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Genetic Reversion in an Acute Myelogenous Leukemia Cell Line from a Fanconi Anemia Patient with Biallelic Mutations in *BRCA2*[1]

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ABSTRACT

A 2-year old boy was diagnosed with Fanconi anemia (FA) and acute myeloid leukemia (AML). A cell line (termed FA-AML1) was established from blast cells obtained after a second relapse after a successful bone marrow transplant. Histochemical and surface marker analysis confirmed that the cells were derived from the myeloid lineage. Cytogenetic analysis revealed multiple chromosomal aberrations, including a ring 7. Stable proliferation of the cultured cells was absolutely dependent on the presence of granulocyte macrophage colony-stimulating factor or interleukin 3. This is the first AML cell line successfully established from a FA patient. Remarkably, FA-AML1 cells appeared to lack the characteristic cellular FA phenotype, *i.e.*, a hypersensitivity to growth inhibition and chromosomal breakage by the cross-linking agent mitomycin C. Genomic DNA from the patient showed biallelic mutations [8415G>T (K2729N)and 8732C>A (S2835STOP)] in the breast cancer susceptibility gene *FANCD1/BRCA2* [N. Howlett *et al.*, Science (Wash. DC), *297:* 606–609, 2002]. In the AML cells, however, the 8732C>A nonsense mutation was changed into a missense mutation by a secondary alteration, 8731T>G, resulting in 2835E, which restored the open-reading frame of the gene and could explain the reverted phenotype of these cells. Loss of the FA phenotype by genetic correction of a FA gene mutation during AML progression may be a common late event in the pathogenesis of AML in FA patients, which may be treatment related. This finding suggests a novel mechanistic principle of tumor progression based on the genetic correction of an early caretaker gene defect.

INTRODUCTION

FA[4] is a rare autosomal recessive disease characterized by multiple congenital abnormalities, progressive bone marrow failure, and predisposition to malignancies, mainly AML and squamous cell carcinoma (1). FA cells are characterized by spontaneous chromosomal breakage, increased accumulation in the G_2 phase of the cell cycle, and increased apoptosis. Furthermore, FA cells are hyperresponsive to the clastogenic and antiproliferative effects of bifunctional alkylating (cross-linking) agents such as MMC and diepoxybutane (which are used for diagnostic chromosomal breakage testing) and cyclophosphamide and Busulfan (used as cytostatics in the treatment of some leukemias). The wide variation of the clinical phenotype of FA patients requires a reliable diagnosis by demonstrating excessive cross-linker-induced chromosomal aberrations or G_2 cell cycle arrest in T lymphocytes or fibroblasts cultured from the patient.

Eight complementation groups have been described in FA (2–4). The genes corresponding to groups A, C, D2, E, F, and G have been cloned (5, 6), whereas the gene defective in groups B and D1 has been identified as *BRCA2* (7). The FA proteins FANCA, FANCC, FANCE, FANCF, and FANCG form a functional multiprotein complex in the nuclear compartment. The nuclear FA protein complex is required for the activation of the FANCD2 protein into a monoubiquitinated isoform, which colocalizes and interacts with BRCA1 in DNA-damage-inducible nuclear foci (8). With the recent identification of *BRCA2* as a FA gene, a picture is emerging of an integrated FA/BRCA nuclear caretaker pathway that protects against the disease features of FA and development of specific malignancies, including AML.

The predisposition of FA patients to malignancies presumably is related to the chromosomal instability feature of the syndrome, but it is unclear why the malignancies mainly involve AML and squamous cell carcinomas. In FA the relative risk of AML, which has been reported to be as high as 15,000× (9), contributes to a strongly reduced average life expectancy, which is currently ~20 years (1).

