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Somatic Mosaicism in Fanconi Anemia: Molecular Basis and Clinical Significance

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Abstract

Approximately 25% of patients with Fanconi anemia (FA) have evidence of spontaneously occurring mosaicism as manifest by the presence of two subpopulations of lymphocytes, one of which is hypersensitive to cross-linking agents (e.g. mitomycin C) while the other behaves normally in response to these agents. The molecular basis of this phenotypic reversion has not yet been determined. We have investigated 8 FA patients with evidence of mosaicism. Epstein-Barr virus-immortalized lymphoblastoid cell lines established from these patients exhibited an IC_{50} for mitomycin C of 25 to >100 n*M* compared to a mean of 2 ± 2 n*M* for 20 nonmosaic FA patients and 49 ± 11 n*M* for 8 healthy controls. In 3 patients who were compound heterozygotes for pathogenic *FAC* gene mutations the molecular mechanism of the mosaicism was investigated by haplotype analysis. The results indicated that an intragenic mitotic recombination must have occurred leading to a segregation of a wild-type allele in the reverted cells and suggested two patterns of recombination. In 1 patient a single intragenic crossover between the maternally and paternally inherited mutations occurred associated with markers located distally to the *FAC* gene; in the other 2 patients (sibs) the mechanism appears to have been gene conversion resulting in segregants which have lost one pathogenic mutation. In 6 of the 8 patients the hematological symptoms were relatively mild despite an age range of 9–30 years.

Key Words

Fanconi anemia
Genetic reversion
Mosaicism
Mitotic recombination
Gene conversion

Introduction

Fanconi anemia (FA) is an autosomal recessive disorder characterized by progressive bone marrow failure and a diversity of somatic abnormalities including hyperpigmentation of the skin (café au lait spots), growth delay, skeletal and renal anomalies, and hypogonadism [1–3]. FA cells exhibit increased spontaneous chromosomal instability and hypersensitivity to chromosomal breakage by cross-linking agents such as diepoxybutane (DEB) and mitomycin C (MMC). There are at least 5 FA complementation groups [4], group A being the most prevalent [5, 6]. The gene for group C has been cloned by expression cloning methodology [7] and mutations in this gene are responsible for an estimated 8% of FA cases worldwide [8] and at least 80% of cases with Ashkenazi Jewish ancestry [9, 10].

Hematopoietic failure is the major life-threatening complication in FA patients, which typically starts between 5 and 10 years of age. The aplastic phase may be followed by a myelodysplastic syndrome and acute myeloid leukemia [1, 11], which is usually fatal. Bone mar-

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row failure may be cured by allogeneic bone marrow or umbilical cord blood transplantation [12, 13]. For FA-C patients without a suitable donor, gene therapy is being explored as a curative option [2, 14].

Hypersensitivity to cross-linking agents, as assessed in fresh phytohemagglutinin (PHA)-stimulated lymphocyte cultures, is currently the best criterion for the diagnosis of FA. Epstein-Barr virus (EBV)-immortalized lymphoblastoid cell lines derived from FA peripheral blood samples usually are also hypersensitive to cross-linking agents, typically exhibiting 10- to 100-fold reduced half-maximal inhibitory concentrations (IC_{50} values) in standard growth inhibition tests compared to cell lines derived from healthy controls. However, over the last few years, it was found that EBV-immortalized lymphoblast lines established from approximately 25% of the FA patients in the EUFAR Cell Repository were MMC-resistant, in spite of the chromosomal breakage test on fresh lymphocytes being positive for DEB or MMC sensitivity. Such resistant cell lines are likely to derive from a subpopulation of B lymphocytes whose disease phenotype has reverted to the wild-type. Indeed, in some of these patients two subpopulations of T lymphocytes were clearly distinguishable in PHA-stimulated peripheral blood cultures, one exhibiting a typical FA-like response to MMC and one behaving like non-FA cells. A particularly well documented case of mosaicism has been reported previously in an FA patient whose lymphocyte cultures revealed a proportion of approximately 60% to be resistant to the chromosome breaking effect of MMC [15]. The possible complications due to the phenomenon of mosaicism for the diagnosis of FA patients have been discussed previously [15–17].

Here we document 8 mosaic FA cases, 2 of whom are brothers, whose EBV-immortalized lymphoblasts were MMC-resistant. Five patients were of an unknown complementation group, while 3 patients were deduced to belong to group C on the basis of mutations found in the *FAC* gene. In the FA-C cases we were able to establish the cause of the reversion by molecular genetic analysis of the *FAC* gene. Our results suggest that (1) reversion of the FA phenotype can occur spontaneously in hematopoietic stem or progenitor cells of FA patients, which may lead to sustained mosaicism of their hematopoietic system, (2) a single reverted stem cell may have the capacity to gradually replace affected progenitor cells leading to proficient clonal hematopoiesis, (3) in compound heterozygous patients phenotypic reversion may be caused by intragenic mitotic recombination generating a wild-type allele at the disease locus; however, additional mechanism(s) are likely to exist.

Materials and Methods

Patients

FA patients were selected from the EUFAR Cell Repository based on relative resistance to MMC of EBV-immortalized lymphoblast lines. Five patients (EUFA192, FA-1, EUFA806, EUFA449, and EUFA450) have been reported on before [15, 16, 18] (EUFA806 was ST in ref. 16). Because of the MMC resistance of the lymphoblast lines the patients could not be classified by functional complementation studies [4]; however, patients EUFA806, EUFA449 and EUFA450 could be assigned to complementation group C on the basis of pathogenic mutations found in the *FAC* gene. Patient EUFA806 was a compound heterozygote for 322delG (exon 1) and 1806insA (exon 14) [10, 19], whereas patients EUFA449 and EUFA450 (sibs) were compound heterozygotes for 322delG and the exon 14 mutation L554P [7, 18]. Clinical data of the patients are summarized in table 1.

