Molecular analysis of Fanconi anemia: the experience of the Bone Marrow Failure Study Group of the Italian Association of Pediatric Onco-Hematology

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ABSTRACT

Fanconi anemia is an inherited disease characterized by congenital malformations, pancytopenia, cancer predisposition, and sensitivity to cross-linking agents. The molecular diagnosis of Fanconi anemia is relatively complex for several aspects including genetic heterogeneity with mutations in at least 16 different genes. In this paper, we report the mutations identified in 100 unrelated probands enrolled into the National Network of the Italian Association of Pediatric Hematoly and Oncology. In approximately half of these cases, mutational screening was carried out after retroviral complementation analyses or protein analysis. In the other half, the analysis was performed on the most frequently mutated genes or using a next generation sequencing approach. We identified 108 distinct variants of the *FANCA, FANCG, FANCC, FANCD2*, and *FANCB* genes in 85, 9, 3, 2, and 1 families, respectively. Despite the relatively high number of private mutations, 45 of which are novel Fanconi anemia alleles, 26% of the *FANCA* alleles are due to 5 distinct mutations. Most of the mutations are large genomic deletions and nonsense or frameshift mutations, although we identified a series of missense mutations, whose pathogenetic role was not always certain. The molecular diagnosis of Fanconi anemia is still a tiered procedure that requires identifying candidate genes to avoid useless sequencing. Introduction of next generation sequencing strategies will greatly improve the diagnostic process, allowing a rapid analysis of all the genes.

Introduction

Fanconi anemia (FA) is a rare hereditary autosomal recessive and X-linked disease characterized by congenital malformations, bone marrow failure, and cancer predisposition.[1] A diagnosis of FA is principally based on careful evaluation of patient history and physical examination in combination with laboratory analyses of sensitivity of peripheral blood lymphocytes to DNA interstrand cross-linking agents such as diepoxybutane or mitomycin C.[2] If the cells demonstrate the characteristic hypersensitivity to diepoxybutane or mitomycin C, identification of the germ-line mutations is important for confirming diagnosis, for genotype-phenotype correlations, and for prenatal and carrier testing. However, a series of additional problems have made the diagnostic procedures in FA much more complicated.

Firstly, at least 16 genes (*FANCA, FANCB, FANCC, FANCD1/BRCA2, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCJ/BRIP1, FANCL, FANCM, FANCN/PALB2, FANCO/RAD51C, FANCP/SLX4*, and *FANCQ/ERCC4*) with a total of 307 exons are responsible for FA,[3-5] thus making sequencing analysis a time-consuming approach. Even the screening of the most frequently mutated genes, such as *FANCA, FANCC*, and *FANCG*, which together account for

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approximately 80% of FA patients worldwide, will not be successful in at least 20%. Secondly, there is a wide spectrum of mutations, mainly private, spanning the entire genes, including intragenic deletions, as frequently observed in FANCA (*http://www.rockefeller.edu/fanconi/*). Since mutational screening would largely benefit from a preliminary knowledge of the candidate gene, complementation by cell fusion or by viral transduction, as well as protein analyses, have been established as screening strategies to identify the putative gene that is defective.[6,7] Thirdly, a false negative or inconclusive chromosomal breakage test might occur in patients who develop hematopoietic mosaicism due to reversion of the cellular FA phenotype.[8,9] This phenomenon arises if a spontaneous genetic event reconstitutes normal protein activity of one allele. Since it is unlikely that the same somatic event will also occur in primary skin fibroblasts, these cells are often used for mutational screening.

In this paper, we report the molecular data of 100 FA families enrolled into the National Network of the Marrow Failure Study Group of the Italian Association of Pediatric Hematology and Oncology. This cohort consists of 76 new families, as well as 24 families that have been described in previous reports.[10,11]

Methods

Patients, cell lines, and DNA samples

Patients with a positive chromosomal breakage test were included in this study for molecular screening of FA genes by the National Network of the Marrow Failure Study Group of the Italian Association of Pediatric Hematology and Oncology. All the subjects or their legal guardians gave written informed consent to the investigation, according to the Declaration of Helsinki. Protocols were approved by the ethics review boards of the institutions that enrolled the patients. DNA was extracted from peripheral blood, lymphoblastoid cell lines and/or primary fibroblasts.

Complementation and Western blot analyses

Lymphoblast cell lines, peripheral blood T lymphocytes or primary fibroblasts were transduced with retroviral expressing the cDNAs for *FANCA, FANCC* or *FANCG* as previously reported.[6] Complementation was considered to occur when the viability of the transduced cells increased by more than 20% that of controls at at least three different mitomycin C concentrations. Western blot analysis of FANCD2 using a monoclonal antibody (Santa Cruz, CA, USA; diluted 1:500) was performed as previously described.[10]

Sequencing analysis and multiplex ligation-dependent probe amplification

The coding exons of the FA genes and their flanking regions were sequenced using a set of oligonucleotides (the primer sequences are available upon request) according to standard procedures.[10] Six samples were first analyzed using the Ion PGM™ system for next generation sequencing of the 16 FA genes according to the manufacturer's protocols (Life Technologies). Variants with minor allele frequency less than 1% were then confirmed by Sanger sequencing as above. No gene was hit by clear pathogenetic mutations except for the disease-causing gene.

