

Relationship between chromosome fragility, aneuploidy and severity of the haematological disease in Fanconi anaemia

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Abstract

Fanconi anemia (FA) is a chromosome instability syndrome, characterized by progressive pancytopenia and cancer susceptibility. Other cellular features of FA cells are hypersensitivity to DNA cross-linking agents and accelerated telomere shortening. We have quantified overall genome chromosome fragility and euploidy as well as chromosomes 7 and 8 aneuploidy in peripheral blood lymphocytes from a group of FA patients and age-matched controls that were previously measured for telomere length. The haematology of FA samples were also characterized in terms of whole blood cell, neutrophil and platelet counts, transfusion dependency, requirement of androgens, cortico-steroids or bone marrow transplantation, and the development of bone marrow clonal cytogenetic abnormalities, myelodysplastic syndrome or acute myeloid leukemia. As expected, a high frequency of spontaneous chromosome breaks was observed in FA patients, especially of chromatid-type. No differences in chromosomes 7 and 8 monosomy, polysomy and non-disjunction were detected between FA patients and controls. The same was true for overall genome haploidy or polyploidy. Interestingly, the spontaneous levels of chromosome fragility but not of numerical abnormalities were correlated to the severity of the haematological disease in FA. None of the variables included in the present investigation (chromosome fragility, chromosome numerical abnormalities and haematological status) were correlated to telomere length. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Fanconi anemia (FA) is a rare autosomal recessive genetic disease characterized by increased spontaneous and induced chromosome instability, a diverse assortment of congenital malformations, progressive pancytopenia and cancer susceptibility, especially acute myeloid leukaemia (AML), but also solid

tumors. FA cells are hypersensitive to cross-linking agents and, to a lesser extent, to ionizing radiation. Other features of FA are abnormal cell cycle regulation, oversensitivity to oxidative stress, high level of apoptosis, overproduction of tumor necrosis factor, deficient induction of P53, and genomic instability (reviewed in [1]).

There are at least eight different genes involved in FA (*FANCA*, *B*, *C*, *D1*, *D2*, *E*, *F* and *G*), all of them but *FANCB* and *FANCD1* have been identified [2–8]. Most of the FA proteins (A, C, E, F, and G) assemble in a nuclear complex that is required for the activation, via

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monoubiquitination, of FANCD2 in response to DNA damage [9]. The recently cloned *FANCD2* gene is the only FA gene conserved in evolution [8] and it is thought to be a key player downstream in the FA pathway where ubiquitinated isoform of FANCD2 moves to DNA damage-induced nuclear foci in association with the double strand break repair protein BRCA1 [9].

Although the exact molecular defect is not known, FA cells are directly or indirectly deficient in DNA repair as they show abnormal rearrangements associated with V(D)J recombination [10] and impaired fidelity in blunt DNA end-joining [11,12]. One of the major consequences of this defect is increased chromosome fragility especially after the treatments with DNA cross-linking agents. Also resembling other chromosome fragility syndromes such as ataxia telangiectasia and Nijmegen breakage syndromes (reviewed in [13]), FA patients show an accelerated telomere shortening [14–16]. Telomeres play an important role in chromosome stability and segregation [17–19]. Elevated frequencies of aneuploidy have been reported in telomerase KO mice with short telomeres [18]. A causative role of telomere shortening in the well-documented X-chromosome aneuploidy in ageing humans has also been proposed [20]. In addition, telomere integrity is also crucial in terms of organismal viability at the haematological level [21,22]. We have therefore, measured chromosome fragility and numerical abnormalities in blood cells from haematologically characterized FA patients and age-matched controls that were previously quantified for telomere length [16]. The aims of this study are to (i) evaluate the spontaneous level of chromosome numerical abnormalities in FA patients compared to control, (ii) unravel whether spontaneous chromosome fragility and numerical abnormalities correlate with the severity of the haematological disease in FA and (iii) investigate a potential modulating role of telomere shortening in these variables.

2. Materials and methods

2.1. Subjects

Blood samples were obtained, with informed consent, from 11 unrelated FA patients during routine clinic visits and from 10 healthy individuals. All

patients belong to complementation group A, as determined by complementation analysis after retroviral mediated gene transfer (data not shown), except two patients whose complementation group is not known for technical reasons. The chromosome fragility analyses were performed in nine of these patients (three males and six females; 10.2 ± 1.5 years old, mean and standard error), and in nine age-matched healthy individuals (four males and five females; 10.3 ± 1.3 years old). Aneuploidy data were obtained from 10 patients (eight of them included in the chromosome fragility study) and six controls (five of them included in the chromosome fragility study). Haematological variables were obtained from all FA patients included in the chromosome fragility study. The telomere length of all patients and controls included in the chromosome fragility study was previously determined by quantitative fluorescence in situ hybridization (Q-FISH) [16]. This study was approved by the University Ethics Committee on Human Research.

2.2. Cell culturing and slide preparation

Five hundred microliter of whole blood were incubated in 5 ml cultures as described elsewhere [20] for 48 and 72 h in order to get metaphases and cytokinesis blocked binucleated cells, respectively. To obtain metaphases, colcemid ($0.1 \mu\text{g/ml}$; Gibco) was added 2 h prior harvesting following standard cytogenetic procedures. Cells were dropped onto clean slides and air-dried overnight. Some of the slides were stained with Giemsa and the remaining were used for telomere length measurement by Q-FISH in an experiment published elsewhere with the same donors [16]. To obtain binucleated cells, cytochalasin-B (Sigma; $6 \mu\text{g/ml}$) was added at 44 h in order to block cytokinesis and identify first division cells by their binucleated appearance. At harvesting time, cell pellets were resuspended in cold 0.075 M KCl and immediately centrifuged. The cells were then fixed in methanol:acetic acid (3:1), dropped onto clean slides and air-dried. The slides were kept at -20°C in the dark until FISH was performed.

2.3. Structural chromosome abnormalities

Slides were stained with 10% Giemsa in phosphate buffer, pH 6.8. A total of 26–100 (50 in most cases)

complete metaphases per sample were analysed for chromosome aberrations including gaps, chromosome and chromatid breaks, acentric fragments and chromosome and chromatid exchanges.