Culture Methods and Chromosomal Breakage Assays

Diagnostic chromosomal breakage testing using PHA-stimulated fresh T lymphocyte cultures was as described previously [15]. Lymphoblastoid cell lines were established by immortalization of B lymphocytes with EBV according to a standard method [21] and cultured as described [4].

Generation of Chinese Hamster Hybrids

Primary diploid fibroblasts were obtained following standard procedures. Somatic cell hybrids from EUFA806 fibroblasts or lymphoblasts fused with Chinese hamster cells were obtained as described previously [22].

Mutation Detection

PCR of exons 1 and 14 of the *FAC* gene was performed as previously described [23]. PCR products of 5 independent amplifications were cloned in pBluescript and 10 randomly picked colonies were sequenced.

Haplotype Analysis

Microsatellite markers were amplified according to standard procedures. PCR was performed in a 15-µl volume containing 100 ng DNA, 0.2 m*M* dATP/dTTP/dGTP, 0.05 m*M* dCTP, 0.02 µl ^{32}P-dCTP (10 µCi/µl), 10 m*M* Tris HCl pH 9.0, 50 m*M* KCl, 0.01% (w/v) gelatin, 0.1% Triton X-100, 1.5 m*M* $MgCl_2$, and 0.2 units SuperTaq (HT Biotechnology LTD); the thermal profile was (30 cycles) 30 s 94°C, 60 s 56°C, and 30 s 72°C. The amplified fragments were separated on a 6% polyacrylamide 7 *M* urea gel and visualized by autoradiography.

Assay of Clonality of Hematopoiesis

Clonality of hematopoiesis was assessed by PCR-based amplification of a polymorphic CAG repeat located on the X chromosome near methylation-sensitive restriction enzyme sites [24]. By comparing the PCR products obtained with and without digestion prior to the amplification the presence of active versus inactive X chromosomes was determined.

Table 1. Clinical features of 8 mosaic FA patients

Patient	EUFA53[a]	EUFA192	FA-1	EUFA631	EUFA554	EUFA806	EUFA449[b]	EUFA450[b]
Sex	M	F	M	M	M	F	M	M
Birth year	1981	1979	1960	1987	1964	1966	1980	1982
Age at diagnosis, years	9	0	7.5	1.3	12	?	6	7
Present age, years	15	16	†	9	†	30	16	14
Complementation group	?	?	?	?	?	C	C	C
Family								
Sibs with FA	1	0	2	?	3	1	1	1
Healthy sibs	1	3	1	?	3	?	1	1
Consanguinity	–	–	–	?	+	?	–	–
Congenital malformations								
Café au lait spots	+	–	+	?	+	+	+	+
Heart	–	+	–	–	–	–	–	–
Thumb	–	+	+	±	–	–	+	–
Radius	–	+	+	–	–	–	–	–
Rib	–	+	–	–	–	+	–	–
Kidney	–	+	–	–	–	–	–	–
Genitalia	–	+	–	–	–	–	–	–
Microcephaly	–	–	?	+	+	?	–	–
Microphthalmia	–	–	+	–	+	–	–	–
Growth	N	R	R	R	–	N	N	N
Hematology[c]								
At time of diagnosis								
Hemoglobin, g/dl	12.6	12.9	?	13.5	12.3	?	?	5.6
WBC, × 10^9/l	4.6	4.8	5.3	8.7	5.5	?	?	2.1
Thrombocytes, × 10^9/l	204	338	98	406	196	?	?	18
HbF, %	?	?	?	0.5	4.3	?	?	14.5
1995/1996								
Hemoglobin, g/dl	14.2	13.5		14.0	5.2	13.2	12.4	8.2
WBC, × 10^9/l	3.9	5.8		7.4	4.2	5.5	3.0	3.3
Thrombocytes, × 10^9/l	197	172		284	170	190	35	7
HbF, %	?	?		?	?	?	3.1	4.4
Bone marrow	N	N		?	H	?	H	H
Treatment								
Transfusion	–	–	–	–	+	–	+	+
Prednisone/oxymetholone	–	–	–	–	+	–	+	+
Bone marrow transplant	–	–	–	–	+	–	–	–
Died at age, years			29		32			
Cause of death			LC		BMT			

– = No; + = yes; ? = no data available; † = patient died; N = normal; R = retarded; H = hypoplastic, hypocellular; LC = lung cancer; BMT = patient died from complications after attempted bone marrow transplantation.

[a] This patient has a monozygous twin brother (EUFA54) who was the first to be diagnosed as having FA because of severe hematological symptoms (hemoglobin 7.3 g/dl, WBC 3.5 × 10^9/l; thrombocytes 29 × 10^9/l); at age 9 years the brother was successfully transplanted with bone marrow from an HLA-identical healthy sib. The possible mosaic status in the brother could thus not be investigated.

[b] EUFA449 and EUFA450 are sibs.

[c] Normal ranges in the 12- to 18-year age group are hemoglobin (g/dl) 12–16 (females), and 13–16 (males), HbF (%) 0.2–1.0, WBC (× 10^{-9}) 4–11, and thrombocytes (× 10^{-9}) 150–400.

Table 2. FA patients with mosaicism for MMC hypersensitivity

Patients	Complementation group[a]	Age	MMC-resistant cells[b], %	IC_{50}, MMC (lymphoblasts)[c], n*M*	Fibroblasts MMC-sensitive?
EUFA53	Unknown	11	49 (39–59)	30	NA
EUFA192	Unknown	3	0[d] (0–4)	NA	Yes
		16	95 (89–98)	30	
FA-1	Unknown	22	69 (59–78)	NA	
		23	NA		Yes
		27	NA	>100	
		28	57 (47–67)	>100	
EUFA631	Unknown	9	37 (28–47)	26	NA
EUFA554	Unknown	32	NA	25	NA
EUFA806	C	19	100[e] (96–100)	49	Yes
EUFA449[f]	C	16	90 (82–95)	50	NA
EUFA450[f]	C	13	0 (0–4)	45	NA
Nonmosaic FA patients (n = 20, ± SD)[g]				2 ± 2	
Healthy controls (n = 8, ± SD)[h]				49 ± 11	

Patients were judged to be mosaic on the basis of (1) two subpopulations present in fresh lymphocyte cultures, one responding as FA and one responding as wild type to MMC challenge, and/or (2) MMC resistance of EBV-immortalized lymphoblasts derived from the patient. NA = Data not available.

