# Investigation of *FANCA* Mutations in Greek Patients

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**Abstract.** *Background: Fanconi anemia (FA) is a rare genetic disease characterized by considerable heterogeneity. Fifteen subtypes are currently recognised and deletions of the Fanconi anemia complementation group A (FANCA) gene account for more than 65% of FA cases. We report on the results from a cohort of 166 patients referred to the Department of Medical Genetics of Athens University for genetic investigation after the clinical suspicion of FA. Materials and Methods : For clastogen-induced chromosome damage, cultures were set up with the addition of mitomycin C (MMC) and diepoxybutane (DEB), respectively. Following a positive cytogenetic result, molecular analysis was performed to allow identification of causative mutations in the FANCA gene. Results: A total of 13/166 patients were diagnosed with FA and 8/13 belonged to the FA-A subtype. A novel point mutation was identified in exon 26 of FANCA gene. Conclusion: In our study 62% of FA patients were classified in the FA-A subtype and a point mutation in exon 26 was noted for the first time.*

Fanconi anaemia (FA) is a rare genetic disorder, characterized by progressive pancytopenia, variable congenital anomalies, susceptibility to malignancies and induced chromosomal instability (1). Age of diagnosis ranges from birth to 50 years, with a mean onset of anemia at eight years. Males and females are equally affected. The incidence internationally is estimated to be approximately 1/300000 and the carrier frequency 1/300. The highest rate is noticed in Ashkenazi Jews (carrier frequency 1/90, incidence 1/30000) and in Afrikaners in South Africa (heterozygote frequency 1/80, incidence 1/22000) (2).

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FA is characterized by progressive bone marrow failure, congenital abnormalities and an increased risk for hematological malignancies and solid tumors. Physical abnormalities are presented in 60%-75% of affected individuals, including short stature, abnormal skin pigmentation, various malformations [skeletal system, eyes, kidneys and urinary tract, hearing loss, cardiovascular, gastrointestinal (GI) and central nervous system], hypogonadism and developmental delay. Progressive bone marrow failure with pancytopenia typically presents in the first decade, often initially with thrombocytopenia or leukopenia (1-4). By age 40 to 50 years, the estimated cumulative incidence of bone marrow failure is 90%, incidence of haematological malignancy (primarily acute myeloid leukemia) 10%-30%, and of nonhematological malignancy (solid tumors, particularly of the head and neck, skin, GI tract, and genital tract) 25%-30% (5-7). In addition to increased spontaneous chromosomal instability, cells from individuals with FA are sensitive to agents that cause interstrand DNA cross-links, such as mitomycin C (MMC), bifunctional nitrogen mustards, diepoxybutane (DEB) and photoactivated psoralens.

On the genetic level there is considerable heterogeneity in FA and 15 subtypes/complementation groups are currently recognised. They all show autosomal recessive inheritance, with the exception of FA-B which is X-linked. Genes and proteins of these subtypes have been identified (*FANCA, FANCB, FANCC, FANCD1, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCJ, FANCL, FANCM, FANCN, FANCO, FANCP*) (1, 2, 4, 7-9).

The FANC proteins cooperate in the Fanconi Anemia/ Breast Cancer (FA/ BRCA) pathway which may be divided in three parts: i) FA core complex, ii) complex of proteins FANCD2 and FANCI (ID complex) and iii) downstream FA proteins (5, 10, 11, 12). The heterodimer ID complex, formed by FANCD2 and FANCI, is monoubiquitinated and then translocated to chromatin, interacting with downstream proteins in the FA pathway in order to repair DNA defects by homologous recombination. In addition to DNA damage, the FA pathway can be activated during the S phase of the cell cycle (13-17). After DNA repair, FANCD2 and FANCI return to the non-ubiquitinated form (11).