Multiplex ligation-dependent probe amplification (MLPA) was performed using the Salsa MLPA Kit with the probe mix P031/P032 for the gene *FANCA*, using the manufacturer's protocol (MRC Holland, Amsterdam, The Netherlands). The separation and quantification of the MLPA products were performed using ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, USA). The MLPA data were analyzed using MRC-Holland Coffalyzer software.

Bioinformatic tools for splicing and missense analysis

The effect of the missense variations was evaluated using four pathogenicity prediction programs, such as PoliPhen-2 (*http://genetics.bwh.harvard.edu/pph2/*), Mutation Taster (*http://www.mutationtaster.org/*), Mutation Assessor (*http://mutationassessor.org/*), and SIFT (*http://sift.jcvi.org*). Since the different software use different prediction scales, we converted each output assigning a value from 0 to 2. Specifically, we assigned values 0, 1, and 2 to the PolyPhen-2 prediction of "benign", "possibly damaging", and "probably damaging", respectively. Values of 0 and 2 were attributed to "polymorphism" and "disease causing" output of Mutation Taster, respectively. To the Mutation Assessor predictions of "neutral", "low", and "medium" we assigned values of 0, 1, and 2, respectively. Finally, when the SIFT output was "tolerated", the value was 0 and for "not tolerated" was 2. A pathogenicity score was obtained by summing the values attributed to the output from the single software.

The *in silico* analyses of splicing mutations were carried out using Human Splicing Finder Version 2.4.1 (*http://www.umd.be/HSF/*) and Splice Site Prediction by Neural Network (NNSplice; *http://www.fruitfly.org/seq_tools/splice.html*). In some cases, reverse transcription PCR was used to confirm alternative splicing.

Results

One hundred patients were enrolled in the study. Eighty patients were of Italian origin, whereas the remaining came from different countries, mainly from the Mediterranean basin. In order to identify the candidate gene, we carried out complementation analysis in 48 lymphoblastoid cell lines established from probands. Using retroviral vectors expressing the *FANCA, FANCG* or *FANCC* genes, we found that 30 probands belonged to complementation group FA-A, 4 to FA-G, 1 to FA-B, and 1 to FA-C (*Online Supplementary Table S1*). In 12 cell lines, the complementation analysis was not successful because survival assays after exposure to mitomycin C and/or cell cycle studies showed wild-type phenotype. In 5 of these cases, fibroblast cells or peripheral blood T lymphocytes allowed us to repeat the analysis and assign 4 probands to group FA-A and 1 to FA-G. Then, mutational screening of candidate genes using multiplex ligation-dependent probe amplification analysis and Sanger sequencing confirmed the complementation data. When the candidate gene was unknown, molecular genetic testing was performed in a tiered approach starting from the screening of *FANCA*, then *FANCG* and *FANCC*. In 6 samples (FA47, FA49, FA58, FA66, FA88, and FA89), Sanger sequencing was preceded by the analysis of all the FA genes using the Ion PGM™ system (Life Technologies) for next generation sequencing approach (Nicchia *et al.*, manuscript in preparation). Finally, in two families, the *FANCD2* gene was analyzed because the expression level of the relative protein was very low compared with controls, suggesting the presence of hypomorphic mutations.[12]

Molecular genetic testing in the 100 FA probands

Table 1. Mutations identified in 100 FA families from Italian population.