[a] Functional complementation analysis, as carried out in our laboratory, can only be done with MMC-sensitive lymphoblast lines and is therefore not feasible with mosaic patients whose lymphoblasts have a reverted phenotype. However, finding pathogenic mutations in the *FAC* gene, as with patients EUFA806, EUFA449 and EUFA450, assigned such patients to group C.

[b] Estimated from the results of a chromosomal breakage test with fresh peripheral blood lymphocytes using a cross-linker concentration that, in a nonmosaic patient, typically causes chromosomal breakage in over 90% of the cells [15]. Percentages of undamaged cells up to 10% at 45 ng/ml were considered not to be indicative of mosaicism. 95% confidence limits are shown in parentheses. The percentage of clastogen-resistant (reverted) cells was calculated by correcting the percentage of undamaged cells for (1) the percentage of undamaged cells observed in a nonmosaic patient and (2) the effect observed in a healthy control studied in parallel.

[c] Concentration of MMC causing half-maximal growth inhibition in a standard test [20]. NA = Lymphoblast line not available.

[d] Case FA-2 in ref. 15.

[e] Results were indistinguishable from those of two healthy controls cultured in parallel [16].

[f] EUFA449 and EUFA450 are sibs.

[g] Based on patients belonging to various complementation groups; there was no significant difference between patients from different groups.

[h] Based on obligatory heterozygous FA gene carriers (FA parents).

Results

Detection of Mosaicism in Lymphocyte Cultures

The experimental approach to demonstrate mosaicism in fresh peripheral blood lymphocyte cultures from FA patients has been described in a previous publication [15], where mosaicism was readily detected in patient FA-1 (at age 22) by assessing chromosomal breakage levels as induced by DEB (10–150 ng/ml), MMC (5–100 ng/ml) or cisplatin (0.2–1.5 mg/ml). At the highest clastogen concentrations the great majority of the healthy control cells were undamaged, while virtually 100% of the cells from a nonmosaic patient had chromosomal damage. FA-1 cells, on the other hand, had 62–69% undamaged cells, depending on the clastogen used. Using this methodology the level of mosaicism in T lymphocytes may be estimated, as summarized for the present patients in table 2.