2.4. Aneuploidy studies

Chromosome numerical abnormalities were studied by multicolor chromosome specific interphase FISH in mononucleated and binucleated cells. The probes used were centromeric specific for chromosomes 7 and 8, and labeled with Spectrum Green (Vysis) and Cy3 (Cambio), respectively. The chromosome 8 probe was heated 5 min at 37 °C and then mixed with the chromosome 7 probe in its commercial buffer before dropping 10 µl onto the slides and denaturing 5 min at 85 °C. After hybridizing at 37 °C overnight, the slides were washed twice at 37 °C for 5 min in 2× SSC, twice in 60% formamide/2× SSC, twice for 5 min at room temperature in 2× SSC and air-dried. The cells were counterstained with 20 µl DAPI (0.1 µg/ml), cover slipped and sealed. Cells were classified according to the number of 7 and 8 chromosome signals. Chromosomes 7 and 8 monosomy ($2n - 1$) and polysomy ($2n + 1$; $2n + 2$), and overall haploidy (n) or polyploidy ($3n$ or $4n$) were evaluated by scoring 2000 mononucleated cells per sample in 72 h cultures. Non-disjunction was evaluated in the same slides by scoring 1000 binucleated cells harboring eight signals (corresponding to two chromosomes 7 and two chromosomes 8 per nuclei in case of a normal binucleated cell) per sample and classified according to the distribution of these signals in the daughter nuclei [23,24]. These analyses were performed with Olympus BX-50 fluorescence microscope equipped with a 100 W mercury lamp and a 1000× magnification objective with iris aperture.

2.5. Haematological variables

Haematological variables were obtained the same day of the extraction of the blood used for cytogenetic studies. These variables include whole blood cell counts, neutrophils, platelets, transfusion dependency, requirement of androgens, cortico-steroids or bone marrow transplantation (BMT), and the development of bone marrow clonal cytogenetic abnormalities, myelodysplastic syndrome (MDS) or AML. None of the patients underwent BMT, suffered AML or pre-

sented bone marrow clonal cytogenetic abnormalities involving chromosomes 7 and 8 before extracting the blood used in the present investigation. The patients were classified as being severe or mild. The criteria for this classification were basically based on the cell counts corrected for the age of the patients and the requirement of specific treatments to recover the low cell counts, either androgens and/or cortico-steroids and/or transfusions and, in the most severe cases, the requirement of bone marrow transplantation after MDS.

2.6. Statistics

The relationship between chromosome fragility or numerical abnormalities and severity of the haematological disease was performed with a one-factor ANOVA. The Student's *t*-test was used to compare FA patients and controls with respect to chromosome breaks and numerical abnormalities. The relationship between chromosome breaks, numerical abnormalities or haematological status and telomere length was determined with a regression analysis. All data were analysed using the statistical packages SPSS and CSS:Statistica™.

3. Results

Detailed data on chromosome structural abnormalities are shown in Table 1. The frequency of chromosome breaks was very low in healthy individuals (four chromatid breaks and one chromosome break in a total of 427 metaphases analyzed, most of them in a single donor). As expected, spontaneous chromosome fragility was detected in FA cells, especially chromatid-type breaks. In 512 metaphases, a total of 68 chromatid-type breaks and 14 chromosome-type breaks were found. Thus, spontaneous chromosome fragility was >10-fold increased in FA patients when compared to controls ($P < 0.001$). The observed interindividual heterogeneity in spontaneous chromosome fragility was not explained by heterogeneous telomere length, since no correlation was found between such variables.

Data on chromosome numerical abnormalities are detailed in Table 2. The simultaneous use of two chromosome specific centromeric probes in different colors allowed us to distinguish monosomy from

Table 1
Spontaneous chromosome breakage in FA patients and age-matched healthy controls

Donor code	Donor age	FA	Cells scored	Chromosome breakage				
				Chromatid type	Chromosome type	Total breaks	Breaks per 100 cells	
1	3	Yes	50	0	0	0	0	
2	14	Yes	100	7	1	8	8	
3	7	Yes	36	9	0	9	25	
4	9	Yes	50	11	5	16	32	
5	11	Yes	100	18	4	22	22	
6	10	Yes	50	1	0	1	2	
7	19	Yes	26	2	1	3	11.5	
8	10	Yes	50	12	2	14	28	
9	9	Yes	50	8	1	9	18	
Mean \pm S.D.							16.3 \pm 11.5	
12	6	Not	50	0	1	1	2	
13	14	Not	50	0	0	0	0	
14	7	Not	50	0	0	0	0	
15	8	Not	27	0	0	0	0	
16	11	Not	50	0	0	0	0	
17	10	Not	50	3	0	3	6	
18	19	Not	50	1	1	2	4	
19	9	Not	50	0	0	0	0	
20	9	Not	50	0	0	0	0	
Mean \pm S.D.							1.3 \pm 2.2	

haploidy and polysomy from polyploidy. In addition, the use of cytochalasin B-induced binucleated cells permitted the study of the segregation of chromosomes by visualizing the reciprocal products of the mitosis [23]. The frequency of non-disjunction of chromosomes 7 and 8 was similar in FA patients and controls ($P > 0.05$). The frequency of chromosome 8 monosomy was again very similar in FA patients and controls ($P > 0.05$). A tendency towards higher frequencies of monosomy 7 in FA patients was observed, but it did not reach statistical significance ($P = 0.07$). Similar non-significant results were obtained for other chromosome numerical abnormalities including haploidy and all types of polysomies and polyploidies. No relationship was found between any of the markers of numerical abnormalities and telomere length. It is therefore concluded that FA patients had normal levels of chromosome numerical abnormalities and that telomere shortening in FA is not associated to any parallel increase in chromosome aneuploidy.

Chromosome abnormalities were also correlated with the severity of the haematological disease in FA

patients in terms of whole blood cell counts, neutrophils, platelets, transfusion dependency, requirement of androgens, cortico-steroids or bone marrow transplantation, and the development of bone marrow clonal cytogenetic abnormalities, MDS or AML. All hematological variables are shown for each FA patient in Table 3. None of the FA patients underwent bone marrow transplantation or presented AML or bone marrow cytogenetic abnormalities involving chromosomes 7 and 8 at the day of blood extraction. To allow meaningful statistical comparisons, and considering that the blood cell counts in children is known to be highly dependent on age, the severity of the hematological disease in FA patients was classified as being mild or severe. A statistically significant positive correlation was found between the degree of chromosome fragility and the severity of the hematological disease ($P < 0.05$; Fig. 1). On the contrary, none of the markers of chromosome numerical abnormalities were correlated to the hematological status of the patients. Finally, no relationship was found between the previously determined telomere length and the

Table 2
Chromosome numerical abnormalities in FA patients and age-matched controls by interphase chromosome-specific centromeric FISH

