Chapter 2

Diagnostic Evaluation of FA

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Definition of Fanconi Anemia

Fanconi anemia is an autosomal recessive disorder associated with a very high frequency of bone marrow failure, leukemia, and squamous cell carcinoma. FA has many other manifestations including, but not restricted to, severe birth defects,[1,2] chromosomal instability, and a defect in DNA repair. Thirteen genes have been identified as of 2008; a few otherwise typical FA patients do not have mutations in the known genes and, thus, more genes await discovery.

The Importance of Early Diagnosis

Early diagnosis of FA permits the exclusion of other diseases and precludes inappropriate management of hematologic disease (aplastic anemia [AA], myelodysplastic syndrome [MDS], acute myeloid leukemia [AML]), and permits appropriate consideration of stem cell transplant, androgens, hematopoietic growth factors or supportive care (see later chapters). Surgical intervention for orthopedic, renal or other anomalies is also optimized if the diagnosis of FA is known. For example, surgeries might be accelerated in order to be completed before the development of significant cytopenias. Physicians can offer targeted cancer surveillance and early, aggressive surgery for solid tumors. Experts can discuss realistic prognoses. Genetic counseling is imperative, because of the 25% risk of FA in each subsequent pregnancy. Opportunities must be provided for family

planning, prenatal diagnosis, and even preimplantation genetic diagnosis.

Index of Suspicion

Physical appearance
The most frequent characteristic birth defects in FA, in descending frequency from approximately 50 to 20 percent, include skin hyperpigmentation and *café au lait* spots; short stature; abnormal thumbs and radii; abnormal head, eyes, kidneys, and ears. These data are from 1,865 case reports in the literature (Alter, unpublished) and are biased by under- and over-reporting because cases in the literature tend to focus on the unusual or more sensational findings. Additional specific types of anomalies in Fanconi anemia patients are listed below. Although these types of anomalies may be present in many other syndromes, FA should be "ruled in" or "ruled out" in patients with these findings. However, at least 25% of known FA patients have few or none of these features.[2]

Examples of Anomalies in Fanconi Anemia[1]
Anomalies are listed in approximate order of frequency within each category, as follows:

Skin: Generalized hyperpigmentation; *café au lait* spots, hypopigmented areas
Microsomia: Short stature
Upper Limbs:
- ***Thumbs:*** Absent or hypoplastic, bifid, rudimentary, attached by a thread, triphalangeal
- ***Radii:*** Absent or hypoplastic (only with abnormal thumbs), absent or weak pulse
- ***Hands:*** Hypoplastic thenar eminence, absent first metacarpal
- ***Ulnae:*** Dysplastic

Gonads:

Males: Hypogenitalia, undescended testes, hypospadias, micropenis

Females: Hypogenitalia, bicornuate uterus, abnormal menses

Other Skeletal:

Head and face: Microcephaly, micrognathia, triangular

Neck: Sprengel, Klippel-Fiel

Spine: Spina bifida, scoliosis, abnormal ribs

Eyes: Small, close-set, strabismus, epicanthal folds, cataracts, astigmatism

Ears: Deaf (usually conductive), abnormal shape, atresia, abnormal middle ear

Renal: Ectopic or pelvic, abnormal, horseshoe, hypoplastic or dysplastic, absent, hydronephrosis or hydroureter

Gastrointestinal: Atresia (esophagus, duodenum, jejunum) imperforate anus, tracheoesophageal fistula

Lower Limbs:

Feet: Toe syndactyly, abnormal toes

Legs: Congenital hip dislocation

Cardiopulmonary: Various structural congenital heart defects.

For a complete listing of possible anomalies in FA, see Young NS, Alter BP. *Aplastic Anemia: Acquired and Inherited*. Philadelphia, PA: WB Saunders; 1994.

