Brazilian Journal of Medical and Biological Research (2005) 38: 669-673
ISSN 0100-879X

Frequency of Fanconi anemia in Brazil and efficacy of screening for the *FANCA* 3788-3790del mutation

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Abstract

Fanconi anemia (FA) is an autosomal recessive genetic disease characterized by progressive bone marrow failure, susceptibility to cancer and multiple congenital anomalies. There is important clinical variability among patients and the knowledge of factors which might predict outcome would greatly help the decision making regarding the choices of treatment and the appropriate time to start it. Future studies of the possible correlation between specific mutations with specific clinical presentations will provide the answer to one of these factors. At our Center we standardized a rapid and precise screening test using a mismatch PCR assay for a specific mutation (3788-3790del in exon 38 of gene *FANCA*) in Brazilian FA patients. We present the results obtained after screening 80 non-consanguineous FA patients referred from all regions of Brazil with a clinical diagnosis of FA supported by cellular hypersensitivity to diepoxybutane. We were able to detect the 3788-3790del allele in 24 of the 80 (30%) FA patients studied. Thirteen of the 80 (16.25%) were homozygotes and 11 of the 80 (13.75%) were compound heterozygotes, thus confirming the high frequency of the *FANCA* 3788-3790del mutation in Brazilian FA patients. The identification of patients with specific mutations in the FA genes may lead to a better clinical description of this condition, also providing data for genotype-phenotype correlations, to a better understanding of the interaction of this specific mutation with other mutations in compound heterozygote patients, and ultimately to the right choices of treatment for each patient with improvement of the prognosis on future studies.

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Research partially supported by St. Jude Children's Research International Outreach Program and by American Lebanese Syrian Associated Charities (ALSAC), Memphis, TN, USA.

Received May 27, 2004
Accepted December 9, 2004

Key words
- Fanconi anemia
- *FANCA*
- 3788-3790del mutation
- Genetic screening

Introduction

Fanconi anemia (FA) is an autosomal recessive genetically and phenotypically heterogeneous disorder, most frequently associated with chromosomal fragility, bone marrow failure, cancer, and congenital abnormalities. These include: developmental anomalies involving mainly the head and face, skeletal malformations particularly of the radial ray, growth retardation, abnormal skin pigmentation, and renal, ocular, genital, and cardiac defects. The cardinal clinical feature is a severe progressive, life-threatening pancytopenia, and hematopoietic stem cell transplantation is the only curative method for restoring normal hematopoiesis. Somatic cell hybrid studies demonstrate that there are at least eleven complementation groups (FA-A, B, C, D1, D2, E, F, G, I, J, and L), all of which have been connected to different genes, except B (1-4). A recent study reported that the FA-B and D1 subgroups result from hypomorphic mutation in *BRCA2* (5). Mutations in a gene of the complementation group A (*FANCA*) accounts for 65% of all cases of the disease, with a highly heterogeneous spectrum of mutations identified throughout the gene in most populations studied. The other most frequent complementation groups are FA-G (10-15%) and FA-C (10-15% of the cases). The worldwide prevalence varies strongly depending on the ethnic background of each group studied (1-3).

The phenotypic heterogeneity of FA can delay or impair clinical diagnosis. The current laboratory diagnostic test consists of cytogenetic analysis looking for chromosomal breakage in response to diepoxybutane (DEB) or mitomycin (6,7). Recently, a new diagnostic test was developed which assays primary lymphocytes for *FANCD2* protein monoubiquitination by immunoblot. It is known that FA proteins, encoded by six cloned genes *(FANCA*, *FANCC*, *FANCD2*, *FANCE*, *FANCF*, and *FANCG*), cooperate in a common pathway to maintain genomic integrity, and that the proteins encoded by *FANCA*, *-C*, *-E*, *-F*, and *-G* form a complex culminating in the activation of *FANCD2* protein into a monoubiquitinated form (1,7-10). The absence of the monoubiquitinated *FANCD2* isoform correlates with the broad diagnosis of FA by the DEB test. A combination of retroviral gene transfer and *FANCD2* protein immunoblotting provides a subtyping assay for patients newly diagnosed with FA, thus allowing the identification of the complementation group to which the patient belongs (7,9).

FA is a condition involving very high risks of bone marrow failure, leukemia and solid tumors (11-14). The first event may be determined by each individual's unique combination of FA genotype, cancer susceptibility modifier genes and environmental risk factors (12). Studies are needed to quantify more precisely the individualized risk of each adverse event.