There is no firm evidence that the distribution of AML subtypes according to the French-American-British classification would be different from that seen in the general population. The most important difference seems to be the age at diagnosis; 64 years in the general population as opposed to 14.8 years in FA patients (10). FA patients with AML have a poor prognosis, with a mean age of death of 15.5 years (9). The reason why treatment of AML in FA patients generally fails is unknown but may relate to specific properties of the leukemic cells and/or to a reduced capacity of the FA patient to tolerate the chemotherapy regimens used. For answering questions related to AML in both FA and non-FA leukemia patients, the availability of stably growing AML cell lines is crucial. A number of cell lines, derived from various French-American-British subclasses of non-FA AML patients, have been described in the literature (11). Here, we report the establishment and partial characterization of the first AML cell line derived from an FA patient.

MATERIALS AND METHODS

Case History. A 2-year-old Japanese boy was referred to the Keio University Hospital Tokyo in June 1996. On admission, he presented with petechiae on the chest and abdomen. Physical examination revealed a short stature (74 cm, <10th percentile), 7.2 kg weight (<3rd percentile), mid-face hypoplasia, Sprengel's deformity, and multiple café-au-lait spots. Initial laboratory examination showed WBC at 16,000/μl with 46% abnormal myeloblasts, Hb at 5.9 g/dl, and platelets at 21,000/μl. He was diagnosed as having an AML (French-American-British classification subtype M2). Cytogenetic analysis of

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[4] The abbreviations used are: FA, Fanconi anemia; AML, acute myeloid leukemia; MMC, mitomycin C; IL, interleukin; GM-CSF, granulocyte macrophage colony-stimulating factor; FA-AML1, FA-derived AML 1; EPO, erythropoietin; TPO, thrombopoietin; M-CSF, macrophage colony-stimulating factor; WST-1, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium, sodium salt; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

the bone marrow demonstrated a number of cell lines with multiple karyotypic alterations, the most consistent being a ring 7(q). FA was suspected from the physical examination and confirmed by a standard cytogenetic chromosomal breakage test using 48 h whole blood cultures and MMC as a cross-linking agent (12). At 60 nM exposure where healthy control cells still have no significant breakage, the patient's lymphocytes had 95% aberrant metaphases. Compared with control children, the clastogenic response in the patient was reported to be 192.7-fold increased (patient AP37P in Ref. 13). The patient was enrolled in a chemotherapy protocol of 100 mg 1-β-D-arabinofuranosylcytosine/m^2 for 7 days and 3 mg of mitoxantrone/m^2 for 5 days. The chemotherapy was not toxic, except for generating a mild mucositis. After complete hematological remission, bone marrow transplantation from his HLA-identical sister was performed August 1996. The preconditioning regimen consisted of cyclophosphamide (40 mg/kg) and anti-thymocyte globulin (10 mg/kg). Cyclosporine and short-term methotrexate were prescribed for prophylaxis of graft *versus* host disease. The regimen was well tolerated without major adverse effects. Myeloid engraftment was achieved at day 9. Fluorescence *in situ* hybridization analysis of sex chromosomes revealed that the blood cells were exclusively from the donor. Although no serious graft *versus* host disease after bone marrow transplantation was observed, relapse occurred in the bone marrow 5 months after the transplant. Reinduction chemotherapy brought a second hematological remission, and donor leukocyte transfusions were attempted twice. However, a second bone marrow relapse occurred in July 1997, and the patient died of leukemia in April 1998.

Establishment of a Stably Growing AML Cell Line. A heparinized peripheral blood sample taken during the second relapse was subjected to culture in February 1998. Mononuclear cells were isolated with Lymphoprep (Nycomed) gradient centrifugation. The cells were washed in PBS and then seeded into 24-well tissue culture flasks (Sumiron MS80240) at 10^6 cells/ml with 2 ml of RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% FCS. Additional supplementation of the medium was with either 10 ng/ml GM-CSF or 10 ng/ml IL-3. Cultures were kept at 37°C under a 5% CO_2-humidified atmosphere. The cells were fed once or twice weekly by partial replacement of spent medium with fresh medium supplemented with GM-CSF or IL-3. Without cytokines, the cells stopped growing in a few days and gradually died. However, continuous growth occurred with GM-CSF or IL-3. These cells could be cryopreserved and successfully recultured after thawing. We designated the cell line FA-AML1. When subjected to routine testing for *Mycoplasma* contamination, FA-AML1 cultures appeared positive for *Mycoplasma fermentans*. Therefore, two more cell lines were established from frozen samples of the original leukemic blasts (FA-AML1A, cultured with GM-CSF, and FA-AML1C, cultured with IL-3), which were found to be free of *Mycoplasma*. Cell line SKNO-1, derived from a non-FA AML (M2) patient [kindly provided by Dr. Noboru Fujinami (SRL, Tokyo, Japan)], was used as a control (14). SKNO-1 cells were cultured in RPMI 1640 with 10% FCS, supplemented with 10 ng/ml GM-CSF. The cell lines used in this study are summarized in Table 1.