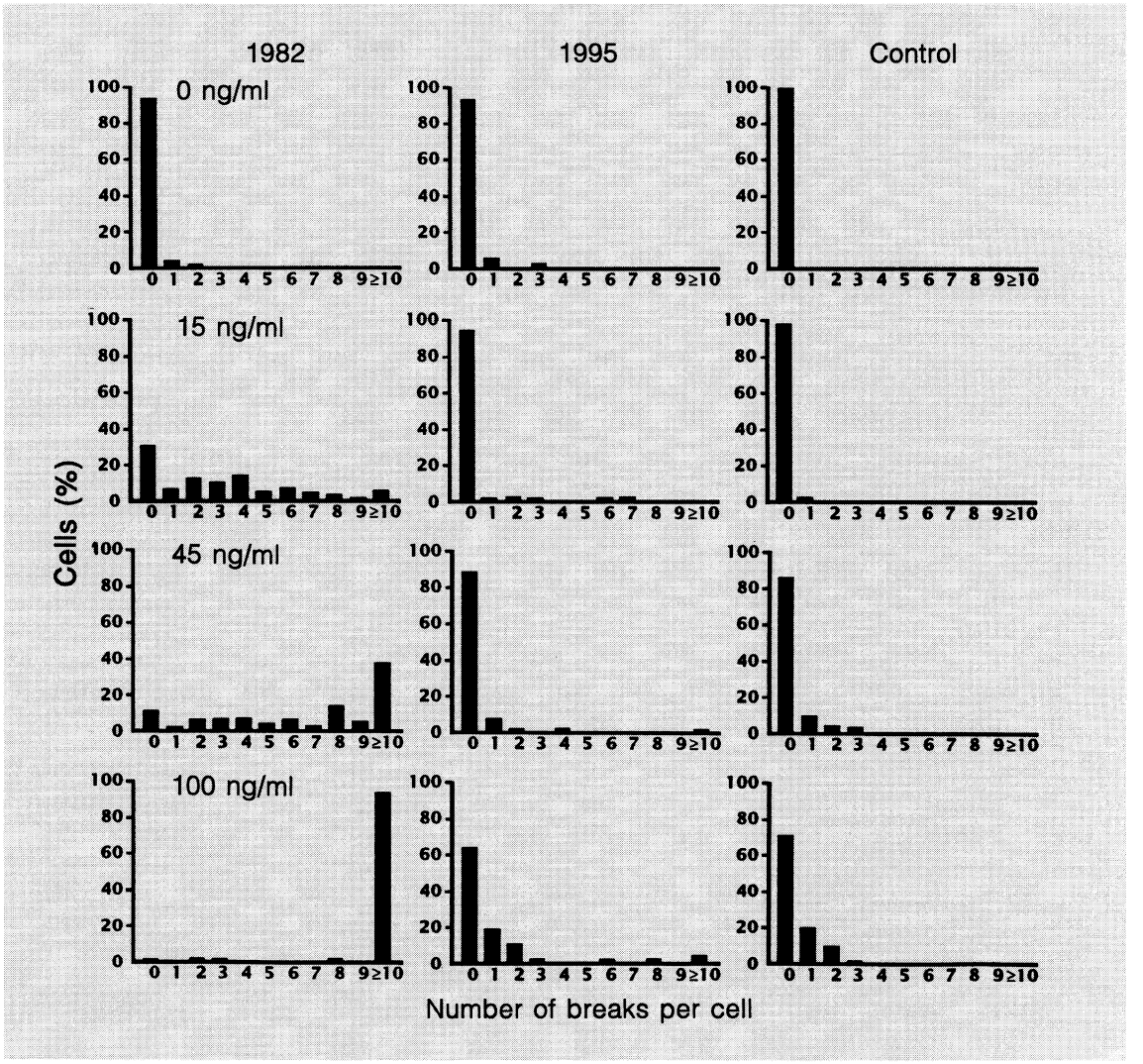

Fig. 1. MMC-induced chromosomal breakage in 72-hour PHA-stimulated lymphocyte cultures from patient EUFA192 at 3 (1982) and 16 years of age (1995), and a healthy control. The concentrations of MMC in the cultures (in ng/ml) are indicated. Per culture, up to 100 cells in metaphase were evaluated for chromosomal breakage. Histograms show the percentage of cells with the indicated number of breaks per cell.

Patients

The 8 patients that yielded a MMC-resistant lymphoblastoid cell line are listed in tables 1 and 2. The MMC resistance of the cell lines is indicated by the IC_{50} values (table 2), which were significantly elevated compared to the cell lines derived from non-mosaic cases. The level of mosaicism as estimated from chromosomal breakage rates in MMC-challenged lymphocyte cultures (see previous section) varied from patient to patient, from no detectable mosaicism (patient EUFA450) to apparently 100% MMC resistance (patient EUFA806); no data are available on patient EUFA554.

Changes in Mosaicism over Time

In 2 patients, EUFA192 and FA-1, it was possible to study the extent of mosaicism on different occasions over a relatively long period of time.

Patient EUFA192. This patient, who was previously reported as case FA-2 [15], was investigated for MMC-induced chromosomal breakage and diagnosed as FA only 1 month after birth and, when reinvestigated at 3 years of age, exhibited no sign of mosaicism [15]. Thirteen years later we established a lymphoblast line from this patient for the purpose of complementation analysis; however, the cell line was MMC-resistant and could therefore not

be used for complementation testing. Since the MMC resistance suggested that the patient might have become mosaic, we repeated the chromosomal breakage test in peripheral blood T lymphocytes. Figure 1 illustrates that at 3 years of age chromosomal breakage was observed in virtually all cells after a 3-day treatment with MMC at 100 ng/ml, whereas at age 16 only a small minority (<10%) of the cells exhibited FA-like high breakage levels, the remaining cells responding as the healthy control. This confirmed that the patient had indeed acquired phenotypically reverted cells, to the extent that almost all MMC-sensitive T lymphocytes were replaced by MMC-resistant ones. To explain this phenomenon we assumed that a pluripotent stem cell in the patient's bone marrow had undergone a stable phenotypic reversion, after which its progenitor cells, because of their proliferative advantage [25, 26], had gradually replaced the affected progenitor cells. Since reversion is supposed to be a rare event that is not likely to occur independently in multiple stem cells, this explanation would predict that the patient's hematopoiesis should have become clonal, or be at least clearly skewed towards clonality. As demonstrated by a PCR-based assay of polymorphic X chromosome markers [24], clonal hematopoiesis in the patient was confirmed. In contrast, fibroblasts cultured from a skin biopsy of the patient were polyclonal (fig. 2).

Patient FA-1. This patient was reported previously [15] as a clear example of mosaicism, with 69% of his T lymphocytes behaving like healthy control cells. During a 5 year follow-up period, to monitor the proportion of reverted cells, no significant change was observed (table 2), indicating that in this patient within this period of time an equilibrium had been reached. Given the relatively long period of time the mosaic status had existed in this patient, we may assume that also in this patient at least one pluripotent stem cell must have undergone stable phenotypic reversion.