Donor code	FA	Non-disjunction		Monosomy		Polysomy		Haploidy	Polyploidy
		Chromosome 7	Chromosome 8	Chromosome 7	Chromosome 8	Chromosome 7	Chromosome 8		
1	Yes	0	2	–	–	–	–	–	–
2	Yes	0	3	16	33	0	2	10	3
3	Yes	–	–	64	39	3	0	10	5
5	Yes	1	0	21	35	1	5	8	14
6	Yes	0	0	48	50	2	2	6	3
7	Yes	7	8	47	46	10	25	9	84
8	Yes	2	1	30	49	0	3	13	6
9	Yes	1	0	42	40	3	3	18	7
10	Yes	0	0	51	74	4	3	11	17
11	Yes	3	2	52	50	3	5	17	3
Mean ± S.D.		1.5 ± 2.2	1.7 ± 2.4	41.1 ± 14.7	46.2 ± 11.4	2.8 ± 2.7	5.3 ± 7.1	11.3 ± 3.8	17.4 ± 26.0
13	Not	4	6	21	41	10	10	13	33
16	Not	4	3	31	48	9	10	17	12
18	Not	0	1	35	53	1	3	13	2
19	Not	2	4	11	29	7	14	17	25
20	Not	1	1	38	45	0	2	6	1
21	Not	4	6	26	32	14	27	27	42
Mean ± S.D.		2.5 ± 1.7	3.5 ± 2.2	27.0 ± 9.1	41.3 ± 9.3	6.8 ± 5.4	11.0 ± 9.1	15.5 ± 6.9	19.2 ± 16.9

Table 3
Haematological variables and severity of the haematological disease in FA patients

Patient code	Neutrophil count ($10^9/l$)	WBC ($10^9/l$)	Hb (g/dl)	Platelets ($10^9/l$)	Transfusion dependency	Corticosteroids	Androgens	BM clonal cytogenetic abnormalities	MDS	BMT after blood extraction	Disease severity
1	3.8	9.6	11.9	335	Not	Not	Not	Not	Not	Not	Mild
2	1.7	3.3	12.8	88	Not	Not	Not	Not	Not	Not	Mild
3	0.4	8.7	9.3	28	Yes	Yes	Not	Not	Yes	Yes	Severe
4	0.7	1.9	8.1	4	Yes	Yes	Not	Yes ^a	Not	Not	Severe
5	0.7	3.6	9.6	31	Not	Not	Yes	Not	Not	Not	Mild
6	3.0	4.8	11.5	86	Not	Not	Not	Not	Not	Not	Mild
7	4.6	3.9	10.5	27	Yes	Yes	Yes	Yes ^a	Yes	Yes	Severe
8	0.7	2.4	8.3	19	Yes	Not	Yes	Not	Not	Not	Severe
9	0.6	3.0	6.6	54	Not	Yes	Yes	Not	Not	Not	Severe

^a Not involving chromosomes 7 or 8; WBC: white blood cell counts; Hb: haemoglobin; BMT: bone marrow transplantation.

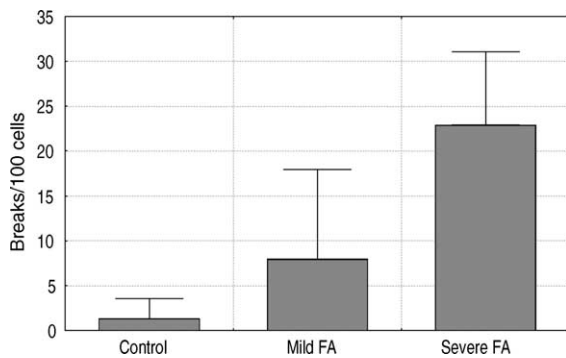


Fig. 1. Spontaneous chromosome fragility in controls and FA patients with mild or severe haematological disease. Means and standard deviations are shown by histograms and bars, respectively.

hematological data indicating that there is no relationship between the severity of the hematological disease and the length of the telomeres in our group of FA patients.

4. Discussion

A comprehensive analysis of chromosome numerical abnormalities have been performed in FA patients and controls that were previously quantified for telomere length by Q-FISH [16]. No increase in any of the markers of chromosome numerical abnormalities was detected in FA patients. Consistent with normal levels of chromosome numerical abnormalities in FA, no relationship was observed between numerical abnormalities and the severity of the haematological disease.

Monosomy 7 is frequently found in the bone marrow of patients with FA and is associated with AML and poor prognosis [25]. However, the same study showed no increase in monosomy 7 in 13 bone marrow samples from nine FA patients, as detected by interphase FISH. This observation together with our data indicating that neither monosomy 7 nor other chromosome numerical abnormalities are found in blood samples from FA patients suggests that chromosome numerical abnormalities in peripheral lymphocytes are not causally related to the haematological disease.

More interestingly, a positive and statistically significant correlation between the severity of the haematological disease and the degree of spontaneous chromosome fragility became evident in spite of the relatively low number of patients included in this study. An heterogeneous complementation group of the FA patients is not expected to explain our results, since most, if not all, FA patients belong to the same complementation group [26]. However, a larger group of patients should be analyzed to determine a possible prognostic value of spontaneous chromosome fragility, as suggested by our results. This analysis would be easy to perform since all FA patients are subject to chromosome fragility studies and are extensively characterized from an haematological point of view. The deleterious effect of chromosome fragility in the evolution of the haematological disease would be supported by the reported observation that spontaneous chromosome aberrations in FA patients are located at fragile sites, oncogenes and breakpoints involved in cancer chromosomal rearrangements, particularly with AML chromosome abnormalities [27].

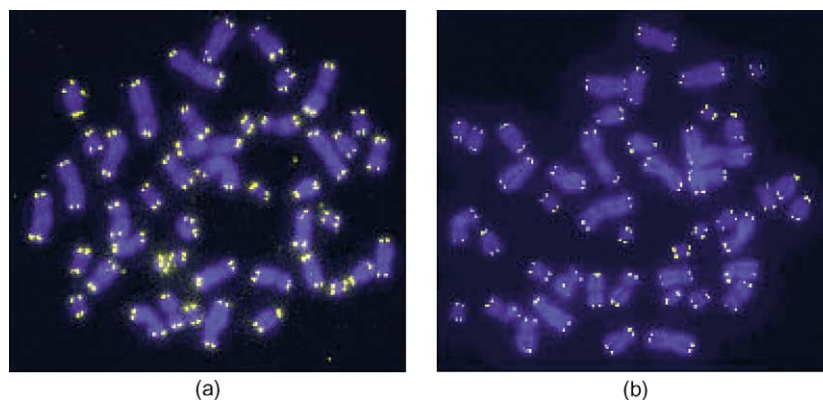


Fig. 2. Metaphase cells after Q-FISH with a PNA telomeric probe: (a) control individual; (b) concurrently processed age-matched FA patient. Note that telomeres in the control are brighter than in the FA patient.

Although average telomere length in the FA patients included in this study was 0.68 kb shorter than in controls ([16]; Fig. 2), no increase in chromosome numerical abnormalities was observed in the FA group. Similarly, no correlation between telomere length and chromosome fragility was detected. A correlation between telomere shortening and aneuploidy has been previously described in telomerase and *ATM*^{-/-} mice with short telomeres using a number of animals similar to the number of patients used in the present investigation [18,28]. However, the degree of telomere erosion in these mouse models is much higher than what we observed in FA patients *in vivo*.