Cell Surface Markers. Cell surface antigens were analyzed by immunofluorescence staining with the monoclonal antibodies Leu1, Leu2a, Leu3a, Leu4, Leu9, Leu12, Leu15, Leu16, HLA-DR, LeuM3, CALLA, and HPCA-2 (Becton Dickinson, Mountain View, CA); WM47, WM54, and TUK4 (Dako, Kyoto, Japan); and TP-80 (Nichirei, Tokyo, Japan). The cells were incubated for 30 min at 4°C with the appropriately diluted monoclonal antibodies. After washing, the cells were reincubated with fluorescent-labeled goat antimouse immunoglobulin for 30 min at 4°C, and after a second wash, fluorescence analysis was performed by flow cytometry (FACScan; Becton Dickinson).

Table 1 *Cell lines used in this study*

Cell line	Cell type	Growth factor requirement	FA/non-FA	FA gene affected	Reference
AML					
FA-AML1	AML (M2)	GM-CSF, IL-3	FA	*BRCA2*	7
FA-AML1A[a]		GM-CSF			
FA-AML1C[a]		IL-3			
SKNO-1	AML (M2)	GM-CSF	non-FA	n.a.[b]	14
LCL[c]					
HSC93	B lymphocytes		non-FA	n.a.	
HSC72	B lymphocytes		FA	*FANCA*	27
HSC62	B lymphocytes		FA	*BRCA2*	7

[a] Independently established from the same patient using frozen samples of primary leukemic blasts.
[b] Not applicable.
[c] EBV-immortalized lymphoblastoid cell lines.

Hemopoietic Growth Factors. Recombinant human IL-3, IL-6, GM-CSF, granulocyte colony-stimulating factor, EPO, stem cell factor, and TPO were kindly provided by Kirin Brewery Co. (Tokyo, Japan). IL-4 was a gift of Ono Pharmaceutical Co. (Tokyo, Japan). IFN-γ was a gift of Shionogi Pharmaceutical Co. (Tokyo, Japan). M-CSF was a gift of Morinaga Milk Co. (Kanagawa, Japan).

Growth Stimulation Assays. Cell proliferation in short-term culture was estimated by a modified version of the colorimetric WST-1 assay. Cells cultured with growth factors were washed once and were resuspended in RPMI 1640 with 10% FCS for 24 h. Cells (2×10^4) were incubated with various cytokines in 0.1 ml for 4–6 days in 96-well microculture plates. At the completion of culture, the starting number of the cells were prepared as reference cells. Ten μl of 3.3 mg/ml WST-1 (Dojindo Co., Kumamoto, Japan) dissolved in 0.2 mM 1-methoxy-5-methylphenazinium methylsulfate with 20 mM of HEPES (pH 7.4) was added to each culture well. After 2 h of incubation with WST-1 at 37°C, the absorbance (A) was measured using a microplate reader (model Benchmark; Bio-Rad) at a wavelength of 450 nm with reference wavelength of 655 nm. Percentage of growth was calculated as (sample A)/(reference cells A) $\times$ 100%.