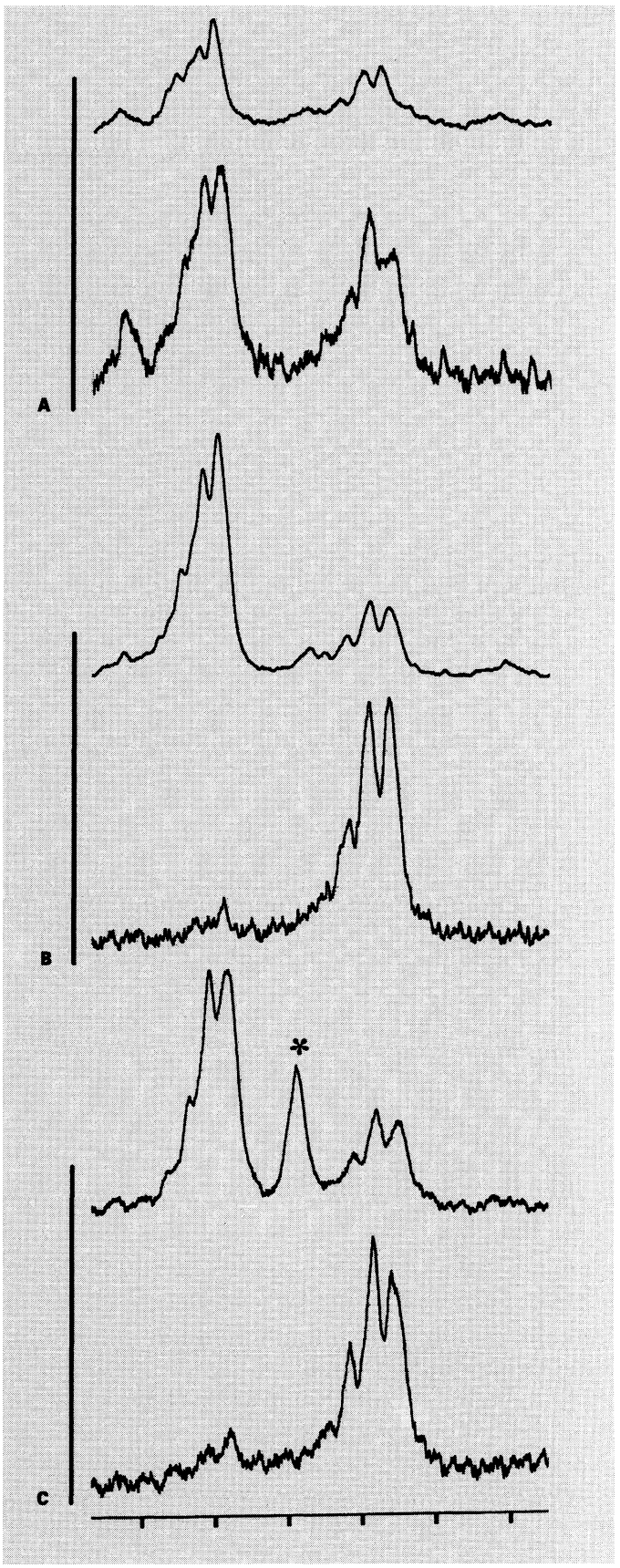

Fig. 2. Clonal hematopoiesis in patient EUFA192. Tracings are shown of PCR products obtained from genomic DNA isolated from fibroblasts (**A**), EBV-immortalized lymphoblasts (**B**) and whole blood (**C**). The upper tracings are without, the lower tracings with *Hpa*II digestion prior to amplification. Digestion with *Hpa*II prevents the allele present on inactive X chromosomes from being amplified. The alleles appear as clusters of bands; the asterisk marks an artifactual by-product of the amplification reaction. In fibroblasts both alleles can be amplified after digestion, indicating that they are a mixture of cells in which either X chromosome is active. Absence of one allele in the amplified DNA from whole blood indicates clonal hematopoiesis. EBV-immortalized lymphoblasts are also clonal and, as expected, have the same active X chromosome as found in whole blood.