Exon/Intron	FANCA cDNA (NM_000135.2)	Number of alleles	FANCA RNA and/or protein (NP_000126.2)	Mutation classification	References
i-1_i6	c.-42-?_522+?del	2	nd	large deletion (no initiation codon)	FA database
i-1_i43	c.-42-?_5481+?del	2	no protein	large deletion (entire gene)	Savino *et al.*, 2003;[10] Centra *et al.*, 1999[13]
1	c.2T>A	1	p.?	initiation codon	Novel
1	c.11C>A	1	p.Ser4*	nonsense	FA database
1	c.50dup	1	p.Arg18Profs*19	small duplication (frameshift)	Novel
1	c.66G>A	1	p.Trp22*	nonsense	FA database
2	c.123_124del	1	p.Lys42Ilefs*21	small deletion (frameshift)	Novel
i2	c.190-1G>T	1	nd	splicing (potential)	Savino *et al.*, 2003[10]
i2_i3	c.190-?_283+?del	1	p.Val64Alafs*43	large deletion (frameshift)	FA database
i3	c.283+3A>C	2	r.190_283del (e3 skipping) p.Val64Alafs*43	splicing (frameshift)	Savino *et al.*, 2003[10]
i3_i20	c.284-?_1826+?del	4	p.Gly95Glufs*31	large deletion (frameshift)	Novel
i3_i4	c.284-?_426+?del	1	p.Gly95Glufs*38	large deletion (frameshift)	Novel
4	c.352G>C	1	p.Ala118Pro	missense	Novel
5	c.457C>T	1	p.Gln153*	nonsense	Novel
5	c.457C>G	1	p.Gln153Glu	missense	Novel
i5_i30	c.523-?_2981+?del	1	p.Ser175Leufs*5	large deletion (frameshift)	Novel
i5_i31	c.523-?_3066+?del	2	p.Ser175_Gln1022del	large deletion (in frame)	Novel
6	c.548G>A	1	p.Trp183*	nonsense	Novel
6	c.549G>A	1	p.Trp183*	nonsense	Novel
i6	c.596+1G>T	1	nd	splicing (potential)	Novel
i7	c.709+1G>A	1	nd	splicing (potential)	FA database
i7	c.710-5T>C	2	r.710_792del (e8 skipping) p.Asp237Glyfs*4	splicing (frameshift)	FA database
7	c.619G>T	1	p.Gly207*	nonsense	Novel
8	c.790C>T	10	r.710_792del (e8 skipping) p.Asp237Glyfs*4	(putative nonsense) splicing (frameshift)	FA database
i8	c.793-1G>C	2	r.793_826del (e9 skipping) p.Val1265Leufs*20 r.[793-1g>c; 792_793ins793-57_793-1] (cryptic site in i8) p.Val265Lysfs*34	splicing (frameshift)	Novel
i9	c.826+3del	3	r.[709+1a>g; 709_710ins709+1_709+30] (cryptic site in intron 7) p.250_251insGlyAlaPheMetThrArgCysGlyPheLeu	splicing (in frame)	Savino *et al.*, 2003[10]
i10	c.893+1G>T	1	r.[893+1g>u; 893_894ins893+1_893+215] (cryptic site in i10) p.Phe879Valfs*19	splicing (frameshift)	Savino *et al.*, 2003[10]
i10	c.893+5G>A	1	nd	splicing (potential)	Novel
i10_i14	c.894-?_1359+?del	1	p.Phe299Profs*72	large deletion (frameshift)	FA database
i10_i33	c.894-?_3348+?del	1	p.Trp298*	large deletion (nonsense)	FA database
11	c.987-990del	2	p.His330Alafs*4	small deletion (frameshift)	FA database
i11	c.1006+1G>T	1	nd	splicing (potential)	Novel
13	c.1115_1118del	2	p.Val372Alafs*42	small deletion (frameshift)	FA database
13	c.1126C>T	1	p.Gln376*	nonsense	Novel
i14	c.1359+1G>C	4	r.1226_1359del (e14 skipping) p.Asp409Glyfs*31	splicing (frameshift)	Savino *et al.*, 2003[10]
i14_i15	c.1360-?_1470+?del	4	r.1360_1470del p.Ala454_Gln490del	large deletion (in frame)	FA database
i14_i20	c.1360-?_1826+?del	1	p.Ala454Serfs*3	large deletion (frameshift)	FA database
i14_i28	c.1360-?_2778+?del	1	p.Ala454_His926del	large deletion (in frame)	Novel

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15	c.1450G>C	1	p.Glu484Gln	missense	Novel
i15_i17	c.1471-?_1626+?del	1	p.Val491_Glu542del	large deletion (in frame)	FA database
i16	c.1567-20A>G	2	r.[1567-20a>g; 1566_1567ins1566+1_1567-1] (frameshift)	(i16 retention) p.Val523Alafs*49	Savino *et al*., 2003[10]
i17_i21	c.1627-?_1900+?del	1	p.Pro543Metfs*6	large deletion (frameshift)	Savino *et al*., 2003[10]
18	c.1645C>T	1	r.1645c>u + r.1627_1715del (e18 skipping) Gln549* + p.Pro543Hisfs*26	nonsense + splicing (frameshift)	FA database
i19	c.1776+7A>G	1	r.1716_1776del (e19 skipping) p.Ile573Serfs*12	splicing (frameshift)	Novel
i19	c.1777-7_1779del	1	nd	splicing (potential)	FA database
i20_i28	c.1827-?_2778+?del	1	p.Arg609Serfs*2	large deletion (frameshift)	FA database
i20_i29	c.1827-?_2852+?del	2	r.1827_2852del p.Ala610_Arg951del	large deletion (in frame)	FA database
21	c.1874G>C	1	p.Cys625Ser	missense	Novel
21	c.1850_1859del	1	p.Leu617Profs*20	small deletion (frameshift)	Novel
22	c.2005C>T	2	p.Gln669*	nonsense	Savino *et al*., 2003[10]
23	c.2051T>C	1	p.Leu684Pro	missense	FA database
25	c.2314C>T	2	p.Gln772*	nonsense	Savino *et al*., 2003[10]
25	c.2290 C>T	1	p.Arg764Trp	missense	FA database
i26	c.2504+2T>C	1	r.[2504+2u>c; 2504_2505ins2504+1_2504+129] (cryptic site in i26) p.Phe836*	splicing (nonsense)	Savino *et al*., 2003[10]
27	c.2535_2536del	1	p.Cys846Glnfs*20	small deletion (frameshift)	FA database
27	c.2574C>G	1	p.Ser858Arg	missense	Savino *et al*., 2003[10]
28	c.2638C>T	1	p.Arg880*	nonsense	FA database
28	c.2738A>C	7	p.His913Pro	missense	FA database
28	c.2770G>T	2	p.Asp924Tyr	missense	Novel
i28	c.2778+1G>A	1	r.2602_2778del (e28 skipping) p.Phe868_His926del	splicing (in frame)	FA database
i28	c.2778+83C>G	1	nd	splicing (potential)	Savino *et al*., 2003[10]
29	c.2840C>G	8	p.Ser947*	nonsense	Savino *et al*., 2003[10]
29	c.2852G>A	5	p.Arg951Gln	missense	FA database
29	c.2812_2830dup	2	p.Asp944Glyfs*5	small duplication (frameshift)	Savino *et al*., 2003[10]
29	c.2851C>T	1	p.Arg951Trp	missense	FA database
30	c.2853dup	2	p.Gln952Alafs*10	small duplication (frameshift)	Novel
30	c.2932C>T	1	p.Gln978*	nonsense	Novel
32	c.3109_3137delinsG	1	p.Pro1037Alafs*14	small indel (frameshift)	Novel
32	c.3164G>A	1	p.Arg1055Gln	missense	FA database
32	c.3239G>A	1	p.Arg1080Gln	missense	FA database
33	c.3263C>T	1	p.Ser1088Phe	missense	FA database
i33	c.3348+1G>A	1	r.3240_3348del (e33 skipping) p.Ile1081Glufs*10	splicing (frameshift)	FA database
34	c.3367G>T	1	p.Gly1123*	nonsense	Novel
35	c.3490C>T	4	p.Pro1164Ser	missense	FA database
36	c.3558dup	7	p.Arg1187Glufs*28	small duplication (frameshift)	Savino *et al*., 2003[10]
37	c.3638_3639del	3	p.Pro1213Argfs*64	small deletion (frameshift)	Savino *et al*., 2003[10]
37	c.3660del	2	p.Asn1221Thrfs*26	small deletion (frameshift)	Novel
37	c.3715_3729del	2	p.Glu1239_Arg1243del (GluGluAsnIleArg)	small deletion (in frame)	FA database
37	c.3692A>C	1	p.His1231Pro	missense	Novel
37	c.3761_3762dup	1	p.Glu1255Argfs*12	small duplication (frameshift)	Savino *et al*., 2003[10]
38	c.3788_3790del	10	p.Phe1263del	small deletion (in frame)	FA database
38	c.3798G>A	1	p.Met1266Ile	missense	Novel
i38	c.3829-9G>A	1	nd	splicing (potential)	Novel
40	c.3971C>T	4	p.Pro1324Leu	missense	FA database