Cytogenetic Analysis. Bone marrow cultures and FA-AML1 cells that had gone through 9, 33, or 60 passages, respectively, were subjected to standard cytogenetic analysis of trypsin-Giemsa-stained metaphase preparations, which were made after a 4-h (37°C) Colcemid treatment. Karyotypes were described according to the International System for Human Cytogenetic Nomenclature (15). Fluorescence *in situ* hybridization with a total chromosome DNA probe for chromosome 7 (Oncor, Inc., Illkirch Cedex, France) and with a probe for the Williams Syndrome region on chromosome 7 [LSI Williams Syndrome (Elastin Gene) Region Probe; Vysis Inc., Downers Grove, IL] was performed according to the manufacturers' instructions.

MMC-induced chromosomal breakage was assessed in FA-AML1A and SKNO-1 cells by inspection of Giemsa-stained metaphase spreads, as described previously (12, 16).

MMC-induced Growth Inhibition. The growth-inhibiting effect of MMC was assessed by growing the cells in the presence of various concentrations of MMC over a period of time that allowed cells without addition of drug to undergo at least three populations doublings (typically 4–8 days), as described previously (17). IC_{50}s are defined as the concentration of MMC causing 50% inhibition of growth.

Mutation Analysis. Mutation screening in *FANCA* was reported previously (13). *FANCC*, *FANCE*, *FANCF*, and *FANCG* were screened for mutations by sequencing fragments amplified from genomic DNA. Mutation screening in *BRCA2* was by denaturing gradient gel electrophoresis, essentially as described previously (18); primers were obtained from Ingeny (Leiden, the Netherlands), and mutations were identified by sequencing of fragments found to be aberrant.

RESULTS

Establishment of a Cell Line from Leukemic Blasts. First, we attempted to grow a cell line from frozen primary leukemic cells obtained just after the patient was diagnosed. However, despite supplementation of the growth medium with GM-CSF or IL-3, the cells failed to grow and gradually differentiated. The attempt was repeated with peripheral blood obtained after the second leukemic relapse, which occurred after successful induction therapy and bone marrow transplantation. The blood sample had a blast count of ~90%. When cultured in RPMI 1640 supplemented with 10% FCS without additional growth factors, the cells ceased to proliferate within 2–3 days. However, with a supplement of 10 ng/ml GM-CSF or IL-3, the cells continued to proliferate for >20 passages and were therefore considered a permanent cell line, termed FA-AML1. After 28 passages, a clone was selected (B2) by limiting dilution of FA-AML1 cells. The clone could grow without feeder cells and clonality was confirmed by

Table 2 *Karyotypic evolution of AML cells in the patient (clinical course) and in vitro (cell lines)*