Hematology

Patients with FA may present with AA, MDS, AML, single cytopenias without another explanation (such as antibodies) or macrocytic red cells without another explanation (e.g., vitamin B12 or folate deficiency). We recommend that FA be considered in all children and young adults with unexplained cytopenias. It is

absolutely imperative to test for FA if a stem cell transplant is planned.

Table 1: Cancer in Patients with FA, Not Transplanted, 1927-2007*

Type of Cancer	Male	Female	Not Stated	Total	Median Age in FA (Range)	Median Age for Sporadic Cancers†
Leukemia:						
Acute myeloid leukemia (AML)	68	59	12	139	13 (0.1-50)	67
Acute leukemia, unspecified	3	9	0	12	14 (9-24)	
Acute lymphoid leukemia (ALL)	3	3	0	6	5 (1-10)	13
Chronic myelomonocytic leukemia (CMML)	0	3	1	4	16 (11, 20)	NA
Solid Tumors:						
Head and neck	15	21	0	36	27 (13-46)	62
Esophagus	3	9	0	12	27 (20-50)	69
Vulva	-	17	-	17	26 (14-38)	68
Cervix	-	6	-	6	24 (22, 25)	48
Breast	0	7	0	7	37 (26-45)	61
Brain	8	11	4	23	3 (0.5-11)	10
Renal Wilms	9	4	3	16	1(0.5-5)	5†
Renal carcinoma	0	1	0	1	36	65
Neuroblastoma	4	1	2	7	0.7 (0.2-1.4)	0.5†
Lung	3	0	0	3	29 (23-34)	70
Stomach	2	0	1	3	28 (21, 35)	71
Lymphoma	1	1	0	2	1.4 (0.3, 2.5)	67
Colon	0	1	0	1	21	71
Retinoblastoma	0	1	0	1	0.3	0.5†
Osteosarcoma	0	1	0	1	7	15†
Bladder	1	0	0	1	38	73
Dermatofibroma	0	1	0	1	20	56
Liver Tumors:						
Adenoma	7	8	0	15	11 (8-48)	NA
Carcinoma	18	10	0	28	14 (5-50)	65

*Data from 1,865 literature cases. 161 patients with leukemia; 11 also had a solid tumor. 181 solid tumors in 166 patients. Twenty-three had 2-4 solid tumors. A hyphen (-) indicates that the cancer type is not possible in males. Ages are in years. If the number of ages is fewer than the number of patients, some data missing. NA=not available. †Median ages for sporadic cancers in pediatric patients where available. Ages for sporadic cancers from SEER. (Alter, unpublished)

The relative risk of AML in FA patients compared to the general population is ~800-fold, and the median age in reported cases is 13 years, with a range from <1 to 50 years of age (Table 1).[3,4] The frequency of MDS is unknown, and the temporal relation between MDS and AML is not clear. However, FA should be considered in patients who are children or young adults and have either diagnosis.

Aplastic anemia is usually the first adverse event in patients with FA, occurring at a median age of around 8-10 years and reaching a plateau by the 20s. Leukemia develops primarily in teenagers and young adults, and solid tumors begin to appear in the 20s and do not level off.[5,6]

Solid Tumors

Patients with FA are at a particularly high risk (hundreds- to thousands-fold) of developing specific solid tumors at unusually young ages, including head, neck, esophageal, and gynecological squamous cell carcinomas, as well as liver tumors (Table 1).[3,4] The risk of head and neck squamous cell carcinomas is even higher in patients who have received a bone marrow transplant.[7] Approximately 25% of reported FA patients with the FA types of cancers were not aware that they had FA until they developed cancer (and sometimes complications from the treatment).[3] This highlights our concern that older FA patients may be significantly underdiagnosed.