The rate of identified mutations in the 15 FANC genes varies, but mutations of *FANCA* gene account for approximately 65% of all cases diagnosed. The *FANCA* gene is mapped to chromosome 16q24.3, spans about 80 kb and consists of 43 exons. Its mutation spectrum is very heterogenous and a number of point mutations, splicing mutations, large intragene deletions, probably Alu-mediated, or insertions have been described. Most defects are point mutations and about 30% are relatively large deletions (2, 4, 9, 17, 18).

We report on the results from a cohort of 166 patients, aged 2 months-40 years old referred to the Department of Medical Genetics of Athens University between 2007-2012 for genetic investigation after the clinical suspicion of FA (myelodysplasia and/or congenital anomalies). Skeletal, heart, pulmonary and kidney defects, developmental delay or squamous cell carcinoma were present in 13/166 patients, while 17/166 had haematological and various congenital anomalies, and 133/166 were referred because of anaemia, thrombocytopenia, pancytopenia, neutropenia and other haematological defects.

## Materials and Methods

*Cytogenetic analysis*. For clastogen-induced chromosome damage, cultures were set up with the addition of MMC and DEB, respectively. The final concentration for MMC was 0.6 μg/ml and 1 μg/ml and cells were cultured for 72 hours. In the second set of cultures, DEB was added 24 hours after initiation at a final concentration of 0,1 μg/ml and cells were exposed to the chemical for 48 hours. A minimum of 150 metaphases were examined and a sample was considered as FA-positive if the percentage of breaks and radial formations detected were 7-10 times higher compared to an aged-matched control.

*Molecular analysis*. Following a positive cytogenetic result, molecular analysis was performed to allow identification of causative mutations. Genomic DNA was extracted from peripheral blood lymphocytes according to the protocol of an automated robotic system (QIAGEN BioROBOT M48; QiaGen, Hilden, Germany). Multiplex ligation-dependent probe amplification (MLPA) was used for the detection of possible *FANCA* gene deletions. Mutation analysis was performed by Polymerase chain reaction (PCR) amplification followed by Enzymatic cleavage mismatch analysis (ECMA), using the SURVEYOR™ Mutation Detection Kit. *FANCA* coding exons 1, 7, 25, 26, 28, 32, 33, 34-35, 36, 37, 38, 39, 40-42, where a higher rate of molecular defects has been recorded, were amplified using primers designed by the Authors (Table I). PCR conditions were applied in order to provide the highest accuracy and specificity in the mutation screening assay. They included the activation of polymerase (Hotstar taq/Multiplex) at 95˚C for 15 min, followed by 33 cycles of denaturation at 95˚C for 1 min, annealing at 55˚C for 1min and extention of primers at 72˚C for 1min and 72˚C for 10 min. Dimethyl sulfoxide (DMSO) was used for exon 1 because of C/G-rich region; annealing of this exon was at 58˚C. Samples demonstrating heteroduplex formation were directly sequenced to allow characterization of molecular defects. Sequencing reactions were performed with M13 universal primers using the DYEnamic ET Dye Terminator Cycle Sequencing kit and products were analyzed on a MegaBACE SNP Genotyping System (GE Healthcare).

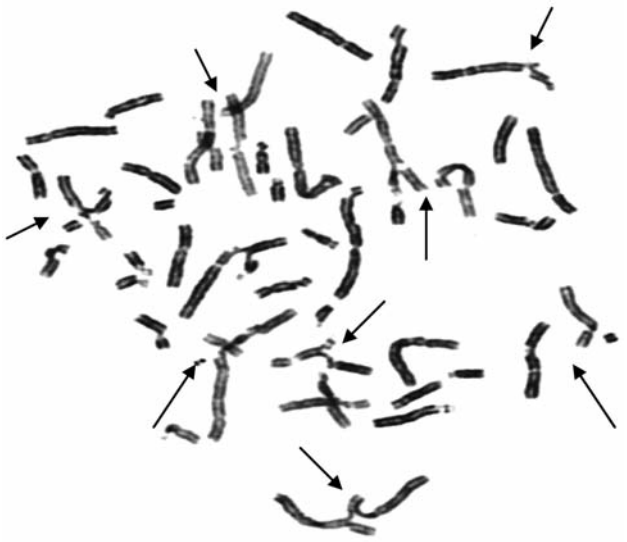

Figure 1. *Metaphase spread from a patient with Fanconi aneamia, after exposure to mitomycin C (MMC), exhibiting multiple chromosomal breaks and radial formations.*

## Results

*Cytogenetic testing*. In 13/166 patients induced breaks and radial formations were noted with both clastogens in 96% of analysed metaphases (Figure 1).