Unlike the study conducted by Leteurtre and co-workers [14], no correlation between telomere shortening and the severity of the haematological disease was observed in our group of patients. Leteurtre and co-workers reported a telomere shortening of 1.75 kb in FA patients compared to controls by terminal restriction fragment (TRF) length analysis in mononuclear blood cells from peripheral blood. However, the genomic measure of telomere length in FA cells by TRF analysis in blood DNA could be artefact-prone in FA since the blood cell counts are highly altered in these patients and it is known that telomere length is highly heterogeneous between blood cell types [29,30]. Considering this high heterogeneity in telomere length between different blood cell types, the extensive shortening of TRF length reported by Leteurtre and co-workers could be partly attributable to the highly altered hematological

status of FA patients, specially those showing a severe hematological disease. The fact that we observed a milder reduction in telomere length in FA by Q-FISH, a technique that specifically detects TTAGGG repeats in a single cell type (PHA-stimulated T-lymphocytes metaphases), would be consistent with this possibility.

The main conclusions that arise from the present work are that chromosome numerical abnormalities are not increased in FA patients when compared to controls and that the spontaneous levels of chromosome fragility, but not of numerical abnormalities was correlated to the severity of the haematological disease in FA. Finally, since the excess in telomere shortening was previously found to be only 0.68 kb in the FA group, the observed telomere erosion does not explain the cellular phenotype and the severity of the hematological disease in our group of patients.

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Quantitative PCR analysis reveals a high incidence of large intragenic deletions in the FANCA gene in Spanish Fanconi anemia patients

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Abstract. Fanconi anaemia is an autosomal recessive disease characterized by chromosome fragility, multiple congenital abnormalities, progressive bone marrow failure and a high predisposition to develop malignancies. Most of the Fanconi anaemia patients belong to complementation group FA-A due to mutations in the FANCA gene. This gene contains 43 exons along a 4.3-kb coding sequence with a very heterogeneous mutational spectrum that makes the mutation screening of FANCA a difficult task. In addition, as the FANCA gene is rich in Alu sequences, it was reported that Alu-mediated recombination led to large intragenic deletions that cannot be detected in heterozygous state by conventional PCR, SSCP analysis, or DNA sequencing. To overcome this problem, a method based on quantitative fluorescent multiplex PCR was proposed to detect intragenic deletions in FANCA involving the most fre-

quently deleted exons (exons 5, 11, 17, 21 and 31). Here we apply the proposed method to detect intragenic deletions in 25 Spanish FA-A patients previously assigned to complementation group FA-A by FANCA cDNA retroviral transduction. A total of eight heterozygous deletions involving from one to more than 26 exons were detected. Thus, one third of the patients carried a large intragenic deletion that would have not been detected by conventional methods. These results are in agreement with previously published data and indicate that large intragenic deletions are one of the most frequent mutations leading to Fanconi anaemia. Consequently, this technology should be applied in future studies on FANCA to improve the mutation detection rate.

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Fanconi anaemia (FA) is an autosomal recessive disease characterized by genomic instability, multiple congenital abnormalities, progressive bone marrow failure and a high predisposition to develop malignancies such as acute myeloid leukaemia and squamous cell carcinoma (Auerbach et al., 1993). FA cells are characterized by chromosomal fragility and are hypersensitive to DNA cross-linking agents such as diepoxybutane and mitomycin C. These features are used as a diagnostic tool (Auerbach, 1993).

To date eight different complementation groups have been described (FA-A, -B, -C, -D1, -D2, -E, -F, -G), and the genes corresponding to seven of these complementation groups (FANCA, FANCC, FANCD1/BRCA2, FANCD2, FANCE,

FANCF and FANCG) have been cloned and characterized (Strathdee et al., 1992; Lo Ten Foe et al., 1996; The Fanconi Anemia/Breast Cancer Consortium, 1996; de Winter et al., 1998, 2000a,b; Timmers et al., 2001; Howlett et al., 2002). It is known that although their protein products do not share significant homology to each other or with other genes in the databases, all of them participate in a common pathway (reviewed in Bogliolo et al., 2002). The FANCA, -C, -G, -E and -F proteins assemble in a multisubunit nuclear complex (de Winter et al., 2000c; Medhurst et al., 2001) required for monoubiquitin-mediated activation of FANCD2 after DNA damage or during S-phase and later targeting to nuclear foci containing BRCA1 and Rad51 (Garcia-Higuera et al., 2001; Taniguchi et al., 2002). This suggests a role for the FA pathway in DNA repair by homologous recombination during S-phase (Taniguchi et al., 2002).

The genetic heterogeneity of FA makes the subtyping of FA patients a difficult task, but over 90% of the FA population worldwide is represented by complementation groups FA-A, -C or -G, FA-A being the most prevalent, accounting for ~ 65% of all FA cases (Kutler et al., 2003). There are, however, regional and ethnic differences. It is estimated that about 80% of all FA patients belong to complementation group FA-A in Spain (Casado et al., 2002) or even more in Italy (Savino et al., 1997). Therefore, a proper detection of FANCA mutations is of critical clinical significance.

The FANCA gene contains 43 exons along a 4.3-kb coding sequence (Ianzano et al., 1997) with a very heterogeneous mutational spectrum, with more than 100 different FANCA mutations described to date, including all types of possible point mutations such as frame-shift mutations, small insertions or deletions, splicing defects, and nucleotide substitutions (The Fanconi Anemia/Breast Cancer Consortium, 1996; Lo Ten Foe et al., 1996; Levran et al., 1997; Savino et al., 1997; Morgan et al., 1999; Wijker et al., 1999). This makes the mutation screening of FANCA a very difficult task. As the FANCA gene is rich in Alu sequences, it was suggested and later reported that Alu-mediated recombination is an important mechanism for the generation of FANCA mutations (Centra et al., 1998; Levran et al., 1998), mainly large deletions that cannot be detected by conventional methods such as SSCP analysis or DNA sequencing. To overcome this problem, a method based on quantitative fluorescent multiplex PCR was developed. This method was used to screen 26 cell lines from FA patients belonging to complementation group A (Morgan et al., 1999), and a high frequency of large deletions was found. In some populations with high prevalence of FA such as the South African Afrikaner, this method has also been employed to scan the FANCA gene, and it was found that exon 12–31 deletion is the most common mutation in this population due to a founder effect (Tipping et al., 2001).