Miscellaneous Conditions

Experts must test for FA if spontaneous chromosome breaks are found during studies for prenatal or postnatal evaluation of genetic conditions (see below). FA should be considered in patients with AML or solid tumors

with excessive sensitivity to chemotherapy or radiotherapy or who are atypically young and lack the usual risk factors for their cancers. Patients with androgen-responsive or ATG/cyclosporine A-non-responsive "acquired" aplastic anemia might have FA. FA should also be considered in individuals with macrocytic red cells and/or increased levels of fetal hemoglobin (Hb F) who do not have a hemoglobinopathy; in males (and perhaps females) with unexplained infertility; and in young patients with liver tumors without the usual viral or alcohol risk factors.

Table 2 outlines the hierarchy of indications for testing for FA, listing those in whom the FA work-up should definitely be done, as well as those in whom it should be highly considered. This table is not restrictive, but rather, is a guide.

Table 2: Indications for Testing for Fanconi Anemia*

Definite:

- Sibling with FA
- Aplastic anemia
- Characteristic birth defects, particularly one or more of abnormal radii and/or thumbs; renal structural anomalies; microophthalmia; microcephaly; *café au lait* spots; features of VACTERL-H such as tracheo-esophageal fistula, imperforate anus, and others (see earlier listing).
- Spontaneous chromosome breaks
- Primary MDS (at a young age)
- Primary AML (at a young age)
- Unusual sensitivity to chemo- or radiotherapy
- Cancer typical of FA at an atypical age, such as HNSCC <50 years old, cervical <30 years old,

anal/vulvar <40 years old (see Table 1)
- Family history consistent with FA or with cancer (e.g., breast cancer)

Consider:
- Single cytopenias
- Macrocytosis unexplained by B12 or folate deficiency
- Liver tumors without alcohol or hepatitis
- Premature ovarian failure <30 years old
- Diminished ovarian reserve <30 years old
- Brain tumor <5 years old
- Wilms tumor <4 years old
- Increased Hb F not otherwise explained
- Male (or female) infertility
- Liver adenomas or hepatomas without alcohol or hepatitis

*Note: Combinations of features are particularly strong indications for testing.

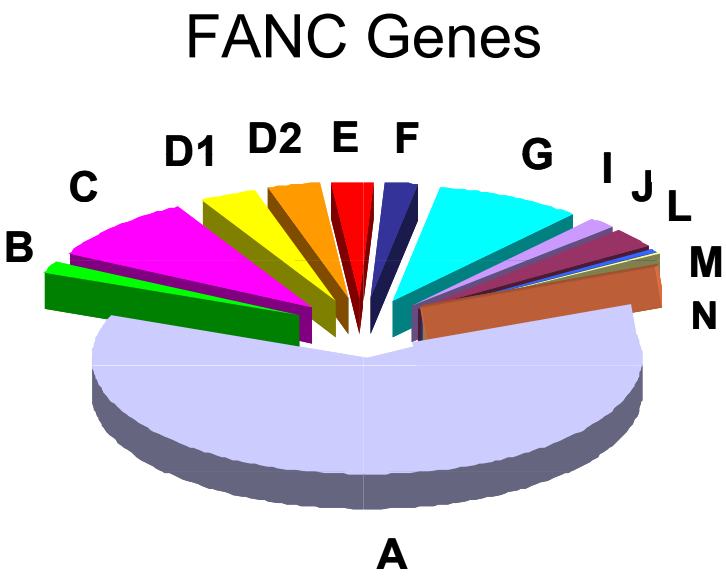

Figure 1: Relative frequency of the FA complementation groups (genes). Modified from Joenje, et al.[8]

FA Genes and DNA Damage Response Pathway

There are currently at least 13 known FA genes (Figure 1 and Table 3).[8]