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i40	c.4011-1G>C	1	r.4011_4033del (cryptic site recognition in e41) p.Ser1337Argfs*80	splicing (frameshift)	Novel
41	c.4029T>G	1	p.His1343Gln	missense	Novel
41	c.4075G>T	1	p.Asp1359Tyr	missense	Savino et al., 2003[10]
42	c.4198C>T	1	p.Arg1400Cys	missense	FA database
42	c.4258G>T	1	p.Glu1420*	nonsense	Novel
i42	c.4261-19_4261-12del	1	nd	splicing (potential)	FA database
i42	c.4261-2A>C	1	nd	splicing (potential)	Novel
Exon/Intron	**FANCB cDNA (NM_152633.2)**	**Number of alleles**	**FANCB protein (NP_001018123.1)**	**Mutation classification**	**References**
3	c.353T>C	1	p.Phe118Ser	missense	Novel
Exon/Intron	**FANCC cDNA (NM_000136.2)**	**Number of alleles**	**FANCC protein (NP_000127.2)**	**Mutation classification**	**References**
2	c.37C>T	1	p.Gln13*	nonsense	FA database
2	c.67del	2	p.Asp23Ilefs*23	small deletion (frameshift)	FA database
8	c.692_694del	1	p.Lys231del	small deletion (in frame)	Novel
15	c.1642C>T	2	p.Arg548*	nonsense	FA database
Exon/Intron	**FANCD2 cDNA (NM_001018115.1)**	**No. of alleles**	**FANCD2 protein (NP_001018125.1)**	**Mutation classification**	**References**
7	c.458T>C	2	p.Leu153Ser	missense	Borriello *et al.*, 2007[11]
12	c.904C>T	1	p.Arg302Trp	missense	Kalb *et al.*, 2007[12]
13	c.1092G>A	1	p.Trp364*	nonsense	
Exon/Intron	**FANCG cDNA (NM_004629.1)**	**Number of alleles**	**FANCG RNA and/or protein (NP_004620.1)**	**Mutation classification**	**References**
4	c.336del	2	p.Arg113Glyfs*39	small deletion (frameshift)	Novel
i7	c.924+1G>A	1	r.778_924del (skipping exon 7) p.Gly260_Glu308del	splicing (in frame)	Novel
i9	c.1144-1G>T	2	r.1144_1168del (cryptic site in e10) p.Phe382Glyfs*13	splicing (frameshift)	Novel
10	c.1182_1192delinsC	2	p.Glu395Trpfs*5	small indel (frameshift)	FA database
10	c.1199_1204del	2	p.Ala400_Ala401del	small deletion (in frame)	Novel
10	c.1223_1226dup	1	p.Ala410Glnfs*10	small duplication (frameshift)	Novel
10	c.1344C>A	2	p.Cys448*	nonsense	Novel
13	c.1715G>A	4	r.1715g>a + r.1637_1760del (e13 skipping) p.Trp572* + p.Asn547Leufs*6	nonsense + splicing (frameshift)	FA database
14	c.1788T>A	2	p.Tyr596*	nonsense	Novel