Taking advantage of this novel method developed by Morgan and co-workers (Morgan et al., 1999), we applied the proposed strategy to screen a group of 25 FA Spanish patients previously assigned to complementation group FA-A by retroviral transduction (Hananberg et al., 2002; Casado et al., 2002). The suggested multiplex PCR was performed with some minor modifications and optimisations to amplify the five most com-

monly deleted exons of the FANCA gene and a control exon from an unrelated gene. After analyzing DNA samples from 25 Spanish FA-A patients, a total of eight different large deletions from one to more than 26 exons were detected, all of them in heterozygous state, confirming that this type of mutation is one of the most prevalent amongst FA-A patients.

Materials and methods

Patients

Blood samples were obtained from 25 Spanish FA patients with informed consent. All of them were previously diagnosed on the basis of their clinical traits and cellular hypersensitivity to diepoxybutane, and assigned to complementation group FA-A by retroviral transduction (Casado et al., 2002). In some cases, lymphoblastoid cell lines established by Epstein-Barr virus infection were used.

Quantitative fluorescent multiplex PCR reaction

Genomic DNA was extracted directly from peripheral blood samples or lymphoblastoid cell lines by salt/chloroform or phenol/chloroform standard methods, respectively. DNA concentration was determined spectrophotometrically and a sample was diluted to 25 ng/ μ l to perform PCR.

Exons 5, 11, 17, 21 and 31 from the FANCA gene were simultaneously amplified together with exon 1 of the myelin protein zero (MPZ1) gene as an external control with respect to the FANCA gene exons in the same reaction. At least four non-FA control samples were included together with the FA sample in every PCR round as normal controls. PCR amplifications were performed in 25- μ l reactions with 125 ng DNA, 1 \times Taq DNA polymerase buffer, 250 μ M each dNTP and 1.8 mM MgCl₂. Primer concentrations and sequences are shown in Table 1. All primers were fluorescently labelled with phosphoramidite 6-FAM dye (PE Biosystems). PCR conditions were an initial denaturation step at 94°C for 5 min, followed by 21 cycles of denaturation at 94°C for 45 s, annealing step at 55°C for 45 s, and extension for 1 min at 72°C. A final extension step at 72°C for 5 min was performed. A 3- μ l aliquot of the PCR product was then mixed with 15 μ l of formamide and 0.2 μ l of Genescan ROX-500 size standard (PE Biosystems). Samples were denatured at 94°C for 5 min and run and analysed on an ABI 310 Gene Analyzer, by means of the GeneScan and Genotyper software, as described in Morgan et al. (1999). Briefly, apart from the FANCA exons (FAAX), another internal control exon from a different gene (exon 1 of the Myelin protein zero; MPZX1) was co-amplified to detect heterozygous deletions. For each of the patients, the peak areas corresponding to the MPZX and each of the five tested FANCA exons were determined in samples from the patient (pt) and from a healthy donor (c). This comparison gave a value called dosage quotient (DQ) that corresponds to the following formula: (ptFAAX peak area/ptMPZX1 peak area)/(cFAAX peak area/cMPZX1 peak area). The DQs range from 0.75 to 1.25 when the DNA copy number of a specific exon is normal, or half/double this value when there is a heterozygous deletion.

Results and discussion

Mutations in the FANCA gene are the most prevalent cause of FA. More than 70 polymorphisms in this gene and over 100 different mutations have been accumulated in the International Fanconi Anemia Registry Database, most of them being in a heterozygous state. This is a clear indication of the complexity of detecting mutations in this gene. Although several studies have focused in finding pathogenic mutations among these patients, in some cases the successful detection rates were poor (Savino et al., 1997) in part due to the high rate of intragenic deletions (Centra et al., 1998; Levran et al., 1998; Wijker et al., 1999). This high rate of intragenic deletions along the FANCA gene sequence is the consequence of Alu-mediated intragenic

Fig. 1. Electrophoretogram showing the peak pattern of the multiplex products from a healthy control (**a**), compared with an exon 5 deletion carrier (**b**). Note that the peak area of exon 5 in the patient is half of the same exon in the healthy individual.

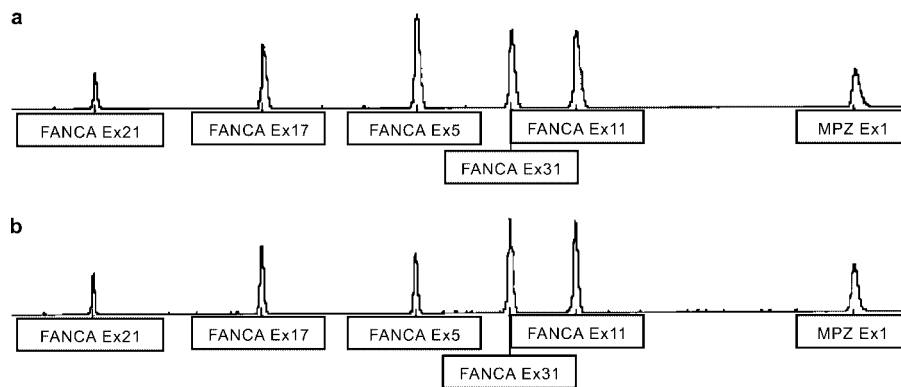


Table 1. Sequence, size and concentration of every primer of the set used

Gene: Exon	Primers (5'→3')	Amplicon size (bp)	Concentration (μ M)
FANCA:			
Exon 5	Forward: ACC TGC CCG TTG TTA CTT TTA Reverse: AGA ACA TTG CCT GGA ACA CTG	250	0.4 0.2
Exon 11	Forward: GAT GAG CCT GAG CCA CAG TTT GTG Reverse: AGA ATT CCT GGC ATC TCC AGT CAG	301	0.4 0.2
Exon 17	Forward: CCA TGC CCA CTC CTC ACA CC Reverse: GTG AAA AGA AAC TGG ACC TTT GCA	205	0.08 0.2
Exon 21	Forward: TAA GCC ATA GCT GAC TTA ATT Reverse: GCA CAA GTC CCA GAG TGG ACA AG	156	0.6 0.2
Exon 31	Forward: CAC ACT GTC AGA GAA GCA CAG CCA Reverse: CAC CGC GCC TGG CAA TAA ATA TC	285	0.08 0.2
Myelin protein zero:			
Exon 1	Forward: CAG TGG ACA CAA AGC CCT CTG TGT A Reverse: GAC ACC TGA GTC CCA AGA CTC CCA G	389	0.2 0.2

recombination (Centra et al., 1998). Here we have applied an improved fluorescent PCR-based method with conditions similar to those proposed by Morgan et al. (1999), using primers that amplify exons 5, 11, 17, 21 and 31 of the FANCA gene. These exons were chosen based on previous observations showing that at least one of these five exons was absent in all FANCA deletions observed to date (Morgan et al., 1999). Some minor modifications in the sequence and concentration of some primers used by Morgan et al. (1999) were introduced to improve the efficiency of the technique.