Table 3: FA Genes and Gene Products

Gene	Locus	Genomic DNA kB	cDNA kB	No. of Exons	Protein kD	Amino Acids	Muta-tions	% of Patients	Genetics
FANCA	16q24.3	80	5.5	43	163	1455	~100	~70	AR
FANCB	Xp22.31	30	2.8	10	95	859	4	rare	XLR
FANCC	9q22.3	219	4.6	14	63	558	10	~10	AR
FANCD1 (BRCA2)	13q12.3	70	11.4	27	380	3418	-	rare	AR
FANCD2	3p25.3	75	5	44	162	1451	5	rare	AR
FANCE	6p21.3	15	2.5	10	60	536	3	rare	AR
FANCF	11p15	3	1.3	1	42	374	6	rare	AR
FANCG (*XRCC9*)	9p13	6	2.5	14	70	622	18	~10	AR
FANCI (KIAA1794)	15q25-26	73	4.5	38	150	1328	~12	rare	AR
FANCJ (BACH1/BRIP1)	17q22.3	180	4.5	20	150	1249	15	rare	AR
FANCL (PHF9/POG)	2p16.1	82	1.7	14	43	375	1	rare	AR
FANCM (Hef)	14q21.3	250	6.5	22	250	2014	1	rare	AR
FANCN (PALB2)	16p12.1	38	3.5	13	130	1186	15	rare	AR

The protein products of eight genes form a complex which permits ubiquitination of the FANCD2 protein, which in turn interacts with downstream FA gene products in the FA/BRCA DNA repair pathway (Figure 2). Three FA genes are associated with breast cancer in heterozygotes: *FANCD1/BRCA2, FANCJ/BRIP1*, and *FANCN/PALB2*.[9]

Laboratory Test Methods to Diagnose FA

Anyone who suspects FA should refer the patient to a hematologist and/or geneticist, who can arrange for an FA test to be performed by a clinically-certified laboratory with the appropriate expertise in FA testing. The specific test may vary by locale. The first test should

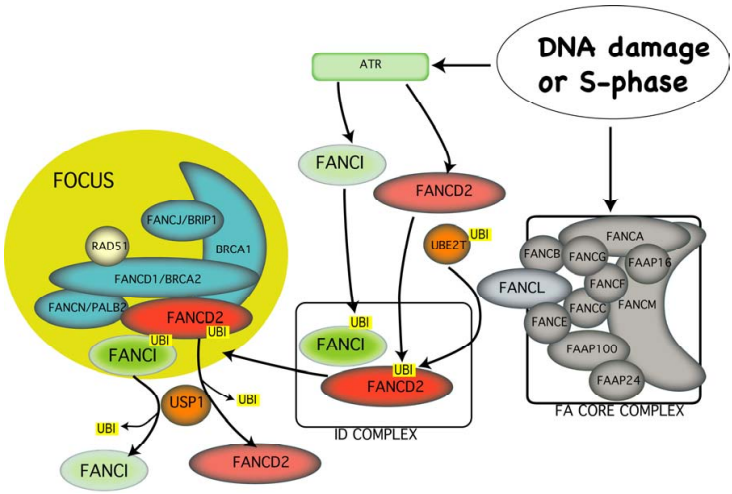

Figure 2: DNA damage response pathway, linking the FA and BRCA pathways. From Grompe and van de Vrugt.[9]

be used as a screening/diagnostic test. If it is positive, the physician should make the appropriate referrals. If it is negative and the level of suspicion of FA is low, no further studies are indicated. If it is negative but the suspicion level is high, then one or more of the next tier of tests should be done. If those are negative and the patient does appear to have an inherited bone marrow failure syndrome, then other disorders must be considered, such as dyskeratosis congenita, Shwachman-Diamond syndrome or Diamond-Blackfan anemia, and specific testing should be performed for each.[1,2,10]

Chromosome breaks in T-lymphocytes

The classical diagnostic test involves detection of chromosomal breakage or aberrations (breaks, gaps, rearrangements, radials, exchanges, endoreduplications) in peripheral blood cells after culture with a T-cell mitogen and a DNA clastogenic (cross-linking) agent, such as diepoxybutane (DEB) or mitomycin C (MMC). Data

are reported as aberrations per cell, as well as percent of cells with aberrations, usually for 20 to 100 cells. The test is most reliable if there is a low concentration of clastogen, which does not produce aberrations in normal controls, as well as a high concentration, which leads to a few abnormal control cells and thus indicates that the reagent is working. There are rare disorders, such as Nijmegen breakage or Roberts syndromes, in which chromosome breakage is positive with DEB or MMC and, yet, the patient does not have FA. If the blood result is normal but FA is still suspected, then a skin biopsy should be done to provide fibroblasts for chromosome breakage analysis in order to evaluate for somatic mosaicism.