The identification of patients with specific mutations in the FA genes may lead to a better clinical description of this condition, also providing data for genotype-phenotype correlations, to a better understanding of the interaction of this specific mutation with other mutations in compound heterozygote patients, and ultimately to the right choices of treatment for each patient with improvement of the prognosis.

At our center we standardized a rapid and precise screening test for a common mutation found in Brazilian FA patients (*FANCA* 3788-3790del mutation) and we present the results obtained after screening a large group of patients diagnosed at the Federal University of Paraná Fanconi Anemia Center, Curitiba, PR, Brazil.

Patients and Methods

Subjects

A total of 80 non-consanguineous FA

patients were enrolled in the study. The patients were seen at our Outpatient Clinic from August 2001 to September 2003, and were referred from all regions of Brazil to our Fanconi Anemia Center. Patient age ranged from 2 to 30 years (median age 10.4 years at the time of this test). Inclusion criteria were a clinical diagnosis of FA supported by cellular hypersensitivity to DEB and availability of adequate clinical information. A peripheral blood sample was obtained from each patient after informed consent. The study was evaluated and approved by the Ethics Committee of Hospital de Clínicas, Federal University of Paraná.

Detection of 3788-3790del mutation

A specific mismatch PCR restriction analysis was used to identify the 3788-3790del mutation in exon 38 of the *FANCA* gene in DNA extracted from peripheral blood from DEB-positive Brazilian FA patients. DNA fragments were amplified using misaligned primers and then digested with *Mbo*II restriction enzyme (15). The normal gene sequence contains two adjacent *Mbo*II sites and the mutant sequence contains only one of them. The reverse primer was designed so that it abolishes the most 3' end *Mbo*II site leaving the normal sequence with one site and the mutant with no *Mbo*II sites.

Normal sequence:...5'-TT^TTC^CTTT
TCTTCT**TCT**CCTTGAT...
3'-AA**A**AGGAACTA...

Mutant sequence:...5'-TT^TTCCTTT
TCTTCTCCTTGAT...
3'-AA**A**AGGAACTA...

*Mbo*II recognition site (TCTTC): underlined; TCT repeat deletion: in bold; ^ = restriction site.

Homozygotes for the 3788-3790 deletion exhibit a 160-bp band on a 2.5% agarose gel; heterozygotes have two bands (160 and 130 bp, and an undetected 30-bp band) and normal individuals have only a 130-bp band and an undetected 30-bp band (Figure 1).

PCR and *Mbo*II digestion

The sequence of the forward intronic primer is: 5'-AGGATTTATGGCCTAGAT GTAAAAA-3' (25 mer) and the sequence of the reverse primer is: 5'-GACGACAGCA GGCCCATCAAGGAAAA-3' (26 mer). The PCR procedure consisted of a 15-µl total volume of 20 ng genomic DNA, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.25 mM spermidine, 0.2 mM dNTPs, 0.4 µM each primer, 0.2 units of *Taq* DNA polymerase, and H_2O. The PCR conditions were: 94ºC for 2 min; 30 cycles of 10 s at 94ºC, 20 s at 60ºC and 20 s at 72ºC; 72ºC for 2 min, and 4ºC unlimited. *Mbo*II digestion: a mix of 1.4 µl $MgCl_2$ (100 mM), at a final concentration of 10 mM, and 0.6 µl *Mbo*II (5 U/µl) per reaction was added to the 15-µl PCR product and incubated for 1 h at 37ºC. Three microliters of 6X loading dye (ethidium bromide) was added and the samples were loaded onto 2.5% agarose gel. Electrophoresis was performed in 0.5X TBE for 30 min. The fragments were visualized under UV light and photographed.

