Detection of somatic mosaicism and classification of Fanconi anemia patients by analysis of the FA/BRCA pathway

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Fanconi anemia (FA) is characterized by congenital abnormalities, bone marrow failure, chromosome fragility, and cancer susceptibility. Eight FA-associated genes have been identified so far, the products of which function in the FA/BRCA pathway. A key event in the pathway is the monoubiquitination of the FANCD2 protein, which depends on a multiprotein FA core complex. In a number of patients, spontaneous genetic reversion can correct FA mutations, leading to somatic mosaicism. We analyzed the FA/BRCA pathway in 53 FA patients by FANCD2 immunoblots and chromosome breakage tests. Strikingly, FANCD2 monoubiquitination was detected in peripheral blood lymphocytes (PBLs) in 8 (15%) patients. FA reversion was further shown in these patients by comparison of primary fibroblasts and PBLs. Reversion was associated with higher blood counts and clinical stability or improvement. Once constitutional FANCD2 patterns were determined, patients could be classified based on the level of FA/BRCA pathway disruption, as “FA core” (upstream inactivation; n = 47, 89%), FA-D2 (n = 4, 8%), and an unidentified downstream group (n = 2, 4%). FA-D2 and unidentified group patients were therefore relatively common, and they had more severe congenital phenotypes. These results show that specific analysis of the FA/BRCA pathway, combined with clinical and chromosome breakage data, allows a comprehensive characterization of FA patients. (Blood. 2005;105:1329-1336)
evaluated the FA/BRCA pathway by analysis of FANCD2 immuno-
blots in a prospective cohort of FA patients. We show that this
analysis, combined with clinical data and chromosome breakage
tests, allows a fine characterization of FA patients by detection of
somatic mosaicism and classification of the disease according to
the level of the FA/BRCA pathway disruption. Clinical correlations
were addressed, considering both the revertant status and the level of
FA/BRCA pathway disruption.

Patients, materials, and methods

Patients and cell lines

From February 2002 to February 2004, 53 FA patients were included in the
study (allografted patients were excluded). All patients were seen at
Saint-Louis Hospital (Paris, France) except 4 patients who were seen
elsewhere in France by expert collaborators and whose samples were sent to
our laboratory together with clinical data. Informed consents were obtained
from the patients and/or their relatives. The study was approved by the
Unique patient numbers were used to refer to FA patients and samples once
the FA diagnosis was established: EGFA (European Group for Fanconi
Anemia) followed by identification number and “-L” for lymphocytes, “-F”
for fibroblasts, and “-E” for Epstein-Barr virus (EBV)–immortalized cell
lines. The diagnosis of FA was based on clinical data, including personal
and familial history, and physical examination, together with chromosome
breakage tests. Main characteristics of the patients are given in Table 1. For
all patients, the following information was available: sex, birthdate, date of
diagnosis, date of entry in the study, description of the clinical phenotype,
and blood cell counts. Short stature was defined by a height below 2
standard deviations (SDs, which corresponds to the 3rd percentile) at
inclusion in the study, or before the use of androgens. Facial appearance
was defined as characteristic or not after examination. Features taken into
account were microcephaly, broad nasal base with micrognathia and
triangular face, and eye abnormalities (microphtalmia and epicanthal folds).
Skin abnormalities were defined by any combination of café-au-lait spots,
hypopigmented spots or areas, and hyperpigmented areas. Renal and
cardiac malformations were evaluated by echography, and limb abnormali-
ties were searched for by examination and X-rays. The extent of the
cardiac malformations were evaluated by echography, and limb abnormali-
ties were searched for by examination and X-rays. The extent of the
malformation syndrome was classified as limited (<3 sites) or extensive (3
or more sites) according to the classification that we used in a previous
study.32 Overall, there were 29 males and 24 females. Median age at
diagnosis, and median age at entry in this study, were 7 years (range, birth to
36 years), and 10 years (range, 2 to 36 years), respectively. At last
examination, 18 patients had short stature (range, −2 SD to −5 SD); among
the others, no patient had an height greater than normal median value,
except for 2 patients treated with androgens. Facial appearance was
characteristic in 43 patients, 42 had skin abnormalities, and limb abnormali-
ties were detected in 28 patients. The malformation syndrome was
extensive in 16 patients and limited in 37.

