Applied Biosystems). Data analysis was performed according to the recommended method (http: //www. mrc-holland.com) on Coffalyser. Net software.

Targeted NGS of FANCA, FANCG, FANCL genes using a customized panel:

FA patients who did not harbor *FANCA* gene deletion were sequenced for the whole coding regions of *FANCA, FANCG, FANCL* and their flanking intronic regions using targeted NGS (Illumina, USA).

Detection of causative variants:

Using the established pipeline (Genomon-exome, http:// genomon.hgc.jp/exome/), sequence reads were aligned to the hg19 reference genome using the Burrows-Wheeler Aligner; variants were detected using our in-house variant caller. Variant allele frequency (VAF) >0.2 (20%) was used as the cut-off value. Following the guidelines published by the American College of Medical Genetics and Genomics, we removed common single-nucleotide polymorphisms (SNPs) showing minor allele frequency values of more than 1% in (i) the ESP6500 exome variant server (the National Heart, Lung, and Blood Institute Exome Sequencing Project, Seattle, WA; http:// evs.gs.washington.edu/EVS/,

as of April 2014); (ii) the 1000 genomes project; or (iii) our in-house SNP database. These variants were considered the causative variants that were previously reported to be pathogenic (category 1) or were other- wise highly expected to cause the associated disorders (e.g., nonsense, frameshift, and splice site variants) (category 2). Other variants of unknown significance such as missense variants without further evidence of pathogenicity were treated as non-diagnostic in this study. For the specific pathogenicity of each variant, we used the Human Genome Mutation Database (http://www.hgmd.cf.ac.uk/, as of March 2014) and performed an extensive search of the literature in PubMed (http://www. ncbi.nlm.nih.gov/pubmed). Finally, causative variants were validated by Sanger sequencing.

RESULTS:

The study included five FA patients (four females and one male), the age range was from 6 to 10 years. All the patients were descending from unrelated consanguineous families. The age of disease onset ranged from 1 to 7 y. Clinical findings of the patients are cafe´ au lait spots and hyperpigmentation observed in all of studied patients in addition to multiple skeletal defects as microcephaly and abnormalities in the thumb. They all suffered from pancytopenia, with average values of 7.7g/dl for hemoglobin, 2.6 x106/ml for RBC, 4x103/ml for WBC, and 45x103/ml for platelet count.

Skin fibroblasts derived from FA patients had normal morphology compared to the control:

The cultured Skin fibroblasts from FA patients showed similar morphological features and cell growth rate observed in those derived from healthy subjects (Figure 1).

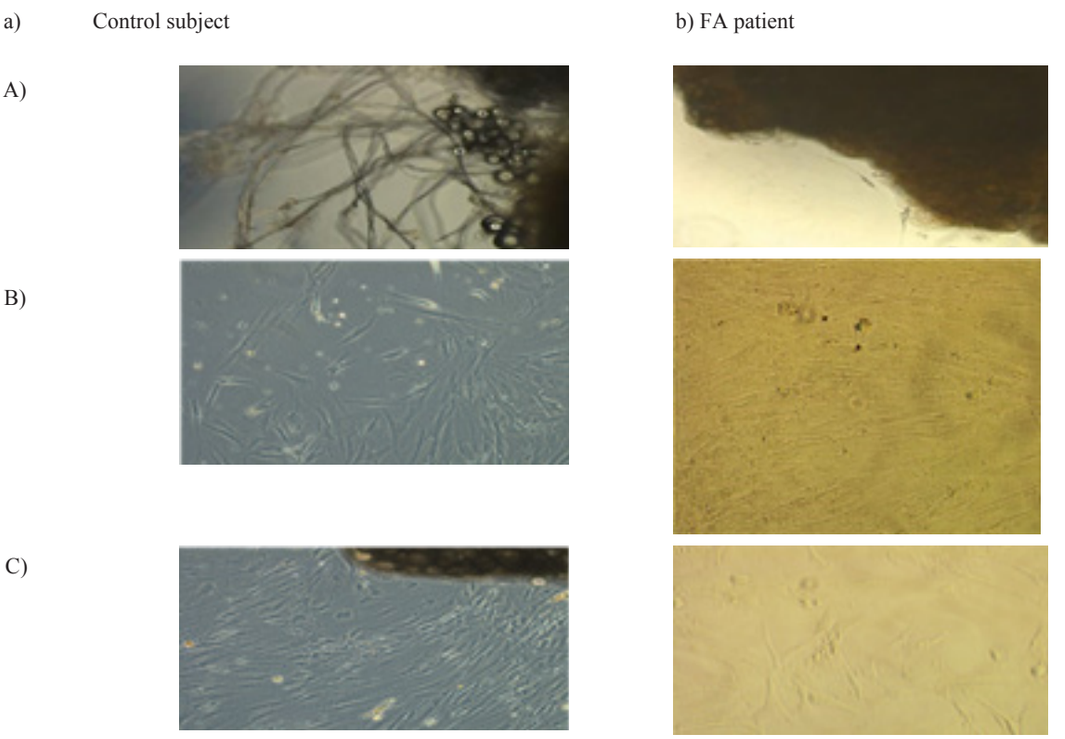

Fig. 1: Images of the stages of growing human skin fibroblasts from a skin biopsy obtained from one of the control subjects (left) and one FA patient (right). A) Skin biopsy day 2. B) Skin biopsy 1 week. C) Skin biopsy 2 weeks.