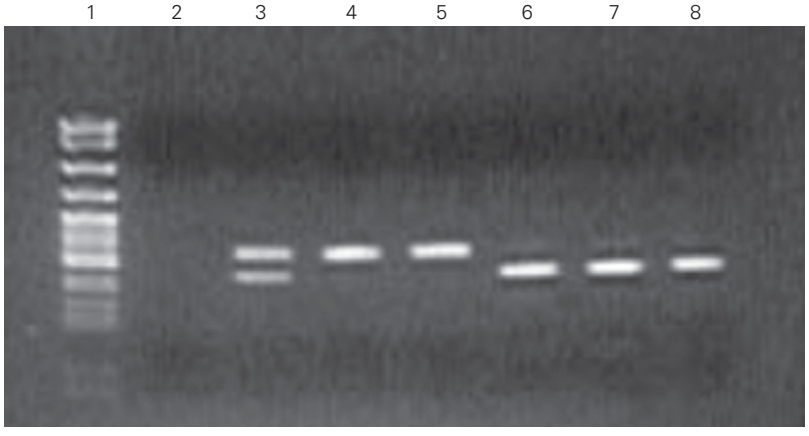

Figure 1. Screening for Fanconi anemia *FANCA* 3788-3790del mutation. *Lane 1*: pBR322; *lane 2*: negative control (water); *lane 3*: amplification of two bands of a heterozygous individual; *lanes 4* and *5*: amplification of the upper band of homozygous individuals; *lanes 6-8*: amplification of the lower band of normal individuals.

Results and Discussion

A total of 80 DNA samples from non-consanguineous FA patients were analyzed. Twenty-four of the 80 (30%) FA patients studied had the 3788-3790del allele, 13 of 80 (16.25%) were homozygotes and 11 of 80 (13.75%) were compound heterozygotes, thus confirming the high frequency of the *FANCA* 3788-3790del mutation in Brazilian FA patients (15).

Investigation of the clinical phenotypes associated with specific mutations in FA is difficult due to the wide heterogeneity of mutations causing FA. The number of patients with the same specific mutation is generally small, and most of them are compound heterozygote individuals.

The *FANCA* gene is highly polymorphic, with more than 100 mutations reported, without a high frequency of a specific allele in other populations (15,16). Most of the pathogenic mutations have been detected in individual patients, making carrier screening difficult, unless the proband has been genotyped for complementation group, and all 43 exons of the gene have been screened for mutations, which is a difficult and time-consuming task.

A deletion mutation in exon 38 of the *FANCA* gene (3788-3790del) was previously found to be present in 9 of 28 (32%) Brazilian FA patients (17). In a study of 350 non-*FANCC* IFAR (International Fanconi Anemia Registry) patients the 3788-3790del mutation was found in 10% of patients and was especially common among the Brazilian patients (15).

Several studies have been done to investigate the possibility of defining risk groups in FA, especially involving the most prevalent complementation groups (FA-A, C, G), by examining the relationship between genotype and phenotype and by dividing patients into complementation or type mutation groups and then comparing the clinical phenotype of these subgroups (1,15).

Analyses of the mutations found in the *FANCC* gene show a relatively small number of characteristic mutations which have specific ancestral origins. Mutation IVS4+4 A>T of the *FANCC* gene is found in more than 80% of FA patients of Ashkenazi Jewish ancestry (18,19). In Asian populations two founder mutations account for most of *FANCG* mutant alleles (20).

A study of the mutations found in 245 patients originating from 24 different countries showed that FA-G patients had more severe cytopenia and a higher incidence of leukemia. Somatic abnormalities were less prevalent in FA-C with a later onset of aplastic anemia, and FA-A patients homozygous for null mutations had an earlier onset of anemia and a higher incidence of leukemia than those with mutations producing an altered protein, indicating that patients with specific mutations in the *FANCA* and *FANCG* genes are in a high-risk group with a poor hematologic outcome and should be considered to be candidates both for frequent monitoring and early therapeutic intervention (13,21).

It is known that *FANCD1* heterozygotes have an increased risk of developing breast, ovarian and other cancers (22), and it has been speculated that heterozygous carriers of mutations in other FA genes - A, C, D2, E, F, and G - may also have an increased cancer risk, perhaps depending on the presence of specific FA mutations.

In the present study, we used a mismatch PCR assay for the 3788-3790del mutation of the *FANCA* gene and we were able to detect this mutation in 30% of Brazilian FA patients and to establish a screening test that, besides being rapid and precise, is very useful and important for the Brazilian population.

Future studies about the correlation of the 3788-3790del mutation and the clinical outcome of the patients may lead to a better knowledge of the clinical picture of these patients and to a better understanding of the

relationship of this specific mutation with other mutations in compound heterozygote patients. Ultimately, they would lead to the right choices of treatment for each patient with improvement of the prognosis. With a large number of patients, it may also be possible to correlate this highly expressed mutation found in the Brazilian population with phenotypic characteristics, natural history of the disease and its behavior related to bone marrow transplant procedures.

Acknowledgments

The authors thank Dr. Arleen D. Auerbach and the staff of the Laboratory of Human Genetics and Hematology, The Rockefeller University, New York, NY, for their collaboration and technical assistance in this study.

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