*Molecular testing*. Molecular analysis by MLPA revealed that 5/13 patients were classified as having the FA-A subtype. Patients no. 1 and 2 were dizygotic twins and compound heterozygotes for the same deletions (exons 1-5 and 7-17) (Table II); patient no. 9 was a compound heterozygote for deletions of exons 1 and 2; patients no. 3 and 8 were carriers of deletions of exons 35 and 7-20, respectively (Table III).

Among the eight remaining patients, three were identified as having FA-A subtype due to point mutations in the *FANCA* gene. Patient no. 6 carried the c.2T>C; p.Met1? in exon 1 and the c.3788_3790delTCT;p.Phe1263del in exon 38. Patients no. 4 and 5 carried c.3348+18A>G in intron 33 (Figure 2) and c.2426 G>A; p.Gly809Asp in exon 26 (Figure 3), respectively. For patient no. 8, c.3788_3790delTCT; p.Phe1263del in exon 38 was the compound defect (Table III).

Out of 26 family members tested for carrier status, 13 presented molecular alterations in the *FANCA* gene, five were heterozygotes for deletions of exons and eight were carriers of point mutations.

Clinical features of patients with FA are presented in Table III.

## Discussion

FA is a genetic condition that strongly predisposes patients to congenital and haematological abnormalities. Early diagnosis

Table I. *Primers (forward/reverse) used in this study of Fanconi aneamia complementation group A (FANCA) gene in Greek patients. Each forward primer had a M13 tail (lowercase letters) to elaborate universal sequencing.*

| Exons | Primers | |
|---|---|---|
| | Forward | Reverse |
| 1 | 5'gtaaaacgacggccagtCCTGGCCGCAGCCAATAGG3' | 5'CCGGCGGAGGCTCTGGCG3' |
| 7 | 5'gtaaaacgacggccagt AGTTGAGCCTTACGTCTG3' | 5'AGAGCTCTTGAGAGCAGA3' |
| 25-26 | 5' gtaaaacgacggccagtTGGATTAGCTGTTGGAGG3' | 5'AACGAGCATGTGTCACTT3' |
| 28 | 5' gtaaaacgacggccagtCTCAGCCACTCACAGTGA3' | 5' GCTGTTCTTGCCTCTGAG3' |
| 32 | 5' gtaaaacgacggccagtGTGCCAGCATACTGCTCT3' | 5'TCTAGGACCGTCATGAGA3' |
| 33 | 5' gtaaaacgacggccagtAAGGAGCAGAGTGTACGC3' | 5'TGCAAGAGCTGCTGTTAG3' |
| 34-35 | 5' gtaaaacgacggccagtACAGCAGCCACTCTGCAT3' | 5'ATGGAGACGTGCTGCAGA3' |
| 36 | 5'gtaaaacgacggccagtCCTGTAGTGGCCTGTAGG3' | 5'TTAGGAGATGACCTTGAG3' |
| 37 | 5'gtaaaacgacggccagtTGGTTGTATGGTTGTAAG3' | 5'GAGAAATAGCACTGATTG3' |
| 38 | 5'gtaaaacgacggccagtTAGAATGACAGCACAGGT3' | 5'CTGGTAAGGTCTGACTTA3' |
| 39 | 5'gtaaaacgacggccagtTAAGACTTACAATAAGCA3' | 5'TGTGCCTCAGCAGCGTGT3' |
| 40-42 | 5'gtaaaacgacggccagtAGCACTGATAATAGGCAG3' | 5'GCTGTCAATTCTCATGTC 3' |