The existence of mosaicism may complicate the FA diagnosis when chromosome breakage tests are used. The percent of cells with aberrations may be more useful than the breaks per cell, because patients with hematopoietic somatic mosaicism (the simultaneous presence of both normal and FA cells in the blood) may have only a few cells with breaks, and the average number of breaks per cell may fall into the normal range. Mosaicism is difficult to diagnose and even to define. Expert hematologists and cytogeneticists define it as a condition in which the peripheral blood lymphocyte breakage is "normal," while skin fibroblasts show clastogen-induced increased breakage. Approximately 10-20% of patients with FA have this result. However, the diagnostic percent of "normal" cells in the blood ranges from "a few," to 20, to 50, to 100%, depending on the laboratory. Low-level mosaicism may develop into high-level mosaicism, and this may be associated with "spontaneous" hematologic improvement. However, the mosaicism measured is in T-lymphocytes, which are long-lived and may not reflect myeloid hematopoiesis.

Final proof requires molecular demonstration of reverse mutation by molecular analyses from myeloid blood cells compared with fibroblasts.

Flow cytometry

Flow cytometry examines cell cycle kinetics and can detect the proportion of cells that are arrested at G2/M after culture with a clastogen such as nitrogen mustard. In contrast with the 100 cells examined microscopically for aberrations, flow cytometry examines thousands of cells and is less labor-intensive and subjective, but it does require sophisticated instrumentation. This test is usually done in a specialized laboratory and is not used nearly as widely as the chromosome breakage assay. Flow cytometry may give a false negative result in FA patients with MDS or AML; experience is limited.

Fibroblasts

Fibroblast cultures are useful for patients who might have hematopoietic somatic mosaicism, for patients following successful bone marrow transplant or for prenatal diagnosis (using chorionic villus cells or amniotic fluid cells). These cells can be used for chromosome breakage analyses or flow cytometry. FA cells often grow poorly, which might provide the first clue that the patient may have FA.

D2 Western blots

Following DNA damage, the complex of upstream FA gene products (A, B, C, E, F, G, I, L) leads to ubiquitination of the product of *FANCD2*, forming a longer protein (D2-L), which can be distinguished from the shorter non-ubiquitinated form (D2-S) on a Western blot with a D2-specific antibody.[11] This relatively inexpensive assay may be useful for screening patients for whom FA is in the differential diagnosis, such as those with radial ray anomalies, short stature, hypogonadism

or *café au lait* spots or for population-based FA incidence studies; however, it is usually only a research tool. FA patients whose gene defect is downstream of *FANCD2* will not be detected with a D2 Western blot.

Complementation analysis

Patient lymphocytes, EBV-lymphoblasts or fibroblasts can be cultured with retroviruses which introduce known normal FANC genes into the patient's cells, leading to correction of the FA cellular phenotype (chromosome breaks or poor growth in the presence of a clastogen). This test is limited to the availability of cloned DNA from known FA genotypes and is performed in a very limited number of primarily research laboratories.