Control cell lines were the FANCd2-deficient PD20 and stably
corrected PD20, and the NBS1-deficient EUFa1020-L and BRCA2-
deficient EUFA2030-L (kindly provided by H. Joenje, Vrije Universiteit
Medical Center, Amsterdam, Netherlands).

Sample preparations and immunobLOTS

Mononuclear peripheral blood cells were purified from fresh blood samples
using Ficoll-Paque Plus (Amersham Biosciences, Saskay, France). 10 × 10
6 cells were cultured at a concentration of 105/mL in RPMI-FBS (RPMI
1640, 10% vol/vol fetal bovine serum [FBS]), and T-cells were stimulated
with phytohemagglutin (PHA) for 72 hours without exposure to a DNA-
damaging agent. For primary fibroblast culture, 4-mm-square skin biopsies
were performed using local anesthesia with Emla (Astra, Rueil, France) and
the Biopsy Punch (Stiefel, Rueil, France). Skin samples were thinly sliced,
and the resulting fragments were adhered on plastic plates for 15 minutes
then covered with modified Eagle medium (MEM) supplemented with
antibiotic, HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid),
MEM nonessential amino acids (Invitrogen, Cergy Pontoise, France), and
20% fetal calf serum (FCS) (GIBCO-BRL Life Technologies, Cergy
Pontoise, France), at 37°C with 5% CO2. Individual growing clones were
picked up after 2 to 3 weeks and expanded. Cells were grown and tested
without exposure to DNA damaging agent. Thirty micrograms of proteins
from PHA-stimulated peripheral blood lymphocytes (PBLs), or 50 μg from
growing nonconfluent fibroblasts, were loaded in a 80 mm × 80 mm
preloaded 3.8% Nu-PAGE Tris-Acetate gel (Invitrogen). Proteins
were migrated; for 85 minutes using a Xcell SureLock Mini-Cell tank
(Invitrogen) then transferred onto nitrocellulose membrane using the Xcell
II Blot module (Invitrogen). After blocking for 2 hours or overnight in 0.5%
milk, a rabbit polyclonal anti-FANCd2 serum (A.D.D.’s laboratory) was
used at 1:1000 for 1 hour. Detection was performed using horseradish
peroxide (HRP)–linked secondary antibodies with the ECL PLUS kit
(Amersham Biosciences). BRCA2 and NBS1 immunoblots were performed
on PHA-stimulated lymphocytes and fibroblasts from patients EGFA008
and EGFA053, and from controls using BRCA2 Ab-2 antibodies (Onco-
genec Research Products, Darmstadt, Germany) and NBS1 antibodies
(ABCam, Cambridge, United Kingdom).

Chromosome breakage tests

Chromosome breakage tests were done as previously described,32 indepen-
dently from the FANCd2 tests. Fresh PBLs were stimulated by PHA for 24
hours and further incubated with and without DNA damaging agent for 48
hours. We used the nitrogen mustard mechlorethamine (Caryolysine;
Synthelabo, Le Plessis Robinson, France) freshly diluted at a final
concentration of 0.05 μg/mL.32 Examination of 100 mitoses (50 with and
50 without DNA damaging agents) allowed the scoring of chromatid breaks
and chromosome breaks as one break, and chromosome rearrangements and
radial as 2 breaks; chromatid gaps were not scored. Results were compared
with healthy controls and positive controls run in parallel. In our laboratory,
cytogenetic diagnosis of FA is based on the combined analysis of several
criteria: (1) the mean number of breaks per metaphase in nitrogen mustard
(NM)–exposed cells (a mean number greater than 2 suggests FA); (2) the
ratio of this mean between NM-exposed cells and nonexposed cells (a ratio
greater than 10:1 is required); (3) the number of NM-dependent breaks per
aberrant mitosis (5 or more breaks in several cells strongly suggests FA);
and (4) the percentage of mitoses with break(s) in NM-exposed cells (a
minimum of 20% of aberrant mitoses is required). If all criteria are
fulfilled, FA is diagnosed. Cases with significantly more breaks than in
normal controls, but less than the Fanconi range, are considered
“ambiguous”; however, this does not necessary rule out the diagnosis of
FA, and additional confirmatory testing may be required. Somatic
mosaicism is suspected when more than 20% of the metaphases in
NM-exposed cells show no break. In primary fibroblasts, chromosome
breakage tests were performed by culturing growing cells with dilutions
of mechlorethamine using 2 exposure times (48 hours and 4 days).
Positivity was determined by comparison with FA and non-FA control
fibroblasts run in parallel (thresholds were lower than those used in
PBLs, and the complete experiments in fibroblasts were run 3 times).28,36