is important, as long term survival depends on it. In future pregnancy, prenatal diagnosis can ensure an unaffected embryo, who may also represent a human leukocyte antigen (HLA)-compatible source of stem cells to be transplanted to an affected sibling. Cytogenetic analysis is the first essential step in the differential diagnosis from other types of anaemia, especially in cases when congenital abnormalities are absent and molecular testing reveals DNA defects, facilitating thus the genetic counselling of the family (1, 2). *FANCA*, as the most frequently affected gene, displays the entire spectrum of genetic alterations, including at least 32% of large deletions correlated to Alu-mediated recombination. The highest rate of *FANCA* molecular defects appears in the Spanish Gypsy population (≈80%) (4, 6, 20, 21).

In the present study, 13/166 patients were diagnosed with FA and, as expected, 62% of them (8/13) belonged to the FA-A subtype. It is interesting to note that 38% of the patients were Roma Gypsies. Both Spanish and Greek Gypsies are Roma, an ethnic group living mostly in Europe which has been genetically traced to a group migrating from north-western India (22). It is also important to mention that of two patients, dizygotic twins, only the boy had VACTERL syndrome. In the literature there is a similar case report but both twins diagnosed with FA had VACTERL syndrome (23).

Molecular defects disclosed included large deletions (3/8) and point mutations (4/8). Exons 1, 7, 25-28, 32-42 of the *FANCA* gene are reported in literature as those with the highest mutational rate in the general population and could therefore be characterized as 'hot spots'. c.3788_3790delTCT; p.Phe1263del point mutation was detected in exon 38 and this is the most frequent FA mutation in Spain (20.7% of all mutated alleles) and in the rest of the world (19, 24). This mutation is shared by 80% of patients from La Palma Island, suggesting a founder effect that explains the extraordinary high frequency of patients with FA in this island (1 in 16000, the highest frequency of FA reported). This mutation also accounts for an elevated percentage (51%) of the mutations found in Brazilian patients.

Table II. *Dizygotic twins, compound heterozygotes for Fanconi aneamia complementation group A (FANCA) gene deletions. Deletion of exons 1-5 is of paternal origin and that of exons 7-17 is of maternal origin. (Multiplex ligation-dependent probe amplification -MLPA- technique). Each ratio corresponds to the fluorescent peak of each amplicon which can be detected by a capillary sequencer. Ratios between the two alleles: 0-0.3→ patient, 0.3-0.7→ carrier of deletion, 0.7-1.3→ normal.*

| Exon of *FANCA* gene | Father | Mother | First twin | Second twin |
|---|---|---|---|---|
| 1 | 0.52 | 0.95 | 0.52 | 0.52 |
| 2 | 0.56 | 0.94 | 0.54 | 0.54 |
| 3 | 0.53 | 1.1 | 0.55 | 0.55 |
| 4 | 0.55 | 1 | 0.48 | 0.48 |
| 5 | 0.55 | 1.18 | 0.57 | 0.57 |
| 6 | 0.99 | 1.08 | 0.99 | 0.99 |
| 7 | 0.98 | 0.4 | 0.42 | 0.42 |
| 8 | 1.1 | 0.36 | 0.6 | 0.6 |
| 9 | 1.12 | 0.51 | 0.52 | 0.52 |
| 10 | 0.98 | 0.5 | 0.53 | 0.53 |
| 11 | 0.97 | 0.6 | 0.52 | 0.52 |
| 12 | 0.91 | 0.48 | 0.48 | 0.48 |
| 13 | 1.1 | 0.42 | 0.54 | 0.54 |
| 14 | 1 | 0.49 | 0.54 | 0.54 |
| 15 | 0.97 | 0.63 | 0.48 | 0.48 |
| 16 | 0.95 | 0.54 | 0.47 | 0.47 |
| 17 | 1.11 | 0.58 | 0.48 | 0.48 |
| 18 | 1.12 | 0.91 | 1 | 1 |
| 19 | 1 | 0.96 | 0.98 | 0.98 |
| 20 | 1.1 | 0.99 | 0.99 | 0.99 |