Mutation testing

Determination of the specific mutation in FA genes is complicated and is done in laboratories with specific expertise. It requires sophisticated methods and involves DNA amplification, sequencing, and detection of large deletions. Many laboratories rely on knowing the complementation group before sequencing, while in some contexts targeted sequencing of candidate genes is more appropriate. One center goes directly to gene sequencing for patients in whom chromosome breakage testing indicates FA: *FANCA* by multiplex ligation-dependent probe amplification (MLPA) for large deletions and full sequencing; *FANCB* by MLPA and full sequencing, if indicated; *FANCC, E, F, G* by denaturing high performance liquid chromatography (DHPLC) and sequencing; *FANCD2* by Western blot; *FANCD2* sequencing if D2 bands are absent; *FANCL* and *FANCM* sequencing if only D2-S is seen; *FANCD1/BRCA2* sequencing, if indicated; *FANCJ/BRIP1* and *FANCN/PALB2* sequencing; and finally *NBS1* and

ESCO2 sequencing for Nijmegen breakage and Roberts syndromes.[12] Mutation testing is used to confirm known cases and for family studies to determine affected or carrier status. Genetic counseling should be included in these processes, because of the complicated explanations and support needed for the families.

Importance of Gene and Mutation Information

Current

The majority of patients worldwide are in the *FANCA* group, in which several hundred mutations have been documented. However, there are several populations in which there is a founder effect, leading to a limited number of specific mutations that can be targeted for genetic diagnoses. These include Ashkenazi Jewish *FANCC* IVS4+4 A>T or *FANCD1/BRCA2* 6174delT; non-Ashkenazi Jewish Moroccan *FANCA* 2172-2173insG or *FANCA* 4275delT; Tunisian *FANCA* 890-893del; Indian *FANCA* 2574C>G (S858R); Israeli Arabs *FANCA* del ex 6-31, *FANCA* IVS 42-2A>C, and *FANCG* IVS4+3A>G; Japanese *FANCC* IVS4+4 A>T; Afrikaner *FANCA* del ex 12-31 and *FANCA* del ex 11-17; Brazil *FANCA* 3788-3790del; Spanish Gypsy *FANCA* 295C>T; and Sub-Saharan African Black *FANCG* 637-643delTACCGCC. Patients from those specific groups can be tested initially for those mutations, and premarital and prenatal testing are possible.

In families in which the proband's mutation is known, mutation testing of family members permits accurate diagnosis of homozygotes and heterozygotes, leading to appropriate medical management and focused genetic counseling. Premarital screening, prenatal diagnosis, and preimplantation genetic diagnosis can be performed. Potential bone marrow transplant donors, such

as siblings who are phenotypically and hematologically "normal," can be accurately genotyped, so that undiagnosed homozygotes will not be used as donors. Patients genotyped as FA who are clinically well can be monitored closely for potential development of aplastic anemia, MDS, leukemia or solid tumors.

We are just beginning to learn about genotype/phenotype correlations. The most severe physical findings, including in some cases features of VACTERL-H syndrome (**V**ertebral anomalies, anal **A**tresia, **C**ardiac anomalies, **T**racheo-esophageal fistula, **E**sophageal atresia, **R**enal anomalies, radial **L**imb anomalies, plus **H**ydrocephalus), were reported more frequently in those with mutations in *FANCC* IVS4+4 A>T, *FANCD1/BRCA2*, *FANCD2*, *FANCG*, *FANCI*, and *FANCN/PALB2*. Early onset aplastic anemia was most common in some patients with *FANCA*, *FANCC* IVS4, *FANCG*, and *FANCI*. Leukemia particularly characterizes *FANCD1/BRCA2* and *FANCN/PALB2*, and the rates of specific solid tumors (medulloblastoma and Wilms tumor) were also extremely high in those with mutations in those two genes. In general, null mutations which produce no protein are more severe than hypomorphic mutations.[13]

Future

Future research is focused on determination of more specific genotype/phenotype outcome correlations, in order to better inform a patient or family with a specific mutation about the risks associated with that mutation. However, since FA gene mutations occur in a milieu of other genes and the environment, this will never be a perfect prediction. Gene-gene, gene-environment, and epigenetic modifiers will continue to challenge physicians and their patients.

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