Nucleotide numbering reflects the FANCA, FANCB, FANCC, FANCD2, and FANCG cDNAs with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence RefSeq NM_000135.2, NM_152633.2, NM_000136.2, NM_001018115.1, and NM_004629.1, respectively. Sequence variant nomenclature has been checked at https://mutalyzer.nl/.

allowed us to identify mutations in the *FANCA* (n=85), *FANCG* (n=9), *FANCC* (n=3), *FANCD2* (n=2), and *FANCB* (n=1) genes (*Online Supplementary Table S1*). Of all the variants identified, 108 were predicted to be potential pathogenetic mutations because they were listed in the FA mutation database (n=62) at *http://www.rockefeller.edu/fanconi/* or because they were novel variants (n=45) not reported in the SNP database.

FANCA mutations

Among the FANCA patients, 27 are homozygous and 58 compound heterozygous for 91 different mutations, which account for 162 of the 170 expected mutant *FANCA* alleles. In 8 families, one parental allele could not be detected.

Large intragenic deletions: multiplex ligation-dependent probe amplification analysis or failure in amplifying specific exons allowed us to characterize 16 different intragenic deletions of *FANCA*, including 5 novel rearrangements. In addition to deletions of the entire gene,[13] we found deletions ranging from a single exon loss, such as exon 4 or 15, to removal of up to 26 exons. Although their breakpoints have not been characterized, the deletions are likely to have occurred at the numerous Alu repeats that are present in the *FANCA* intronic sequences.[14]

Nonsense, initiation codon and frameshift mutations: as pathogenetic alterations, we identified 16 nonsense and 13 frameshift mutations due to small deletions (n=7), duplications (n=5), or indel (n=1) (Table 1). PCR analysis on cDNA reveals that two predicted nonsense mutations, c.790C>T and c.1645C>T, were associated with skipping of exon 8 and 18, respectively. Whereas in the first case, the skipping was complete (*Mattioli et al., submitted manuscript*), in the second we identified two distinct products based on the presence or absence of exon 18. Thirteen of the alterations described here are novel, as is the alteration

Table 2. Pathogenicity prediction of 21 *FANCA* amino acid substitutions based on genetics and bioinformatic tools.

NM_000135.2 (SNP)	Protein (MAF as %)[a]	N. of FA alleles (this study)	N. of FA alleles (FA mutation database)	Pathogenicity on genetic basis[e] (patient ID)[f]	Pathogenicity score using bioinformatics tools
		MAF (%) among FA alleles[b]			
c.1450G>C	p.Glu484Gln	1	0	VUS	8
c.2851C>T	p.Arg951Trp	1	14	P	8
c.3490C>T	p.Pro1164Ser	4	1	P	8
c.4198C>T (rs149851163)	p.Arg1400Cys (1000G: 0.05) (ESP: 0.02)	1	4	P	8
		0.25 (P=0.04)			
c.1874G>C (rs139235751)	p.Cys625Ser (1000G: 0.14) (ESP: 0.23)	1	1[c]	NP (FA56)	7
		0.10			
c.2852G>A	p.Arg951Gln	5	8	P	7
c.3239G>A	p.Arg1080Gln	1	1	P	7
c.3971C>T (rs182657062)	p.Pro1324Leu (1000G: 0.05) (ESP: 0.00)	4	5	P	7[g]
		0.46 (P=0.003)			
c.2051T>C	p.Leu684Pro	1	3	P	6[h]
c.2290 C>T	p.Arg764Trp	1	1	P	6
c.3164G>A	p.Arg1055Gln	1	1	P	6
c.352G>C	p.Ala118Pro	1	0	VUS	5[i]
c.4075G>T	p.Asp1359Tyr	1	1	P	5
c.3692A>C	p.His1231Pro	1	0	VUS	4
c.3798G>A	p.Met1266Ile	1	0	VUS (FA79)	3
c.457C>G	p.Gln153Glu	1	0	VUS (FA65)	2
c.2770G>T	p.Asp924Tyr	2	0	VUS (FA35)	2
c.2574C>G (rs17233141)	p.Ser858Arg (1000G: 0.50) (ESP: 0.53)	1	5[d]	NP	1
		0.31			
c.2738A>C	p.His913Pro	7	4	P	1[i]
c.3263C>T (rs17233497)	p.Ser1088Phe (1000G: 3.34) (ESP: 5.92)	1	3	NP (FA45)	1
		0.20			
c.4029T>G	p.His1343Gln	1	0	VUS	0

[a]*MAF, Minor Allele Frequency based on data reported from 2,184 alleles in 1000G (1000 Genome database at http://www.1000genomes.org/) and from 12,996 alleles in ESP (Exome Sequencing Project at http://evs.gs.washington.edu/EVS/) at the time of submission of this paper.* [b]*Frequency estimated on 1960 FA alleles from 980 FA-A patients, 85 from this study and 895 reported in the mutation database (http://www.rockefeller.edu/fanconi/) at the time of submission of this paper. Only FA* vs. *controls p-values (data from both 1000G and ESP) ≤ 0.05 are reported.* [c]*Reported erroneously as c.1873G>C (p.Cys625Ser) by Castella* et al., *2011.*[15] [d]*Wijker* et al., *1990 (1 allele); Tamary* et al., *2000 (4 alleles).*[16,17] [e]*P: pathogenetic; NP: non-pathogenetic; VUS: variants of uncertain biological significance.* [f]*Patients carrying other two pathogenetic mutations (Table 1S).* [g]*Functional studies in Adachi* et al., *2002; Ameziane* et al., *2008.*[7,18] [h]De novo *mutation identified in patient FA48 (Table 1S).* [i]*Mutation lost in revertant lymphoblastoid cell lines (Table 3).*

of the first initiation codon (c.2T>A).