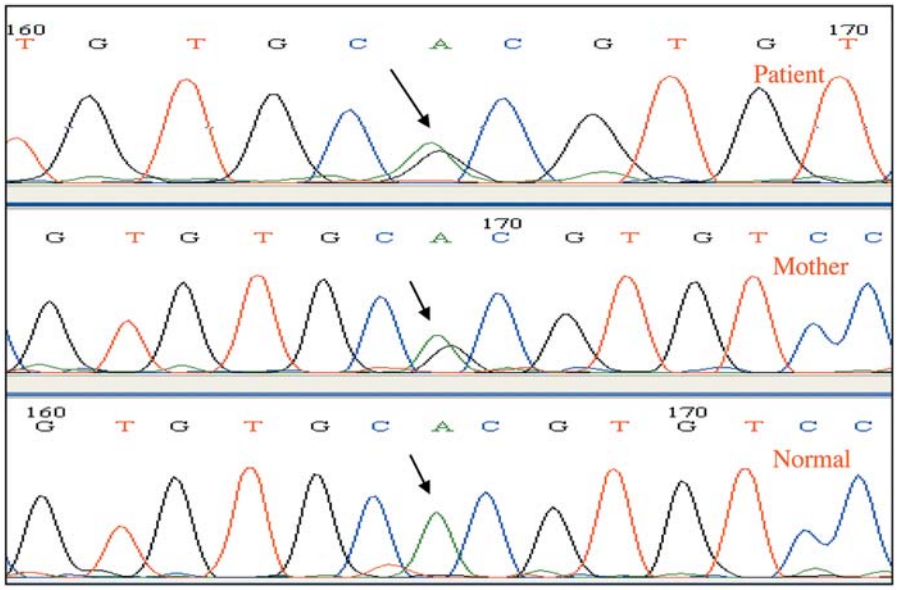


Figure 2. *Point mutation c.3348+18A>G in intron 33 of the Fanconi aneamia complementation group A (FANCA) gene.*

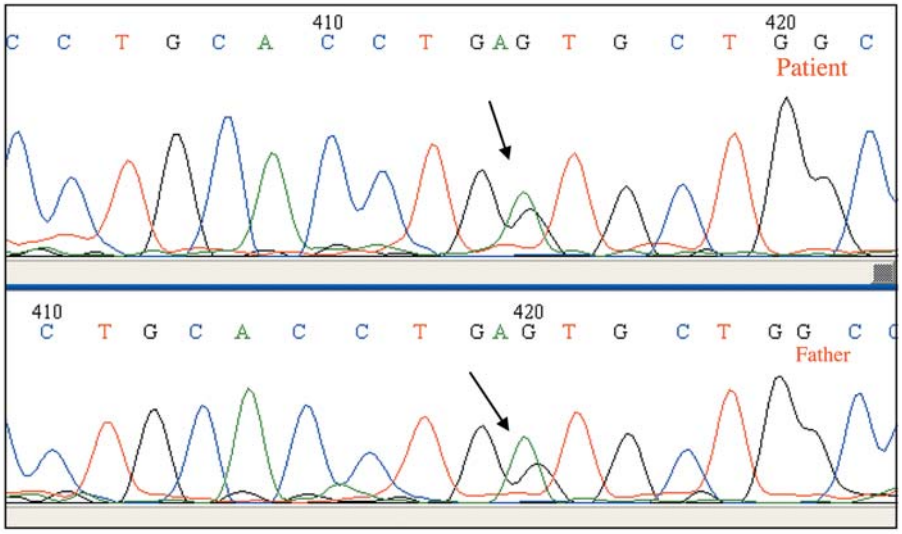


Figure 3. *The novel point mutation c.2426 G>A; p.Gly809Asp was identified in exon 26 of the Fanconi aneamia complementation group A (FANCA) gene.*