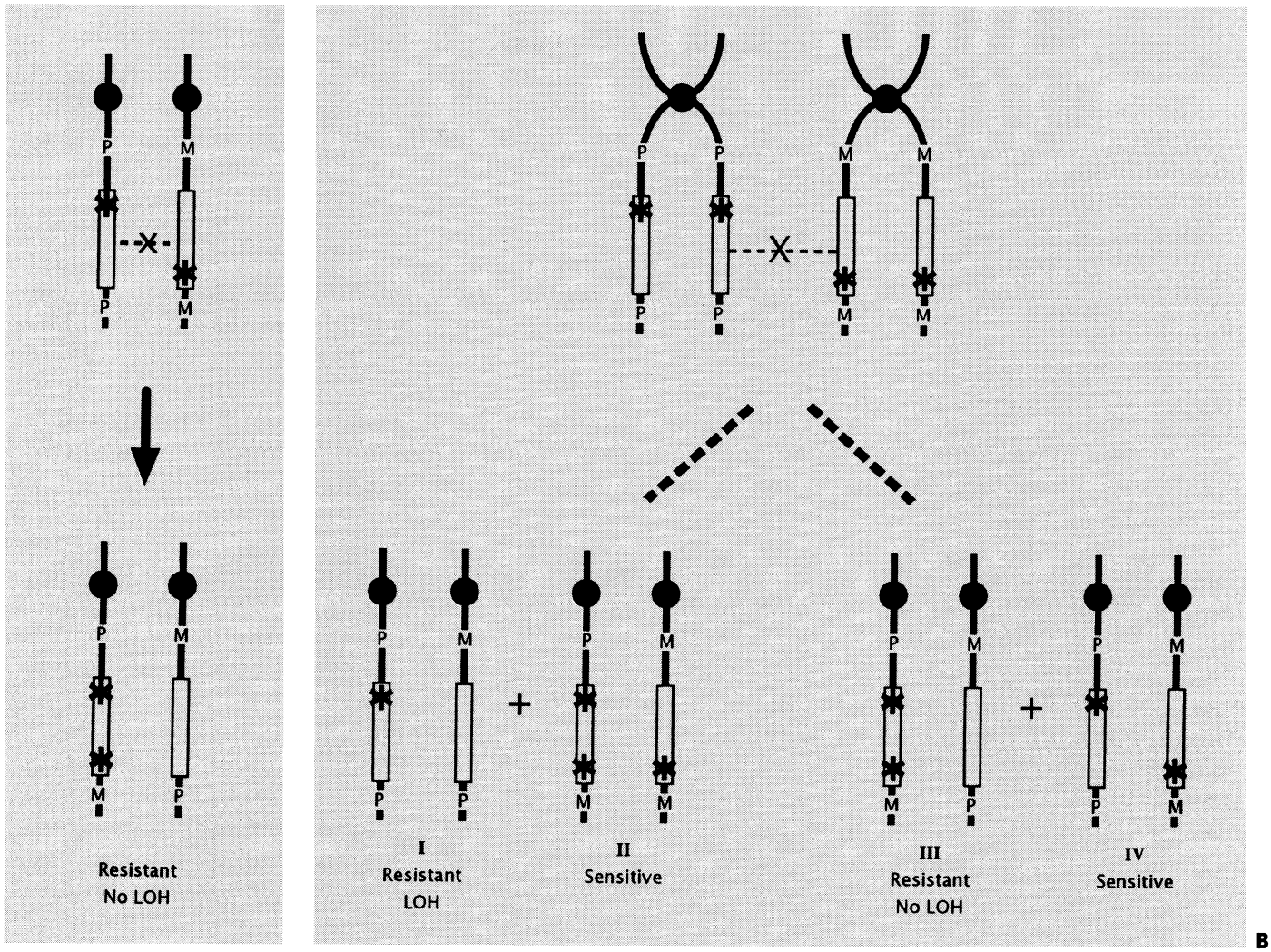

Fig. 3. Cellular genotypes segregating after intragenic somatic crossover, depending on DNA replicative status at the time of recombination. Before (**A**) and after DNA replication (**B**). ● = Centromere; X = site of recombination. The asterisks in the boxed area symbolize the two *FAC* frameshift mutations 1806insA and 322delG in the *FAC* gene. Segregants with a wild-type allele are resistant to MMC. P = Paternal chromosome; M = maternal chromosome. LOH = loss of heterozygosity for markers distal to the *FAC* gene. For revertants with only one mutation (type I segregants) LOH is observed for distal markers. Absence of LOH in EUFA449 and EUFA450 lymphoblasts is indicative of gene conversion (see text).

Genetic Basis of Phenotypic Reversion in FA-C Patients EUFA806, EUFA449 and EUFA450

Patient EUFA806. This patient was described previously [patient ST in 16] as an example of a mosaic FA patient with practically 100% of her lymphocytes behaving like normal control cells. Classification of this patient as FA-C was based on finding mutations in the *FAC* gene. The patient was found to be a compound heterozygote for the exon 1 mutation 322delG [7, 10] and the exon 14 mutation 1806insA [19]. Whereas fibroblasts from the patient were sensitive to MMC, an EBV-immortalized lymphoblastoid cell line derived from the patient was MMC-resistant (table 2); the same level of resistance was observed with DEB and cisplatin. Both mutations were detected in the fibroblasts as well as in the MMC-resistant lymphoblasts. A mitotic crossover between the mutated sites in compound heterozygotes was considered a plausible mechanism to explain the occurrence of phenotypically reverted FA cells (fig. 3). If an intragenic mitotic recombination event between the two *FAC* mutations was

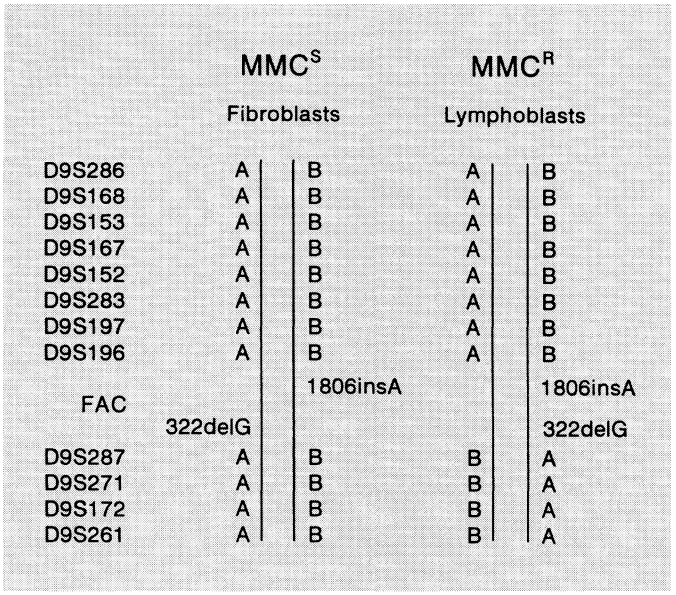

Fig. 4. Haplotypes flanking the *FAC* alleles on chromosome 9 in (MMC-sensitive) fibroblasts and phenotypically reverted (MMC-resistant) lymphoblasts from FA-C patient EUFA806. Association of either mutant allele with its haplotype ('phase') was deduced from the haplotype found in fusion hybrids containing single copies of the human chromosome 9. The haplotype switching in the lymphoblasts indicates that a mitotic recombination event must have been responsible for phenotypic reversion. All markers shown were informative.

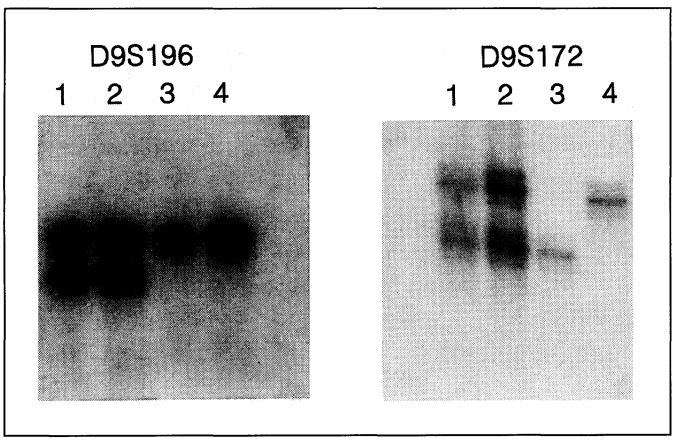

Fig. 5. Association of polymorphic markers flanking *FAC* proximally (D9S196) or distally (D9S172) establishes the orientation of *FAC* towards the centromere (see fig. 4). 1 = MMC-sensitive fibroblasts; 2 = MMC-resistant lymphoblasts; 3 = 322delG-containing chromosome 9 from a fibroblast/rodent hybrid; 4 = recombinant wild-type allele-containing chromosome 9 from a lymphoblast/rodent hybrid. The wild-type allele has the proximal haplotype of the 322delG chromosome and the distal part of the 1806insA chromosome. The exon 14 mutation (1806insA) must therefore be oriented towards the centromere.