Splicing mutations: all the intronic variants (n=22) were considered as putative splicing mutations. Twelve of 22 were characterized functionally from the patient's cells at the RNA level. In addition to 4 mutations (c.283+3A>C; c.893+1G>T; c.1567-20A>G, and c.2504+2T>C) that we have previously described,[10] 5 additional alterations (c.710-5T>C, c.1359+1G>C, c.1776+7A>G, c.2778+1G>A, and c.3348+1G>A) were associated with the skipping of exons 8 (*Mattioli C et al. submitted manuscript*), 14, 19, 28, and 33, respectively (Table 1). In the presence of c.793-1G>C, PCR on cDNA revealed two products, a faint band lacking exon 9 and another product containing the last 57 nucleotide of intron 8 due to the usage of an intronic cryptic splice site. Similarly, cryptic splice sites in intron 7 and exon 41 were activated when the natural sites were destroyed by c.709+1G>A or c.4011-1G>C, respectively.

Small inframe and missense mutations: we identified 23 variants of uncertain pathogenetic significance: two in-frame deletions and 21 putative missense mutations (Tables 1 and 2). The two in-frame deletions

Table 3. Revertant *FANCA* mutations in 10 different lymphoblastoid cell lines.

Patient ID	Mutations detected in peripheral blood and/or fibroblast cell lines	Mutations detected in lymphoblast cell lines	Type of events reversing the cellular phenotype
FA2	c.2738 A>C[a] (p.His913Pro)	c.2738 A>C[a] + c.2737 C>G (p.His913Ala)	*De novo* potential compensatory mutation
FA50	c.352G>C (p.Ala118Pro) c.3715_3729del	wt c.3715_3729del	
FA51	c.790C>T c.2840C>G	wt c.2840C>G	
FA57	c.790C>T c.3367G>T	wt c.3367G>T	
FA58	c.3388_3790del c.826+3del	wt c.826+3del	Back mutations
FA59	c.66G>A c.1006+1G>T	wt c.1006+1G>T	
FA68	c.190-1G>T c.790C>T	wt c.790C>T	
FA70	c.596+1G>T c.1450G>C	wt c.1450G>C	
FA73	c.1777-7_1779del c.2851C>T	wt c.2851C>T	
FA80	c.3558dup[a]	c.3558dup[a] + c.3576_3580dup (restoration of open reading frame)	*De novo* compensatory mutation[b]

[a]*Homozygous status;* [b]*Waisfisz* et al., *1998.*

(p.Glu1239_Arg1243del and p.Phe1263del) and 14 missense variants were enlisted in the FA database (*http://www.rockefeller.edu/fanconi/*). However, 5 of these 14 amino acid substitutions were also present in SNP databases (Table 2), thus questioning their pathogenicity. We regarded c.4198C>T (rs149851163) and c.3971C>T (rs182657062) as pathogenetic ("P") variants because their frequency was significantly greater within the FA population than in control populations as in 1000 Genomes (*http://www.1000genomes.org*) or Exome Sequencing Project (*http://evs.gs.washington.edu/EVS/*). On the contrary, we assigned SNPs rs139235751 (c.1874G>C), rs17233141 (c.2574C>G), and rs17233497 (c.3263C>T) to the non-pathogenetic variant group ("NP"), as they were relatively common even in controls (Table 2). The remaining 9 out of 14 missense variants present in the FA mutation database but not in the SNP databases were regarded as "P". The remaining 7 out of the 21 amino acid substitutions were novel variants of uncertain biological significance (VUS).

Pathogenicity of these mutations was also evaluated using 4 different bioinformatic tools and summing their outputs in a unique value ranging from 0 to 8 (see Methods). Although the predictive programs have to be taken cautiously, 10 of 11 variants previously classified as "P" received a score ≥4. In this range, there are three VUS and a "NP" variant (rs139235751) that is located downstream of a clear pathogenetic mutation in FA56. With the only exception of c.2738A>C (p.His913Pro), the variants previously considered as "NP" or VUS instead received a value of less than 4. Of note, 4 of these variants were identified in patients carrying other certain pathogenetic alterations of *FANCA* (Table 2).