We initially established the FA complementation group in all our samples by retroviral complementation to later select those belonging to the complementation group FA-A. Using DNA from these patients (either from blood samples or cell lines), the multiplex PCR reaction was performed. The rationale is that the area of the amplification peak of a given exon should be half the expected size in case of a heterozygous deletion. Figure 1 shows a representative example of an electrophoretogram of an exon 5 deletion carrier and Table 2 shows a numerical example of a typical result of a patient with a heterozygous exon 21 deletion. After analyzing 25 FA-A samples, a total of eight large deletions containing from one to more than 26 exons were detected, all of them in heterozygosis (Fig. 2). Thus, one third of the patients carried a large intragenic deletion, emphasizing the relevance of this type of mutation in FA-A patients.

Because of the very nature of the approach used here, the second pathogenic deletion was not detected. None of the mutations found in this work seem to be novel mutations, although this should be confirmed with a more accurate analysis using RT-PCR and polymorphic marker typing to map the approximate endpoints of the deletions. The percentage of heterozygous deletions described here is similar to that previously reported by Morgan and co-workers (1999). It is important, however, to keep in mind that although we have detected FANCA deletions in 32% of the patients, it is possible that the deletion frequency is even higher. Obviously, the dosage assay would have missed small deletions in the 5' half of the gene between exons that we tested, and also deletions that occur 3' to exon 31.

While detection of point mutations or small insertions/deletions is well established, large genomic deletions constitute a technical problem and it might be difficult to develop a cheap, easy and reliable quantitative method. This is a serious limitation as this type of mutation constitutes an important fraction of the mutations described not only in FA-A patients, but also in several malignancies, although their prevalence is yet underestimated due to the lack of a robust detection method (Armour et al., 2002). Several genes such as MSH21 or BRCA1 might be also affected by large heterozygous deletions and, in both cases, a similar method named quantitative multiplex PCR of short fluorescent fragments or other methods have been developed



Fig. 2. Scheme of the large deletions detected in the 25 samples analyzed, along the schematic figure of FANCA gene exons. The amplified exons are shown in grey colour. Continuous lines refer to the exons that are deleted and stippled lines refer to the fact that the exact break points are not detected by this method.

Table 2. Results of a dosage multiplex assay of an exon 21 deletion carrier

Exon	Peak area		Dosage quotient					
	Healthy donor	FA patient	MPZX1	FAAX5	FAAX11	FAAX17	FAAX21	FAAX31
MPZX1	62062	17523	–	1.28	1.17	1.05	2.30	1.07
FAAX5	36569	8083	0.78	–	0.91	0.82	1.80	0.84
FAAX11	56996.5	13794	0.86	1.09	–	0.90	1.97	0.92
FAAX17	19361	5218	0.95	1.22	1.11	–	2.19	1.02
FAAX21	23321	2864	0.43	0.56	0.51	0.46	–	0.47
FAAX31	14868	3914	0.93	1.19	1.09	0.98	2.14	–

for this same purpose (Charbonnier et al., 2000; Casilli et al., 2002; Wang et al., 2002, 2003). We proposed that the approach described here could also be applied to the above mentioned genes by just adapting the primers and PCR conditions. Most methods used to date are based on PCR although other techniques should be considered using cytogenetic tools or Southern-blot (Armour et al., 2002). Although PCR is basically a qualitative technique, several modifications can be introduced to get quantitative or semi-quantitative results, for example by using control samples and internal controls to coamplify and compare with the sample under study, in a manner similar to the method described here.

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*A common founder mutation in FANCA
underlies the world highest prevalence of
Fanconi anemia in Gypsy families from Spain*

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Abstract

Fanconi anemia (FA) is a genetic disease characterized by bone-marrow failure and cancer predisposition. Here we have identified Spanish Gypsies as the ethnic group with the world highest prevalence of FA (carrier frequency of 1/64-1/70). DNA sequencing of the *FANCA* gene in 8 unrelated Spanish Gypsy FA families after retroviral subtyping revealed a homozygous *FANCA* mutation (295C>T) leading to FANCA truncation and FA pathway disruption. This mutation appeared specific for Spanish Gypsies as it is not found in other Gypsy FA patients from Hungary, Germany, Slovakia and Ireland. Haplotype analysis showed that Spanish Gypsy patients all share the same haplotype. Our data thus suggest that the high incidence of FA among Spanish Gypsies is due to an ancestral founder mutation in *FANCA* that originated in Spain less than six hundred years ago. The high carrier frequency makes the Spanish Gypsies a population model to study FA heterozygote mutations in cancer.

Introduction

Fanconi anemia (FA) is a rare genetic syndrome characterized by various congenital abnormalities, bone-marrow failure and cancer predisposition¹. Recent results of somatic cell hybridisation, protein association and other functional complementation studies indicate that there are at least 11 complementation groups in FA (A, B, C, D1, D2, E, F, G, I, J, and L), each connected with a distinct disease gene^{2,3}. The products of the genes *FANCA*, *C*, *E*, *F*, *G*, *L* and possibly *I* interact in a nuclear core complex required for FANCD2 activation by monoubiquitination at residue K561, a key post-translational modification in the FA pathway⁴. Active FANCD2 then functions downstream to maintain the genome integrity by mechanisms which are still not well understood⁵⁻⁷.

FA is a rare autosomal recessive disease with an overall prevalence of 1-5 per million and an estimated carrier frequency of 1/200-1/300 in most populations⁸. This is also true in Spain which has about 40 million inhabitants and 122 FA patients registered in the Spanish FA Research Network (SFARN) register. Some ethnic groups have a higher prevalence of FA due to founder effects and isolation. Two previously reported examples are the Afrikaner population of South Africa and the Ashkenazi Jewish population with a carrier frequency of 1/77 and 1/90, respectively⁹. Some geographic clusters also exist in Italy with a similar high prevalence¹⁰⁻¹¹. Here we report an even higher prevalence of FA in Spanish Gypsies. A total of 31 FA Gypsy patients are currently registered in the SFARN register indicating that one quarter of all Spanish FA patients belong to this ethnic group. As there are approximately 500.000 to 600.000 Gypsies in Spain¹², the estimated frequency of carriers is 1/64-1/70, the highest ever reported in the world. Here we report the genetic characterization of FA in this ethnic group.