responsible for the phenotypic reversion, a wild-type allele should exist in the patient's lymphoblasts. To examine this possibility the two *FAC* alleles had to be studied separately. To this end, we fused the patient's sensitive fibroblasts as well as her resistant lymphoblasts with Chinese hamster cells to produce hybrids, which, due to random chromosome loss, contained single copies of the human chromosome 9. Fusions involving the patient's fibroblasts yielded 20 clones, which were investigated for the presence of the human chromosome 9 by microsatellite typing. Ten of the hybrid clones had retained both copies of chromosome 9, the remaining 10 having only one copy. Remarkably, all of the latter clones apparently had retained the chromosome carrying the 322delG mutation, indicating preferential loss of the chromosome 9 having the 1806insA mutation. Nevertheless, the results are consistent with the two mutations in the fibroblasts residing on different chromosomes. Fusions of the patient's MMC-resistant lymphoblasts with a Chinese hamster cell line resulted in 23 hybrid clones containing two chromosomes 9 and only one clone with a single human chromosome 9. This chromosome, which contained neither the 322delG nor the 1806insA mutation, apparently carried a wild-type *FAC* allele.

If the wild-type allele had indeed originated from a single mitotic crossover, a switch of haplotypes was to be expected at the disease locus. Haplotype analysis using polymorphic CA-repeat markers flanking the *FAC* locus was carried out in the patient's fibroblasts, her MMC-resistant lymphoblasts and the fusion hybrids containing a single chromosome 9. Comparison of the pattern found in fibroblasts with that in the fibroblast/hamster fusion hybrid established which haplotype was associated with each of the two mutations (fig. 4). In the lymphoblast/hamster hybrid the wild-type allele apparently had parts of both haplotypes, i.e. the proximal part of the 322delG haplotype (including markers on the p-arm) and the distal part of the 1806insA haplotype. This result indicated that an intragenic recombination event had generated a wild-type allele in the MMC-resistant lymphoblastoid cell line. These results also permitted the deduction that the 3′ end of the *FAC* gene (containing the 1806insA mutation) must be oriented towards the centromere of chromosome 9 (fig. 5).

Since heterozygous carriers of a FA mutation have a normal (non-FA-like) response to cross-linkers, the intragenic recombination event deduced to have occurred in the patient's hematopoietic system and leading to the production of a wild-type allele appears to provide an adequate explanation for the observed phenotypic reversion to MMC resistance.

Patients EUFA449 and EUFA450. These patients, who are siblings, are compound heterozygotes for mutations in exons 1 and 14, that is 322delG and L554P [7, 10, 18], respectively. These mutations were found in genomic DNA isolated from the patients' peripheral blood samples. EBV-immortalized lymphoblastoid cell lines established from the patients were both found to be MMC resistant. Screening for the mutations in the lymphoblasts revealed that in both cell lines the exon 1 mutation 322delG was not present. If, as in case EUFA806, a single intragenic crossover had been responsible for this result, loss of heterozygosity would be expected for polymorphic markers distal to the gene (fig. 3). However, all distal markers investigated had retained heterozygosity [results not shown]. Therefore, the recombination mechanism of gene conversion has most likely been responsible for the loss of the 322delG mutation [27].

Discussion

Possible Causes of Mosaicism

Theoretically, mosaicism in FA patients might originate from genetic changes at the disease locus or at modifier genes capable of alleviating the disease phenotype. With respect to changes at the disease locus, two types of event may be distinguished. First, during development, an individual cell of a heterozygous carrier of an FA gene mutation may acquire a pathogenic mutation in the second allele causing all descendants from this cell to be affected by FA. Depending on the age at which this happens it will affect many or only a minor proportion of the individual's cells. This may be called 'forward mosaicism'. The second possibility may occur in a homozygously affected patient in whom one or more cells reverse to a heterozygous carrier state by the generation of a wild-type allele at the disease locus ('reverse mosaicism'). Where the FA gene mutation(s) are unknown in a patient, it is not currently possible to determine how the mosaicism originated. Clearly, as shown by our data, in the 3 patients who were found to belong to group FA-C by mutation screening (EUFA806, EUFA449 and EUFA450) the generation of a wild-type allele at the disease locus is most likely to have been responsible for their mosaicism. Forward mosaicism is a formal possibility in the unclassified patients EUFA192 and EUFA631, since these patients were singleton cases and there are no data to confirm that they inherited two mutated FA alleles from their parents. However, all other cases were from multiplex families, which strongly suggests that they originated from homozygously affected zygotes. Ultimate proof for 'reverse mosaicism' in these cases, however, requires the determination of their complementation group, finding mutations in the corresponding gene and confirming the presence of these mutations in the parents.

Detection of Mosaicism in FA Patients

Hypersensitivity to chromosomal breakage by cross-linking agents is the essential criterion to confirm the diagnosis in a patient suspected of having FA [28]. This test is normally carried out with 72 h PHA-stimulated peripheral blood cultures, incubated with and without a cross-linking agent, like DEB or MMC, which are added at a concentration that evokes a significant clastogenic response in FA but not in non-FA individuals. This test readily detects clastogen-resistant cells, when occurring at relatively high levels [15], but is not suitable for detecting low levels of mosaicism (e.g. 20% or less). However, EBV-immortalized cell lines established from low-level mosaic patients may still reveal MMC resistance. Since FA lymphocytes are more difficult to immortalize than non-FA lymphocytes, lymphoblastoid cell lines established from mosaic patients will preferentially derive from the subpopulation of reverted cells, even if reverted cells occur as a minority, i.e. not detectable by chromosomal breakage analysis (e.g. patient EUFA450). In view of the clinical implications that may be associated with the phenomenon of mosaicism in FA, it may be advisable to establish EBV-immortalized lymphoblastoid cell lines from all FA patients routinely, and to test their level of cross-linker sensitivity for possible low-level mosaicism.