Molecular characterization of FANCA mosaicisms supporting the pathogenetic role of p.Ala118Pro and p.His913Pro: the lymphoblast cell lines of 12 FA patients with characteristic clinical features and confirmed diagnosis of FA showed no sensitivity to diepoxybutane. Therefore, we hypothesized they had undergone mutation reversion(s) leading to normal cellular phenotypes. Using DNA extracted from primary skin fibroblasts or peripheral blood cells, we identified germ-line mutations (*Online Supplementary Table S1*) and then, analyzing the diepoxybutane resistant cell lines, we revealed the nature of the phenotypic reversions in 10 cases (Table 3). Remarkably, in the cell lines obtained from patients FA51 and FA57, the heterozygous c.790C>T germ-line substitution was not detectable. Consistent with these data, *FANCA* exon 8, which is spliced out in the transcript from the allele with c.790C>T (*Mattioli C et al., submitted manuscript*), was correctly included in the mRNA from the cell lines (*data not shown*). The fact that the reversion of this point mutation occurred independently in two different cell lines suggests that this location/mutation makes a reversion more likely to happen. Another 4 single base mutations, c.352G>C, c.66G>A, c190-1G>T, and c.596+1G>T, reverted back to the corresponding normal alleles in patients FA50, FA59, FA68, and FA70, respectively. In 2 patients, small genomic deletions (c.3388_3790del and 1777-7_1779del) were mutated back to a wild-type allele, events that are likely to have occurred through gene conversion. Moreover, the analysis in the revertant cell lines allowed us to support the pathogenetic nature of 2 missense mutations, c.352G>C (p.Ala118Pro) and c.2738A>C (p.His913Pro). For the first alteration, phenotypic normalization of the cell line was indeed associated with the disappearance of the missense variant. Although the second c.2738A>C (p.His913Pro) missense variant accounts for 11 *FANCA* alleles (Table 2), the analysis in patient FA2 further supported its pathogenic role. The patient was homozygous for this *FANCA* alteration but in her reverted cell line we also identified a heterozygous de novo c.2737C>G mutation, leading to a new amino acid substitution at the same codon (p.His913Ala). Although both p.His913Ala and p.His913Pro are predicted to be well tolerated, at least on the basis of bioinformatics analyses, the association of a normal cellular phenotype

with the disappearance of the original mutation could be considered a strong argument for classifying this missense alteration as pathogenic.

In 2 (FA61 and FA99) of the 12 diepoxybutane resistant cell lines, we were not able to establish the nature of the phenotypic reversion. In FA61, the second mutant *FANCA* allele was not detected using DNA from the patient's peripheral blood. In FA99, both the germline FANCG mutations identified in primary fibroblast cells were also revealed in the lymphoblastoid cell line. Although we could have missed a *de novo* compensatory mutation, we cannot exclude the possibility that a mitotic crossover event could have reconstituted a wild-type FANCG allele.

Founder effect of FANCA mutations: most of the *FANCA* mutations were only detected once (n=61) or twice (n=17) in the 162 characterized FANCA alleles, confirming again the wide spectrum of private mutations in *FANCA* (*http://www.rockefeller.edu/fanconi/*). Nevertheless, 5 *FANCA* mutations were relatively frequent, accounting together for 26% (42 of 162) of the *FANCA* alleles identified in our Italian series (Table 1). Considering the geographical origins of the families, we speculated that the c.790C>T (n=10) and c.2840C>G (n=8) mutations originated in the Campania region of Italy. A third potential founder effect, c.2738A>C (n=7), occurred in Sicily. Consistent with their frequency in the FA mutation database (*http://www.rockefeller.edu/fanconi/*), the other 2 more common alterations, c.3558dup (n=7) and c.3788_3790del (n=10), are widely found around the world.

FANCG mutations

Patients from nine families were either homozygous (n=8) or compound heterozygous (n=1) for 9 alterations of the *FANCG* gene, with 7 of 9 being novel mutations (Table 1). Three alterations were frameshift mutations due to small deletions, insertions or duplications. Another 3 were nonsense mutations, one of which c.1715G>A (p.Trp572*) was also associated with an aberrant PCR product resulting in an alternative splicing with skipping of exon 13. Two nucleotide substitutions (c.924+1G>A and c.1144-1G>T) affected the "G" of the donor and the acceptor splice site of introns 7 and 9.

The first was associated with skipping of exon 7 whereas the other with recognition of a cryptic acceptor splice site in exon 10. Finally, one mutation was an in wframe deletion of 6 nucleotides at a highly conserved region, as seen at *http://www.ncbi.nlm.nih.gov/homologene/3402*.

Mutations in other Fanconi anemia genes

In addition to patients belonging to groups FA-A and FA-G, we identified mutations in other genes, but only in a few cases. In addition to families with mutations of *FANCD2*, which were associated with a low expression level of the protein,[11,12] we found sequence alterations of the *FANCB* and *FANCC* genes. A new missense mutation (p.Phe118Ser) of *FANCB* was detected in an FA male belonging to complementation group FA-B, and its deleterious effect is suggested by bioinformatics tools.

Three patients had mutations in the *FANCC* gene. Two were homozygous for nonsense or frameshift known mutations whereas the third was a compound heterozygote for the p.Gln13* mutation and a novel (p.Lys231del) amino acid deletion. The latter is reported as SNP (rs3831244), without any frequency value.

Although Lys231 is a well-conserved residue (*http://www.ncbi.nlm.nih.gov/homologene/?term=FANCC*), the pathogenetic effect of its deletion remains uncertain.