Results and Discussion

In order to genetically characterize FA in this ethnic group, blood samples were obtained from FA patients and carriers, all previously diagnosed on the basis of their clinical features and chromosome hypersensitivity to diepoxybutane (DEB) essentially as described elsewhere¹³⁻¹⁵. This study was duly approved by the Universitat Autònoma de Barcelona Ethical Committee for Human Research. The affected families were not clustered in any specific geographic region but scattered throughout Spain. All of the Spanish Gypsy patients studied by FANCD2 immunoblotting lacked monoubiquitinated FANCD2 (Fig. 1a), indicating that the genetic defect was up-stream of FANCD2 in the FA core complex. Genetic subtyping by retrovirus mediated gene transfer showed that all the Spanish Gypsy patients examined belong to the complementation group FA-A (Fig 1b). Quantitative fluorescence multiplex PCR analysis¹⁶⁻¹⁷ indicated that no large deletions involving the most frequently deleted *FANCA* exons (5, 11, 17, 21 and/or 31) were present in Spanish Gypsy patients (data not shown) although this type of mutation has been found in a quarter of the Spanish non-Gypsy FA-A patients previously analysed¹⁷. DNA sequencing of *FANCA* uncovered a novel homozygous truncating mutation in exon 4 (295C>T; Q99X) in eight unrelated Spanish Gypsy FA patients (Fig. 1c; Table 1). One of these Spanish Gypsy families was of Portuguese origin (Table 1). This mutation was not found in a Spanish Gypsy patient of Romanian descent, in Spanish non-Gypsy FA-A patients or in healthy individuals, and all FA obligate carriers analysed were heterozygous for this mutation (Fig.1c, Table 1 and data not shown). The truncating nature of the mutation was confirmed by FANCA immunoblotting with antibodies detecting the C-terminus of FANCA (Fig. 1d). While approximately 20% of all Spanish FA patients show somatic mosaicism (data not shown), none of the Gypsy

patients were mosaic, suggesting that the pathogenic mutation is difficult to revert by a mutation *in cis*, as expected for a base substitution.

Afro-American FA patients from Ghana also have a truncating mutation in exon 4 of *FANCA* but involving a different codon. The Ghana mutation is associated with a severe clinical phenotype (A.D. Auerbach, personal communication). However, analysis of the registered clinical and cellular data obtained from all Spanish FA patients in an SFARN standardized manner, the clinical phenotype of the Spanish Gypsy patients is similar to other Spanish FA-A patients with similar average number of congenital abnormalities and age of onset of impaired hematopoiesis. Only the severity of the congenital abnormalities was slightly higher in Spanish Gypsies. At the cellular level, the phenotype is also quite similar. This is true for the spontaneous frequency of breaks per cell (0.36 ± 0.16 in 5 Spanish Gypsy patients versus 0.22 ± 0.03 in 18 non-Gypsy Spanish FA-A non-mosaic patients), the number of breaks per cell after DEB (3.1 ± 0.8 in 7 Spanish Gypsy patients versus 3.6 ± 0.4 in 20 non-Gypsy Spanish FA-A non-mosaic patients) and the percentage of aberrant cells after DEB (76.7 ± 17.3 in 7 Spanish Gypsy patients versus 74.6 ± 14.6 in 20 non-Gypsy Spanish FA-A patients). The survival of blood T-cells at 33nM of MMC is even higher ($P=0.014$) in Gypsy patients (33.1 ± 1.87 % in 5 Gypsy patients versus 21.2 ± 1.5 in 27 non-Gypsies FA-A patients). The fact that a similar mutation in FA patients of Ghanese decent is associated with a severe phenotype suggests that other ethnically-related genetic factors such as *trans*-acting polymorphic genes are probably modulating the clinical evolution in FA patients. Such effect has been shown even for an identical mutation, IVS4+4 in *FANCC*, in Ashkenazi and Japanese populations.

There are genetic evidences that the Gypsies originally migrated from Northern India to the West in the early-middle ages, and then in a second XIVth century

migration, known as the Aresajipi migration, from Eastern Europe to Western Europe¹². They arrived to Spain through the Pyrenees in the early XVth century, with the first documented presence in Barcelona in 1425¹⁸. The genealogy of Gypsies is highly complex but they are divided in three principal tribal groups, all genetically related: the Roma, the Munush (also called Sinti) and the Gitanos (also known as Cales), the latter mainly concentrated in Spain and France¹⁸⁻¹⁹. In order to define whether the identified mutation is unique in Spain or present in the whole ethnic group and brought to Spain, we performed a world-wide search of FA Gypsy patients outside Spain. Although millions of Gypsies live in neighbouring countries such as France, Germany, Italy and, especially in East European countries such as Romania, Slovakia or Hungary, only 3 non-Spanish Gypsy FA-A patients were found in Hungary (1 family), Germany (1 family) and Slovakia (1 family). In addition 3 patients from Ireland belonging to the Irish Travellers ethnic group were also identified. The Irish Travellers have been often confused with traditional Gypsies. However, in spite of cultural exchange and some intermarriage, the Irish Travellers remain a distinct people. The fact that FA is usually associated to consanguinity and the above mentioned intermarriage of Irish Travellers with Roman Gypsies, made us include these Irish family in this study. DNA sequencing revealed that none of these patients share the mutation with the Spanish Gypsies (Table 1). Therefore, the *FANCA* 295C>T mutation is specific of Spanish Gypsy patients.

We finally performed an extensive haplotype analysis in order to determine a common ancestral origin of the Spanish Gypsy mutation. Haplotype analysis was carried out by PCR-based techniques as previously described, by using four polymorphic microsatellite markers flanking *FANCA*, D16S3026, D16S3407, D16S3121 and D16S303⁹⁻²⁹. The results indicate that all Spanish Gypsy patients, including the one of Portuguese origin, had exactly the same homozygote haplotype that

is clearly distinguishable from the haplotype of Spanish Gypsy carriers, the non-Gypsy FA-A Spanish patients, and the non-Spanish Gypsy patients or Irish Travellers (Table 1, and data not shown). This result clearly indicates the Spanish Gypsy mutation is associated to a specific haplotype and that all Spanish Gypsy patients studied share a common ancestry.

In conclusion, the data presented here strongly suggest that the high incidence of FA among Spanish Gypsies is due to a founder ancestral mutation in *FANCA*, leading to protein truncation and FA pathway disruption. This mutation probably originated less than six hundred years ago in a Gypsy family that migrated to Spain and spread by consanguinity all over the Iberian peninsula, including Spain and Portugal. Consanguinity and a common ancestry are well-known feature of this ethnic group²¹. The extremely high incidence of FA carriers among Spanish Gypsies and the easy detection of their associated mutation/haplotype makes this ethnic group a population model to study the role of heterozygous FA mutations and the FA pathway in both sporadic and hereditary cancer. Several studies are underway in our laboratory in this context.