Determination of complementation groups

In a subset of patients (FA core patients with reversion), subtyping was
performed in the fibroblasts according to the FANCd2-based rapid FA
subtyping assay.33 Briefly, stably expressing cell lines were established
using the pMMP retroviral vectors containing the FANCA, FANCC, and
FANCg cDNA, and empty vector.17 Growing fibroblasts from patients and
control fibroblasts of known groups were transduced with each of the
supernatant in parallel. After 48 hours, cell lysates were analyzed by
FANCd2 immunoblot. In 12 nonrevertant FA core patients, subtyping was
determined in fibroblasts, blood, or EBV cell lines (FA-A, n = 11; FA-G, n = 1).

Statistical analysis

All analyses were performed with PRISM (GraphPad, San Diego, CA) and
were 2 tailed. Categorical variables were compared using the chi-square
### Table 1. Clinical and biological data in 53 FA patients

<table>
<thead>
<tr>
<th>Patients</th>
<th>Sex</th>
<th>Extent of malformation</th>
<th>Age at diagnosis, y</th>
<th>Age at study, y</th>
<th>Blood cell counts</th>
<th>Bone marrow karyotype</th>
<th>PBL breaks</th>
<th>PBL FANCD2</th>
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<tr>
<td>FA-D2</td>
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<td>EGFA050</td>
<td>M</td>
<td>E</td>
<td>0.3</td>
<td>4</td>
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<td>2.1</td>
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### Unidentified downstream group

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<th>Age at study, y</th>
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### FA core

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<th>Age at diagnosis, y</th>
<th>Age at study, y</th>
<th>Blood cell counts</th>
<th>Bone marrow karyotype</th>
<th>PBL breaks</th>
<th>PBL FANCD2</th>
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<td>L</td>
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<td>6</td>
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<td>1.2</td>
<td>10</td>
<td>10</td>
<td>N</td>
<td>+ 1</td>
</tr>
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</table>

Revertant patients are indicated in italics. Blood cell counts have been determined before any transfusion. WBC indicates white blood cells; ANC, absolute neutrophil count; Hb, hemoglobin; Plt, platelets; E, extensive malformation syndrome as defined in “Patients, materials, and methods”; L, limited; NA, information not available; N, no detected abnormality; ABN, clonal chromosomal abnormalities; *, positive; †, negative; AMB, ambiguous at the time of the study but formerly found positive at diagnosis; 2, detection of both the short and the long monoubiquitinated FANCD2 isoforms; 1, one single short isoform; and 0/2f, no FANCD2 bands or 2 faint bands in long exposure (Fig 3A).
Results

Comparison of PBLs and fibroblasts using FA/BRCA tests detects somatic mosaicism

Clinical data, chromosome breakage tests, and FANCD2 immunoblots were analyzed in all the 53 FA patients, and results are summarized in Table 1. Most patients demonstrated abnormal FANCD2 patterns in PBLs, confirming the specific FA/BRCA pathway defect. Specifically, a single FANCD2 small isoform (FANCD2-S) but no large isoform (FANCD2-L) was detected in 42 patients (Figure 1A), whereas no FANCD2 bands were detected in 3 patients (D2 group; see classification of FA patients in the next subsection); in total, n = 45 patients out of 53. Consistently, positive chromosome breakage tests were found in these cases, except 1 patient who presented with acute myeloblastic leukemia (EGFA015; no more analysis could be done in this patient). Strikingly, 8 additional patients had normal FANCD2 patterns in PBLs (ie, the 2 FANCD2 isoforms), demonstrating an ability of PHA-stimulated lymphocytes to monoubiquitinate the FANCD2 protein. Four of these patients (EGFA012, -013, -039 and -050) exhibited increased chromosomal breakage in a subset of mitoses (20% to 56%), suggesting somatic mosaicism (Table 2). In the 4 remaining patients (EGFA006, -008, -047, and -053), chromosome breakage tests were negative or ambiguous (Table 2). Primary fibroblasts were grown from skin biopsies in all the 8 patients in order to obtain constitutional cells. Abnormal FANCD2 patterns were found in 6 out of the 8 fibroblast samples, demonstrating functional FA reversion in the PBLs (Figure 1B; Table 2). In the 2 remaining questionable patients (EGFA006 and -053), primary fibroblasts showed normal FANCD2 patterns but positive chromosome breakage tests, suggesting somatic mosaicism (reversion in unidentified group patients; see Table 2). Clinical records showed that these 2 patients had positive breakage tests in PBLs at diagnosis, 8 and 10 years before the present analysis, respectively, which is consistent with emergence of somatic mosaicism since this time. Altogether, the combined data from PBLs and fibroblasts using FANCD2 and chromosomal breakage tests allowed us to detect reversion in the PBLs of 8 (15%) FA patients out of 53. Dilution experiments of normal PBLs within FA PBLs showed that the threshold for detection of reversion by FANCD2 Western blot was approximately 15% of revertant cells by sample (data not shown).