←

Figure 4. *Hypoplasia of the right thumb and aplasia of the left thumb.*

Table III. *Clinical features and molecular investigation of patients with Fanconi anaemia.*

| Patient no. | Age | Clinical features | Mutations | | Carriers in the family |
|---|---|---|---|---|---|
| | | | MLPA | PCR/ECMA/Sequencing | |
| 1 (dizygotic twin of patient 2) | 6 Years | Microcephaly, hypermelanosis of the skin, petechias and pancytopenia | 1-5 and 7-17 | - | 2/2 |
| 2 (dizygotic twin of patient 1) | 6 Years | VACTERL Syndrome, small stature, microcephaly, ocular ptosis, hypermelanosis of the skin, radial ray defect and pancytopenia | 1-5 and 7-17 | - | 2/2 |
| 3 (Roma Gypsy) | 6 Years | Anaemia, thrombocytopenia | Carrier, deletion of exon 35 | - | 1/5 |
| 4 | 9.5 Years | Small stature, petechias, anaemia, microcephaly, radial ray defect, hyperpigmentation | | Carrier c.3348+18A>G in intron 33. | 1/4 |
| 5 (Roma Gypsy) | 1.5 Years | Aplasia of thumb, anaemia, horseshoe kidney, congenital cardiopathy (the parents are second cousins) | - | Carrier of the point mutation c.2426 G>A; p.Gly809Asp in exon 26. | 1/2 |
| 6 | 5 Years | Anaemia, thrombocytopenia, radial ray defect, short stature, hypopigmentation | - | Carrier of two point mutations c.2T>C; p.Met1? in the exon1 and c.3788_3790delTCT; p.Phe1263del in exon 38. | 4/6 |
| 7 | 6 Years | Haematological deffects | - | - | - |
| 8 | 12 Years | Haematological deffects | Carrier, deletion exons 7-20 | Carrier of the point mutation c.3788_3790delTCT; p.Phe1263del in exon 38. | - |
| 9 (Roma Gypsy) | 2.5 Years | Hypoplasia of the right thumb and aplasia of the left thumb (Figure 4) | Compound heterozygous for deletions of exons 1 and 2 | - | 2/5 |
| 10 | 7 Years | Thrombocytopenia, anaemia, neutropenia, radial ray deffect | - | - | - |
| 11 | 3 Years | Radial ray deffect | - | - | - |
| 12 | 17 Days | Aplasia of the right radius and thumb, hypogonadism, dysplastic kidney | - | - | - |
| 13 | 30 Years | Anaemia, thrombocytopenia, MDS, aplasia of thumbs, café au lait spots | - | - | - |

The point mutation c. 3348+18A>G in intron 33 has been reported only once while c.2426 G>A; p.Gly809Asp in exon 26, to our knowledge, is reported for the first time in the present study. The mutation is already registered at the Leiden Open Variation Database (LOVD) as a novel mutation and functional studies should be performed to allow

characterization of its pathogenic impact (24). The carrier of this point mutation was a Roma Gypsy with typical features of FA born to consanguineous parents (second cousins) after an uneventful pregnancy (Table III).

In patient nos. 3, 4 and 5 a second molecular defect remains to be identified in order to elucidate the pathogenic mechanism underlying the presence of FA (Table III). The compound mutation may be located on the rest exons of *FANCA* gene where causative alterations are less frequent (19). Previous reports have disclosed that Roma Gypsies of Spanish origin share the 295C→T mutation in exon 4, which in homozygotes, leads to *FANCA* truncation (20). This mutation was, however, not screened because the present study design of FA was based on the high mutational rate recorded for the general population and not specifically that for the Roma Gypsies (19). Further analysis is needed in order to identify the second defect in the *FANCA* gene in the patients and their relatives.

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