Mechanisms of Phenotypic Reversion

Mosaicism due to mitotic recombination has been described in Bloom syndrome (BS), both in vitro [29] and in vivo [30]. Lymphocytes from BS patients typically exhibit much increased levels of sister chromatid exchanges. As in FA, about 20% of BS patients appear to be mosaics, in particular those whose parents do not share a common ancestor and therefore are more likely to be compound heterozygotes. In such patients a minority of lymphocytes are detected with a normal level of sister chromatid exchanges. Ellis et al. [30] found that in 5 out of 11 such mosaic patients loss of heterozygosity had occurred for polymorphic microsatellite markers distal to the gene *BLM*, which is consistent with an expected 50% of this type of segregant (fig. 3B), assuming that recombination occurs after the DNA has been replicated (see below).

The FA-C patients reported here illustrate that intragenic mitotic recombination can also be a cause of mosaicism in compound heterozygote FA patients. Two types of recombination could be inferred from the genotypes of the reverted cells: (1) a single intragenic crossover between the maternally and paternally inherited mutations, associated with haplotype switching for markers located distally to the disease gene (patient EUFA806), and (2) gene conversion, leading to segregants having lost one pathogenic mutation (patients EUFA449 and EUFA450). Both types of recombination are compatible with their occurrence after the DNA having been replicated, as postulated by Ellis et al. [30] for BS; however, they are also compatible with the recombination having taken place in prereplicative DNA (fig. 3A).

The inferred recombination mechanisms require a compound heterozygous genotype in the patients. Ellis et al. [30] found 3 cases with mosaicism among 68 (4%) of their BS patients who were judged to be homozygous by descent at the *BLM* locus, implying that a mechanism other than intragenic somatic recombination must have been responsible for mosaicism in these BS cases. Such cases may represent the involvement of modifier genes that, when altered in a specific way, may be capable of alleviating the cellular disease phenotype. Patient EUFA554, who was born in a consanguineous multiplex family, might be an analogous case in FA. Thus, also in FA mechanisms other than intragenic mitotic recombination are likely to exist that can generate phenotypically reverted cells.

Spontaneous in vivo reversion to normal of an inherited mutation in a patient with adenosine deaminase deficiency was recently reported [31]; however, the molecular mechanism underlying this reversion has not been established.

Likelihood of Reversion and Level of Mosaicism

The phenomenon of phenotypic reversion has also been observed in ataxia telangiectasia (AT), where lymphoblastoid cell lines from some patients appear to have lost their AT phenotype [32]. FA, BS and AT share spontaneous chromosomal instability as a cellular feature, which in AT is associated with a phenotype of hyperrecombination [33]. In FA an additional phenomenon might explain the apparently high proportion of reverted cells in some patients: hematopoietic progenitor cells originating from a reverted stem cell are likely to have a proliferative advantage over poorly proliferating homozygous FA progenitors [25, 26], leading to progressive replacement of the affected progenitor cells. Clonal hematopoiesis in patient EUFA192 and the hematological condition of this patient suggest that MMC-resistant cells are derived from a single reverted pluripotent stem cell and that the probability of reversion in stem cells may be quite low. The discordant course of the disease in the monozygous twin patients EUFA53 (mosaic; no hematological symptoms) and EUFA54 (severe symptoms; transplanted; however, no information available on mosaicism) again suggests a low probability for mosaicism to occur. On the other hand, in the siblings EUFA449 and EUFA450 the same type of genetic reversion (gene conversion) occurred independently, suggesting that the probability of reversion cannot be exceedingly low.

Course of the Disease in Mosaic Patients

The severity of the disease phenotype in FA-C patients has been correlated with the type of mutation, the IVS4+4A→T mutation being associated with a severe and the 322delG mutation with a relatively mild course of the disease [10, 34]. A possible correlation between course of hematological disease and (level of) mosaicism has not been studied systematically. Our limited data suggest a negative correlation between level of mosaicism and severity of hematological symptoms. Most of the patients with clearly demonstrable levels of MMC-resistant T lymphocytes (table 2) had no or only very mild hematological symptoms, in spite of an age range of 9–30 years [table 1; cf. ref. 1].

Clinical Implications

Our data suggest that mosaicism might be associated with a relatively mild hematological course, even though more research would be necessary to substantiate this point. Below, we discuss some considerations that might be relevant for the clinical management of mosaic FA patients in relation to bone marrow transplantation (BMT), gene therapy and leukemia. BMT of FA patients with HLA-identical donors has been increasingly successful after the original conditioning treatment developed for non-FA patients had been attenuated to meet the increased sensitivity of FA patients to cyclophosphamide [12]. In spite of this, BMT fails in over 30% of such transplants. It is conceivable that mosaic patients would do less well in BMT than nonmosaics, since the attenuated ablation protocol may fail to eliminate the phenotypically reverted cells, which may subsequently compromise engraftment.

In FA-C patients undergoing gene therapy, CD34+ cells are transduced ex vivo with a wild-type *FAC* gene-expressing retroviral vector. Reinfusion into the patient is

intended to generate a mosaic bone marrow in which corrected stem cells initially coexist with affected stem cells, but are expected to gradually take over hematopoiesis. Such an approach would, however, create little benefit for a mosaic patient whose bone marrow has already undergone the desired change spontaneously. Clonal hematopoiesis in patient EUFA192 suggests that spontaneous or gene therapeutic correction of a single pluripotent stem cell may be sufficient to restore proficient hematopoiesis.

Implications for Leukemia

FA patients are prone to develop cancer, in particular acute myeloid leukemia, presumably as a result of the chromosomal instability inherent in the disease phenotype [1–3, 35]. Leukemia in FA patients is extremely difficult to manage clinically and is usually fatal. The presence of phenotypically reverted cells in mosaic patients makes this situation even more complicated. The use of cross-linking agents (e.g. cyclophosphamide, cisplatin) for chemotherapeutic treatment should be carefully considered, as such agents will differentially affect the survival of the FA and non-FA cells present in a mosaic patient.

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