Analysis of blood cell counts demonstrated that patients with FA reversion had significantly higher blood cell counts compared to nonrevertants (Table 3). Moreover, with a median follow up of 5 years (range, 1 to 27 years) of the 8 revertant patients in our institution, we did not observe any evolution to bone marrow aplasia or leukemia (Table 2; Figure 2). Two revertant patients, aged 21 and 34 years old, developed mouth dysplasias which are currently being treated and followed for detection of progression to squamous cell carcinoma. In nonrevertant FA patients, 31 out of 45 had aplasia (absolute neutrophil count [ANC] < 1 × 10^9/L, hemoglobin [Hb] < 80 g/L [< 8 g/dL], and/or Platelet [Plt] < 50 × 10^9/L, P < 10^-5; Table 1), 1 patient had acute myeloblastic leukemia, and 1 had a severe myelodysplasia. This small number of leukemia and severe myelodysplasia cases during the time of the study precluded statistical analysis. No mouth dysplasia or other cancers were detected in the nonrevertant FA patients at the time of the study.

FANCD2 immunoblot analysis identifies the level of disruption of the FA/BRCA pathway and allows classification of FA patients

Considering PBL data in nonrevertant patients and fibroblast data in revertant patients, FANCD2 immunoblot patterns allowed determination of the level at which the FA/BRCA pathway is disrupted. In 47 (89%) out of 53 patients, including 5 FA revertant patients, one single, short, nonubiquitinated FANCD2 isoform was detected (Figure 1). These patients were therefore classified as “FA core,” corresponding to the upstream inactivation of the pathway. The 5 revertant FA core patients were shown to be complementation group A by retroviral transduction of their fibroblasts (Table 2). In 4 patients (EGFA014, -021, -050, -066), including 1 revertant (EGFA050), no FANCD2 bands were observed in several independent samples (Table 1; Figure 3A). When Western blots were overexposed, 2 barely detectable bands could be seen indicating faint residual levels of the protein, similar to the FA-D2 reference cell line PD20.6,10 These patients were therefore classified as “FA-D2.” In 2 additional patients (EGFA008, EGFA053), no FANCD2 abnormalities were detected by immunoblot in either the PBLs or fibroblasts (Figure 3B). Two distinct primary fibroblast clones from patient EGFA006, and 4 from patient EGFA053, were tested with similar results (data not shown). Together with their strong FA clinical features, including typical FA face and thumb abnormalities in both patients, short stature in patient EGFA053, positive chromosomal breakage testing in fibroblasts, and a history of a positive breakage test in PBLs at diagnosis, these 2 patients were provisionally classified as FA of unidentified group (Table 2). These findings are consistent with an inactivation of the FA/BRCA pathway downstream of FANCD2. Considering reports on D1/BRCA2 and NBS1 mutated phenotypes,7,11 we analyzed the PBLs and primary fibroblasts of these 2 patients by BRCA2 and NBS1 immunoblots but detected no alteration in level of protein expression (Figure 3B).