Discussion

In FA, as well as in many other genetic diseases characterized by genetic heterogeneity, the molecular diagnosis is a complex and not always successful issue. First of all, there are at least 16 different FA genes responsible for the disease. Complementation analysis would help in identifying the disease-causing gene before molecular analysis, as was partially done in this study. Alternatively, one could focus on *FANCA*, which alone accounts for 63% of the FA mutations listed in the FA mutations database (*http://www.rockefeller.edu/fanconi/*). Indeed, most (85%) of our patients have mutations on *FANCA*. As in Spain,[15] this percentage is higher than reported in the FA database, suggesting that only very rare mutational events have hit the non-*FANCA* gene in populations of the Mediterranean basin. Consistent with data from literature, the mutations we found are mainly private, including 45 novel variants, though there is evidence for founder effects in the regions of Campania and Sicily. The screening of *FANCA* is, however, complicated not only by the large size of the gene and by the absence of mutational hot spots, but also by the presence of large intragenic, often heterozygous, deletions. For these reasons, *FANCA* alleles will escape the analysis, as occurred in 6% of the alleles. If *FANCA* is not altered, the other FA genes are analyzed, starting from those more frequently mutated in a specific population. In our cohort, 9% of families have mutations in the *FANCG* gene. Few mutations were identified in the *FANCC, FANCD2,* and *FANCB* genes, with the support of complementation or protein analysis, or next generation sequencing approaches.

The presence of hematopoietic revertant cells in FA is another aspect that can make the diagnostic process in FA even more complex. Almost 25% of lymphoblastoid cell lines revert their phenotype becoming cross-linking agent resistant.[19] In our case series, the frequency of mosaicisms in the EBV-transformed cell lines is lower than that reported. One reason might be that our cell lines are not maintained in culture for long, thus preventing the selection of revertant clones. Regarding the mechanism leading to the reconstitution of allele encoding for functional proteins, intragenic crossover, gene conversion, back mutation and compensating *in cis* mutations have all been observed in revertant cell lines and sometimes in patients' peripheral blood cells,[8,9,19] suggesting a possible improvement in bone marrow function.

A further limiting aspect of the molecular diagnostic process in FA is the detection of a relatively high number of variants of unknown significance leading to amino acid substitutions (22% of the *FANCA* variants identified in this study). Whereas the deleterious effects of large deletions, nonsense or frameshift mutations are usually clear, the pathogenetic role of missense alterations is not so obvious. Variants present in mutation databases, but not included in SNP databases, are usually considered pathogenetic. However, as long as projects, such as 1000 Genomes (*http://www.1000genomes.org/*) or Exome Sequencing Project (*http://evs.gs.washington.edu/EVS/*), are ongoing, even the rarest pathogenetic variants will be detected and listed in the SNP databases. This could be the

case of p.Arg1400Cys or p.Pro1324Leu, which are identified in the FA cohorts but also in a few healthy individuals in heterozygous state who could be potential asymptomatic carriers.

Bioinformatic tools have been developed to help predict the effect of missense variants on protein function using straightforward physical and comparative consideration. Even though the software is very quick and easy to use, it can give rise both to false negatives and false positives.[20]

However, combining multiple prediction tools, we can obtain important information on potential pathogenicity of the missense mutations, whose functional studies are not usually suitable in many molecular genetic laboratories. Therefore, using both genetic and bioinformatic data, we tried to classify the missense mutations on the basis of their predicted pathogenicity. Interestingly, for most of the variants there was concordance between the two approaches.

However, the pathogenicity of missense variants should be proven by experimental evidence. Functional studies are even more important in clinical conditions where the identification of missense alleles cannot guarantee the correct assignment of a complementation group, thus preventing families carrying these variants from molecular prenatal diagnosis. Therefore, complementation analysis was another suitable tool that allowed us to confirm that *FANCA* was the gene affected in 5 patients (FA2, FA4, FA23, FA25, and FA44) who were homozygotes or compound heterozygotes for missense variants. In contrast, the assignment to group FA-A of patient FA10 was not certain despite the fact that the missense variations identified were classified as pathogenetic by both genetic and bioinformatic tools.

Of the missense mutations identified in this study, only 2 (p.Pro1324Leu and p.Asp1359Tyr) had been previously investigated in overexpression conditions, showing that they are mild mutants, reconstituting the FA pathway partially.[7,18] Taking advantage of molecular data from revertant cell lines, we were also able to support the deleterious effect of p.Ala118Pro and p.His913Pro. Moreover, p.Leu684Pro was identified as a *de novo* mutation in FA48 (*Online Supplementary Table S1*), strongly suggesting its pathogenetic role.

Complementation analyses and functional studies are time-consuming and complex procedures not usually available in molecular genetic testing laboratories. In the era of next generation sequencing strategies, the molecular diagnosis of genetic diseases such as FA, whose genes explain the disease in almost all the cases, would greatly benefit from these innovative technologies. For this reason, mutations will be identified in the majority of cases without any pre-screening, thus limiting complementation and functional studies only when the nature of the variants does not guarantee an accurate molecular diagnosis. Massive parallel sequencing approaches have already been successfully used.[21,22] In our experience, we are validating the Ion PGM™ system (Life Technologies) and have identified mutations in 6 cases, including 2 belonging to group FA-C. Since preliminary data are very encouraging, we are confident to be able to apply this procedure not only in the routine of molecular screening in FA but also for re-sequencing those cases (3 in our experience) who, having been analyzed only partially, are still without a molecular diagnosis.

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