Immunodetection of FANCA protein

One band at an approximate molecular weight (MW) of 65 kDa. This band had the same intensity in all the patients and the control samples. Another band with an approximate MW 25 kDa. This band was detected at reduced intensity in patients P3 and P4, in comparison with the other patients and the control (Figure 2) indicating that this band is for FANCG or FAAP24.

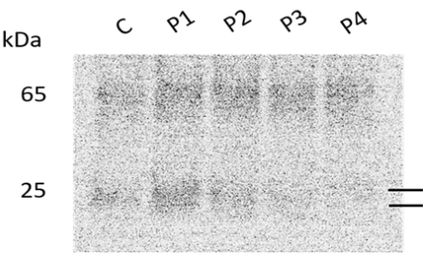

Fig. 2: Western blot of FA core complex protein in skin fibroblasts from FA patients using anti-FANCA antibody.

Multiplex ligation-dependent probe amplification (MLPA) results

We started mutation analysis of FA patients by applying the MLPA test for the detection of possible *FANCA* gene deletions revealing the two patients harbored homozygous deletions in Exon 15 of the *FANCA* gene (Figure 3).

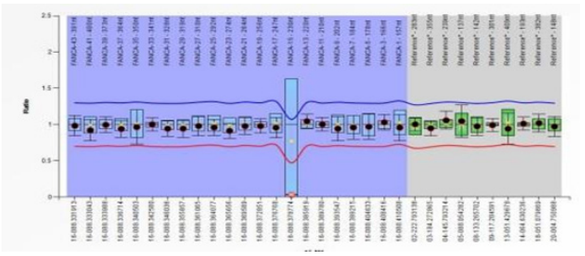

Fig. 3: Multiplex ligation-dependent probe amplification analysis (MLPA) output of one probe mix (MLPAP031) from the Coffalayzer.net software, showing *FANCA* exons and reference loci (R) values. Revealing homozygous intragenic deletion of exon 15 in the *FANCA* gene in the proband. The inter sample normalization of deleted adjacent exons is under the threshold of 0.7.

Immunodetection of FANCA protein revealed the presence of reduced intensity band in patients P3 and P4, in comparison with the other patients and the control at band with an approximate molecular weight of 25 kDa. This result was confirmed by applying MLPA technique in the same patients, which indicate the presence of intragenic deletion mutation of exon 15 in *FANCA* gene and this may explain the difference in the band intensity between these two patients compared with other patients and control.

Molecular results of Target NGS customized panel

FA patients where no mutation could be detected by MLPA technique were further studied by a targeted customized panel including *FANCA, FANCG* and *FANCL,* details of all detected variants were summarized in (Table 1).

Description of the mutations is based on cDNA sequence, corresponding to the A of the ATG translation initiation codon of the reference sequence GenBank reference sequences: FANCA, NM_000135.2 & FANCL, NM_018062.4.

Table 1: Results from Screening for causative gene variants in Five FA Patients

Patients	FANC gene	Exon/ Intron	DNA change	Protein change	Mutation	Reference
(P 1)	FANCA	Exon 23	c. 2143 G >T	p.E715*	Nonsense	Huang *et al*. (2018)
(P2)	FANCA	Intron 3	rs891323617 c.189+1G>A, (g.782G>A) (IVS2+1 G>A	altered splicing of pre-mRNA	Splice donor	Steinberg-Shemer *et al*.(2020); Moghrabi *et al*. (2009); Baralle and Baralle (2005)
(P 3)	FANCA	Exon 15	Ex.15 del.	Deletion	Exon Deletion	Amouri *et al*. (2014); Ameziane *et al*. (2008)
(P 4)	FANCA	Exon 15	Ex.15 del.	Deletion	Exon Deletion	Amouri *et al*. (2014); Ameziane *et al*. (2008)
(P 5)	FANCL	Exon 9	c.703T>C	p.S235P	Missense	Nykamp *et al*. (2017).

DISCUSSION

In this study, we employed various methods for subtyping studied FA patients, including immunodetection, MLPA and Targeted NGS customized panel. All these methods were useful in identifying pathogenic mutations in all studied patients.