Clinical features of the FA-D2 (EGFA014, -021, -050, and -067) and unidentified group patients (EGFA006 and -053) were analyzed. All the 6 patients had extensive congenital abnormalities, compared to 10 out of 46 FA core patients (P < 10^-5; Table 1). Furthermore, these patients were diagnosed early, at ages of 3 months, 4 months, 2.8 years, 3 years, and 6 years.
When FANCD2 in fibroblasts is monoubiquitinated to normal FANCD2 monoubiquitination in PBLs indicates FA reversion. When fibroblasts demonstrate an FA pattern (FA core or FA-D2), FA-D2 group. Patients with FANCD2 monoubiquitination in PBLs patients. Absence of detection of the FANCD2 protein suggests nated FANCD2 isoform and are considered nonrevertant FA core immunoblot. Most patients demonstrate one single nonubiquiti-

pathway in order to detect functional FA reversion in PBLs and to shown in Figure 4. This is based on the analysis of the FA/BRCA tests. From this study, we propose an evaluation strategy for FA, as examination, FANCD2 immunoblot, and chromosome breakage

Discussion

We prospectively analyzed a series of FA patients by clinical examination, FANCD2 immunoblot, and chromosome breakage tests. From this study, we propose an evaluation strategy for FA, as shown in Figure 4. This is based on the analysis of the FA/BRCA pathway in order to detect functional FA reversion in PBLs and to classify patients. First, FA patients’ PBLs are tested by FANCD2 immunoblot. Most patients demonstrate one single nonubiquiti-

nated FANCD2 isoform and are considered nonrevertant FA core patients. Absence of detection of the FANCD2 protein suggests FA-D2 group. Patients with FANCD2 monoubiquitination in PBLs are further investigated by FANCD2 immunoblot in fibroblasts. When fibroblasts demonstrate an FA pattern (FA core or FA-D2), FANCD2 monoubiquitination in PBLs indicates FA reversion. When FANCD2 in fibroblasts is monoubiquitinated to normal levels, chromosomal breakage tests in fibroblasts can be performed if necessary to confirm FA, and BRCA2 or NBS1 abnormalities should be investigated by immunoblotting and/or molecular analy-

sis. Remaining patients with hypersensitivity to DNA crosslinks but in an unidentified complementation group are provisionally classified as “unidentified downstream group” patients. This evaluation strategy is rapid and cost-effective, and in most FA cases it allows confirmation of FA diagnosis, detection of potential FA reversion in PBLs, and classification of FA patients. It further facilitates determination of the complementation group and

| Table 2. Fanconi anemia patients with reversion (somatic mosaicism) |
|-----------------|----------------|----------------|----------------|----------------|
| Patient no.     | Age, y         | Group | PBL breaks | FANCD2 test | Breaks, fibro | Follow-up, y |
|                 |                |       | 0          | 1           | ≥ 2           |              |
|                 |                |       | NM− NM+ NM− NM+ NM− NM+ | PBL Fibro | Breaks, fibro |              |
| EGFA012         | 6              | A     | 46 27 3 2 1 21* | POS | 2 1 ND | 4 |
| EGFA013         | 21             | A     | 47 40 2 0 1 10* | POS | 2 1 ND | 13 |
| EGFA039         | 10             | A     | 44 22 5 6 1 22* | POS | 2 1 ND | 1 |
| EGFA050         | 4              | D2    | 49 37 1 4 0 9* | POS | 2 0 ND | 4 |
| EGFA008         | 23             | A     | 50 46 0 4 0 0 | NEG | 2 1 ND | 10 |
| EGFA006         | 8              | Unid  | 49 48 1 2 0 0 | NEG | 2 2† POS | 3 |
| EGFA047         | 34             | A     | 49 48 1 1 0 1 | NEG | 2 1 POS | 27 |
| EGFA053         | 10             | Unid  | 48 44 2 3 0 3* | AMB | 2 2† POS | 6 |

Patients are ordered in this table as presented in the text. Results shown in this table were obtained during the course of the present study (ps). Unid indicates unidentified downstream group(s). Chromosome breakage tests performed with (NM+) and without (NM−) exposure to nitrogen mustard; nos. of mitosis with 0, 1, and ≥ 2 breaks are indicated; conclusions of the chromosomal breakage tests are indicated as POS, positive, NEG, negative, AMB, ambiguous result. FANCD2 tests: 2, both the short and long FANCD2 isoforms were detected using FANCD2 immunoblot; 1, only the FANCD2-S isoform was detected; 0, no FANCD2 protein was detected; †, 2 and 4 independant primary fibroblasts clones were tested in patients EGFA006 and EGFA053, respectively. Breaks fibro : breakage tests in primary fibroblasts; nd, not tested. The association of a FANCD2 tests: 2, both the short and long downstream group(s). Chromosome breakage tests performed with (NM+) and without (NM−) exposure to nitrogen mustard; nos. of mitosis with 0, 1, and ≥ 2 breaks are indicated; conclusions of the chromosomal breakage tests are indicated as POS, positive, NEG, negative, AMB, ambiguous result. FANCD2 tests: 2, both the short and long FANCD2 isoforms were detected using FANCD2 immunoblot; 1, only the FANCD2-S isoform was detected; 0, no FANCD2 protein was detected; †, 2 and 4 independant primary fibroblasts clones were tested in patients EGFA006 and EGFA053, respectively. Breaks fibro : breakage tests in primary fibroblasts; nd, not tested. The association of a