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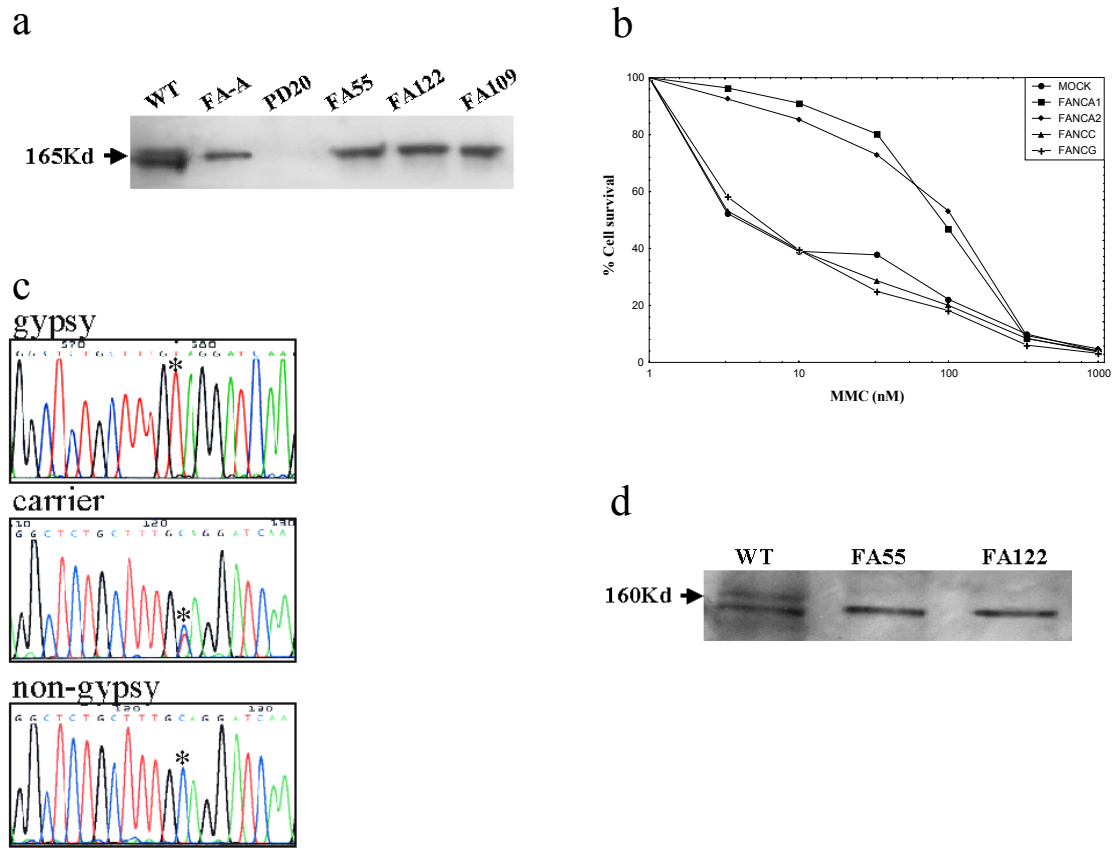
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Table 1 : Mutational and haplotype analysis of Fanconi anemia Gypsy patients

Patient	Residence	Origin	Tribal grup ^a	Mutational analysis	haplotype ^b
FA55	Spain	Spain	Cales	Homozygote 295C>T	1/1
FA87	Spain	Spain	Cales	Homozygote 295C>T	1/1
FA103	Spain	Spain	Cales	Homozygote 295C>T	1/1
FA108	Spain	Spain	Cales	Homozygote 295C>T	1/1
FA109	Spain	Spain	Cales	Homozygote 295C>T	1/1
FA113	Spain	Spain	Cales	Homozygote 295C>T	1/1
FA122	Spain	Spain	Cales	Homozygote 295C>T	1/1
FA123	Spain	Portugal	Cales	Homozygote 295C>T	1/1
FA163	Spain	Romania	Rom	ND ^c /ND	2/2
VU483	Holland	Hungary	Rom	1263delF/ND	2/3
VANA	Slovakia	Romania	Rom	Homozygote IVS11+2 del TAGG	4/4
KZFE	Germany	Germany	Sinti	Homozygote 1267C>T	5/5
EJM-737	Ireland	Ireland	Irish Travellers	Homozygote ex11-14 deletion	6/6
JM-512	Ireland	Ireland	Irish Travellers	Homozygote ex11-14 deletion	6/6
KMM-336 ^d	Ireland	Ireland	Irish Travellers	Homozygote ex11-14 deletion	6/6

^aThe genealogy of Gypsies is complex but they are divided in three principal tribal groups, the Rom, the Cales (or Gitanos) and the Munush (or Sinti). Irish Travellers are often confused with Roma Gypsies but their remain a separate ethic group (see Discussion); ^bArbitrary numbers based on the analysis of 4 highly polymorphic microsatellite markers flanking *FANCA*. Each chromosome/allele is defined by an haplotype block including the four markers. All Spanish patients but the one of Romanian origin were homozygous for a given haplotype block (1) and all the other patients but VU483 were homozygous for an specific but different haplotype block (2, 4, etc..). ^cD: mutation not detected; the 295C>T mutation was disregarded in the last 7 patients of the table. ^dThis patient corresponds to cell line EUFA/VU544

Figure 1



Legend to Figure 1

Genetic characterization of FA in Spanish Gypsy patients. a) FANCD2 immunoblotting revealed two isoforms in a wild-type cell (WT), no signal in a FANCD2 deficient cell line (PD20) and a non-ubiquitinated isoform in a FA-A cell line or in cell lines derived from Spanish Gypsy patients 55, 122 and 109, indicating that the genetic defect is upstream FANCD2 monoubiquitination. Immunoblotting experiments were performed following standard Western blot method with a commercially available antibody against FANCD2 (Santa Cruz Biotech, CA). b) Subtyping by retrovirus mediated cDNA gene transduction in a cell line derived from a Spanish Gypsy FA patient. The complementation group was determined by retroviral transduction of cDNA of FA genes essentially as previously reported²². Briefly peripheral blood T cells were proliferated in the presence of a monoclonal antibody anti-CD3 and anti-CD28. Afterwards, cells were transduced with retroviral vectors containing *FANCA* (two vectors), *FANCC*, *FANG* cDNA and control vector (mock) and then evaluated for MMC hypersensitivity to detect phenotypic cellular correction. Only retrovirus encoding wild-type *FANCA* cDNAs led to reversion of the cellular hypersensitivity to mitomycin C (MMC) indicating that the patient belong to complementation group FA-A. c) Spanish Gypsy FA patients with a 295C>T base change, as detected by DNA sequencing, leading to a stop codon (Q99X). All obligate carriers were heterozygote for this mutation. d) FANCA immunoblotting with an affinity purified antibody against the C-terminus of the FANCA protein in two Spanish Gypsy FA patients 55 and 122 and a wild-type cell line. FANCA signal (upper band) is not present in the Gypsy patients consistent with the truncating nature of the Spanish Gypsy mutation. The lower band is unspecific signal that serves as internal control and that is equally present in all cell extracts.