Patients with FA may reveal obvious with symptoms including short stature, skin findings such as café-au-lait skin lesions and hyper- or hypo- pigmentation which was observed in the studied patients, in addition to multiple skeletal abnormalities as microcephaly and abnormalities in the thumb was also detected in the studied patients Progressive bone marrow failure with pancytopenia was typically present within the first decade, often presenting with thrombocytopenia or leukopenia. The clinical presentations of our studied patients are similar to FA reports in different populations (**Freire et al., 2018; Bianchi *et al*., 2018; Soulier, 2011; Eiler *et al*., 2008**).

The clinical features of FA are highly variable, even among individuals within the same family or among patients within the same complementation group. Genetic testing for the diagnosis of FA is considered medically and clinically necessary when the clinical signs and symptoms are present. Molecular subtyping is critically needed for the accurate diagnosis and clinical management of FA patients (**Esmail *et al*., 2016; Olson, 2020; Bogliolo *et al*., 2020; Mori *et al*., 2019; Solanki *et al*., 2016**). In the current

study we attempted to establish a preliminary strategy for the molecular investigation of FA patients based on immunodetection of the deficient protein of the FA core complex in skin fibroblasts. Skin fibroblasts samples had been cultured from biopsies taken from FA patients and control samples. We started with the immunodetection of FANCA protein as the majority of FA patients (85%) harbor mutations in *FANCA* gene among FA patients worldwide. The *FANCC* and *FANCG* are the next generally mutated genes where they are responsible for 20% of FA patients (**Shahid *et al*., 2020; Castella *et al*., 2011**). Mutations in remaining FA genes are rare and each gene mutation accounts for 1%–3% of cases (**Nalepa and Clapp, 2018; Callen *et al*., 2005**).

The result of immunodetection of FA core complex proteins using an antibody for FANCA revealed two bands. One band at an approximate molecular weight 65 kDa which could represent FANCG protein. The other band at molecular weight 25 kDa could represent FA associated peptide 24 (FAAP24). It has been reported that the FA core complex proteins are co-immunoprecipitated with each other as well as with FAAPs (**Jung *et al*., 2020; Ciccia *et al*., 2007**). FANCA protein is considered the largest member of the FA core complex and it is needed for its stability (**Yokoi *et al*., 2019**). We hypothesize that a mutant *FANCA* gene would result in a truncated or reduced level of FANCA protein resulting in degradation of the FA core complex. Patients 3 and 4 who had the same mutation in *FANCA* gene had reduced levels of FANCG and FAAP24 compared to the control and the other patients. Patient 2 had a splice mutation which might have not any effect on the FANCA protein and this could explain the observed normal pattern.

In the current study we applied (MLPA) method for the screening of deletion mutations in *FANCA* gene, which revealed intragenic homozygous deletions in Exon 15 of *FANCA* gene in two patients. The results obtained from immune detection of FANCA protein with different intensity in two FA patients was confirmed by applying MLPA technique in the same patients which indicate the presence of intragenic deletion mutation.

This deletion was previously reported on FA Tunisian patients thus **Amouri *et al* (2014)** in his study demonstrated that exon 15 deletion in *FANCA* exists with frequency of 54% among FA-A patients (42 patients were homozygous and four were compound heterozygous). Otherwise, similar to our results, the same deletion was reported in one European FA patient (**Ameziane *et al*., 2008**).

Another molecular tool used during this work was NGS targeted panel sequencing, which allows the sequencing and detection of human pathogenic variants in short time (**Negahdari *et al*., 2020; Ataei and Nazari, 2020; Toksoy *et al*., 2020**).

In the present study, we identified two genes among studied FA patients *FANCA* and *FANCL*, two different *FANCA* gene mutations both were previously reported, one of them was a nonsense mutation c. 2143 G >T (p.E715*) detected in exon 23 of the gene. This mutation was previously reported in an American patient affected with Lung squamous cell carcinoma (**Huang *et al*., 2018**), while in our study this mutation is detected in an FA patient for the first time.

The second *FANCA* gene detected mutation was a Splice donor type c.189+1G>A (g.782G>A, rs891323617). This mutation was previously mentioned in three different studies one of these studies was done on 111 FA patients from Israel and this mutation was detected in one patient from Arab Muslim descent, which is interestingly in agreement with our patient descent (**Steinberg-Shemer *et al*., 2020**). The same mutation was also reported in an American FA patient (**Moghrabi *et al*., 2009**) and another European one (**Baralle and Baralle, 2005**).

Concerning the second *FANC* gene, a missense mutation in exon 9 of *FANCL* gene c.703T>C (p.S235P) was detected, this mutation was previously reported in one American FA patient (**Nykamp *et al*., 2017**).

CONCLUSION

In conclusion, immunodetection can guide the targeted molecular diagnosis of FA in an economic way saving the cost and time of sequencing the 22 FA genes. However, it is recommended to include all the FA antibodies with the extension of the study on more patients hopefully with large cohorts from every gene mutation.to set this diagnostic strategy giving a basis for future molecular diagnostic work-ups for Egyptian FA patients.

CONFLICT OF INTEREST

There are no conflicts of interest.

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