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nated FANCD2 isoform and are considered nonrevertant FA core patients. Absence of detection of the FANCD2 protein suggests FA-D2 group. Patients with FANCD2 monoubiquitination in PBLs are further investigated by FANCD2 immunoblot in fibroblasts. When fibroblasts demonstrate an FA pattern (FA core or FA-D2), FANCD2 monoubiquitination in PBLs indicates FA reversion. When FANCD2 in fibroblasts is monoubiquitinated to normal

Table 3. FA reversion is associated with higher blood cell counts

<table>
<thead>
<tr>
<th>Age, y</th>
<th>Group</th>
<th>PBL breaks</th>
<th>FANCD2 test</th>
<th>Breaks, fibro</th>
<th>Follow-up, y</th>
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<tr>
<td></td>
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<td>0</td>
<td>1</td>
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<td>ANC, × 10⁵/L</td>
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<td>Hb, g/L</td>
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For revertant, n = 8; for nonrevertant, n = 45. MCV indicates mean corpuscular volume.

*Nonsignificant.
identification of the constitutional FA mutations by molecular analysis. Ultimately, reverse genetic events may be searched for by molecular analysis to demonstrate molecular reversion and somatic mosaicism.

We found that comparison of fibroblasts and PBLs using FANCD2 immunoblot allowed easy detection of functional reversion in the PBLs of 5 FA-core patients by positively and specifically showing the ability of the PBLs to monoubiquitinate FANCD2. In one FA-D2 patient, FA reversion was detected in PBLs by the normal level of FANCD2 expression compared with fibroblasts. In 2 patients from unidentified complementation groups, we had to analyze chromosome breaks in fibroblasts to confirm the constitutional FA phenotype and subsequently to detect functional reversion in the PBLs by comparison. Altogether, FA reversion was found in the PBLs of 8 (15%) of 53 FA patients (Figure 4). We demonstrated that FA reversion as detected here was associated with significantly higher blood cell counts, as was previously suggested from case reports.23,26,27 Clinical records in the 8 revertant patients showed higher blood cell counts, as was previously suggested from case reports.23,26,27 Clinical records in the 8 revertant patients showed

**Figure 2. Blood cell curves in FA revertant patient EGFA047.** Blood cell count curves from patient EGFA047 are shown over a 27-year follow-up in our institution. A total of 213 recorded blood cell counts were analyzed from age 6 years to 34 years (from diagnosis to present study). Starting values at diagnosis are indicated. PIt, platelets \( \times 10^{12}/L \); Hb, hemoglobin (g/dL); ANC, absolute neutrophil counts \( \times 10^{9}/L \).

Leukemia may also develop from residual nonrevertant FA cells,25 and in non-FA patients an acquired FA defect might cause FA-like leukemia.41

Once constitutional cells were analyzed in all patients (PBLs in nonrevertant patients, and fibroblasts in revertant patients), the FANCD2 status allowed a classification based on the level of the FA/BRCA pathway inactivation (Figures 4-5). Most patients (\( n = 47; 89\% \)) were defined as "FA core," consistent with the known high prevalence of the FA-A, and to a lesser extent, FA-G and FA-C groups.3,17,20 4 unrelated patients (8%) were classified as FA-D2, suggesting that this group might be more common than previously appreciated.3,17 Two of these patients were further analyzed by DNA sequencing, and FANC2 mutations were detected, confirming D2 group (A.S., data not shown). In addition, 2 patients were provisionally considered as FA of unidentified downstream group (4%), considering their strong clinical FA features, normal FANC2 monoubiquitination but chromosome breaks in fibroblasts, and a history of positive breakage tests in PBLs in infancy. No personal or familial cancer history was found in these patients now aged 9 and 10 years, and no BRCA2 or NBS1 failure and neoplastic events, including leukemia. Notably, clonal karyotypic abnormalities were detected in 7 FA patients whose PBLs were found to be nonrevertant, including one with acute myeloid leukemia and one with severe myelodysplasia at the time of the analysis (Table 1). However, these chromosome abnormalities were detected in mitoses from unstimulated bone marrow but not in the PHA-stimulated PBLs, and therefore the FANCD2 status of the bone marrow clonal cells is elusive. Addressing the FA/BRCA pathway in these clones will be of interest, considering recent work associating leukemic progression to reversion.40

Figure 3. Analysis of FA-D2 and unidentified downstream group FA cells. (A) FANC2 immunoblots detect the FA-D2 group. (i) No FANC2 protein was observed at normal exposure of the immunoblot in sample EGFA014-L (top arrow). Long exposure revealed 2 barely detectable FANC2 bands (bottom arrow), indicating residual levels of the FANC2 protein, as previously shown in the reference D2 cell line PD20.5,10 Ponceau S staining is shown as loading control. Sequencing data further confirmed mutations in the FANC2 gene in this patient (A.S., data not shown). (ii) FANC2 immunoblot of PHA-stimulated primary fibroblasts and EBV-immortalized cells from FA-D2 patient EGFA021. (B, i) Unidentified group patients EGFA006 and EGFA053 have normal FANC2 patterns in both their primary fibroblasts and lymphocytes (EGFA005-L; not shown). (ii) BRCA2 and NBS1 immunoblots detected no abnormalities in the fibroblasts of these 2 patients. Eufa243 and Eufa1020 EBV cell lines were used as positive controls for BRCA2 and NBS1 deficiency, as indicated.
protein abnormalities were detected. Although extensive DNA analysis must be done to definitely rule out BRCA2 gene mutation and discard D1 group, it is likely that these cases belong to additional downstream group(s), such as the FA-J group, which has been defined recently by complementation analysis.\(^4\) Notably, the 6 FA-D2 and unidentified downstream group patients had severe phenotype with extensive congenital abnormalities and early diagnosis (Table 1), consistent with pleiotropic functions of the FANCD2 and downstream proteins.\(^9,14-16,42\) Interestingly, 3 of these 6 patients had FA reversion, compared to 5 out of 47 FA core patients, and these 3 patients were diagnosed in infancy. A severe specific cellular defect might favor early reversion, due to a high level of genetic instability and apoptosis.\(^42,43\)

The present study also allowed evaluation of the FANCD2 immunoblot as a diagnostic test in FA patients. We found that FANCD2 Western blot is a rapid and specific FA test, as previously reported, and it can be used to screen broad patient populations.\(^34\) In rare FA cases, it appeared that the chromosome breakage test was more sensitive as a diagnosis test than the FANCD2 immunoblot, due to the ability to detect minor residual nonrevertant populations in PBLs (n = 4 cases of the present study), and because of unidentified downstream group (n = 2 cases; Table 2). Therefore, we believe that the chromosomal breakage test remains the standard to-date for initial FA diagnosis. On the other hand, it has been reported that increased chromosomal breakage may also be detected in other syndromes with FA-like features, such as Nijmegen breakage syndrome.\(^11\) A normal FANCD2 pattern in these patients would show that more investigations are required. In addition, in unassigned patients with negative or ambiguous FA tests in PBLs but a strong clinical suspicion of FA, fibroblast analysis can be essential to search for FA with reversion.\(^30\) To that purpose, we found it much more convenient to analyze primary fibroblasts in first line by FANCD2 immunoblot than by chromosome breakage test or MMC sensitivity. Finally, primary fibroblasts from allografted FA patients can also be analyzed by FANCD2 immunoblot to identify FA subtype (n = 3 allografted FA patients not included in this study, including one further FA-D2 patient; data not shown).

In conclusion, we found that the specific analysis of the FA/BRCA pathway by FANCD2 test gives helpful information in addition to the chromosome breakage data. Most important, it allows easy detection of FA reversion, and it pinpoints the level at which the FA/BRCA pathway is disrupted, including the FANCD2 protein itself. We propose that the FANCD2 analysis might be used as a research center approach, in combination with the chromosome breakage analysis, for a comprehensive characterization of FA and FA-like patients.

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References


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