Clinical course:	
96/06/04 (1y11m): admission to the hospital; diagnosed as FA with AML (FAB-M2).	
5/17	46,XY,r(7q)
8/17	46,XY,r(7q),add(18)(q2?)
1/17	46,XY,r(7q),der(9)t(4;9)(q21;q22),+mar1
1/17	46,XY,r(7q),del(X)(q2?),−4,der(9)t(4;9)(q21;q22),?add(14)(q2?),add(18)(q2?1),+mar1
2/17	46,XY
2y1m: allogenic bone marrow transplantation from HLA-matched sibling (female, non-FA) was performed.	
2y2m:	
20/20	46,XX
97/01/09 (2y6m):	
2/14	46,XY,r(7q),add(8)(p23)
1/14	46,XY,r(7q),add(18)(q23)
2/14	46,XY,r(7q),add(13)(q34),add(18)(q23)
2/14	46,XY,r(7q),t(10;10)(p11.2;q11.2)
4/14	46,XY,r(7q),t(4;9)(q21;q22),add(18)(q23)
3/14	46,XX
97/07/10 (3y):	
1/10	46,XY,r(7q),add(18)(q23)
3/10	46,XY,r(7q),add(13)(q34),add(18)(q23)
2/10	46,XY,r(7q),t(10;10)(p11.2;q11.2),del(16)(q22)
4/10	46,XX
97/11/17 (3y4m):	
7/14	46,XY,r(7q),add(13)(q34),add(18)(q23),+mar1
2/14	46,XY,r(7q),add(13)(q34),add(17)(q23),add(18)(q23),+mar1
1/14	46,XY,r(7q),add(6)(q25),add(13)(q34),−14,add(17)(q23),−18,add(18)(q23),+mar3
1/14	46,XY,r(7q),t(2;3)(q31,q27),t(4;9)(q12;q22),del(16)(q22),add(18)(q23),+mar1
1/14	46,XY,r(7q),t(2;3)(q31,q27),t(4;9)(q12;q22),−14,add(18)(q23),+mar2
1/14	47,X,−Y,r(7q),add(1)(p36),t(2;3)(q31,q27),t(4;9)(q12;q22),add(14)(p11),del(18)(q21),+mar3
1/14	46,XY,r(7q),t(10;10)(p11.2;q11.2),del(16)(q22),+mar1
3y7m: start of cell culture	
3y9m: patient deceased	
Cell lines:	
FA-AML1, p. 9,	
20/20	46,XY,r(7q),dup(1)(p32p13),t(2;3)(q31;q27),t(4;9)(q21;q22),add(12)(p13),t(12;20)(q14;p13),add(15)(q24),dup(17)(q21q23),add(18)(q23)
B2, p. 33,	
20/20	46,XY,r(7q),add(1)(p13),t(2;3)(q31;q27),t(4;9)(q21;q22),add(12)(p13),t(12;20)(q14;p13),add(15)(q24),dup(17)(q21q23),add(18)(q23)
B2, p. 60,	
19/20	46,XY,r(7q),dup(1)(p32p13),t(2;3)(q31;q27),t(4;9)(q21;q22),add(12)(p13),t(12;20)(q14;p13),add(15)(q24),dup(17)(q21q23),add(18)(q23)
1/20	45,X,−Y,r(7q),dup(1)(p32p13),t(2;3)(q31;q27),t(4;9)(q21;q22),add(12)(p13),t(12;20)(q14;p13),add(15)(q24),dup(17)(q21q23),add(18)(q23)

cytogenetic analysis (Table 2). B2 cells expressed both GM-CSF and IL-3 receptors, as analyzed by fluorescence-activated cell sorting, and after 40 passages in medium with GM-CSF, they proliferated equally well with IL-3 (data not shown). FA-AML1 cells were maintained in suspension culture for >24 months (>60 passages), with a population doubling time between 30 and 48 h and were thus considered the first successfully established permanent AML cell line from a FA patient.

Morphology. By phase-contrast microscopy, suspensions of FA-AML1 cells appeared monocellular and homogeneous in size and shape (rounded), although Wright-Giemsa stain revealed a considerable variation in the nuclear cytoplasmic ratio. Nuclei were relatively large and often lobulated, with multiple nucleoli/cell. Myeloperoxidase staining was positive in ~3% of the cells, whereas α-naphthyl butyrate esterase and naphthol AS-D chloroacetate esterase staining were 100% negative. Electron microscopy confirmed that most of the cells had considerable nuclear segmentation with multiple nucleoli. Furthermore, they showed well developed primary granules, mitochondria, and endoplasmic reticulum in the cytoplasm (results not shown). The morphology of the FA-AML1 cells did not seem to be distinct or unique in any particular respect when compared with other leukemic cell lines.

Cytogenetic Studies. On admission, cytogenetic analysis of bone marrow samples revealed 15 of 17 cells to be aberrant by showing a ring chromosome 7 in addition to several other aberrations (Table 2). Ring 7 persisted in all of the samples examined during subsequent follow-up, whereas subsequently, an addition of the long arm of chromosome 18 (add18q23) appeared in most of the clinical samples. These two aberrations in addition to several others were also consistently observed in FA-AML1 cells after 9, 33, and 60 passages in the clonally derived B2 cells (Table 2), indicating that the cell line was cytogenetically rather stable and that it closely resembled the primary leukemic blasts. The independently established cell lines FA-AML1A and FA-AML1C appeared to have a grossly similar karyotype that included ring 7 and several of the marker chromosomes, indicating that they had originated from the same leukemic blast population as FA-AML1. Fluorescence *in situ* hybridization with a whole chromosome probe for chromosome 7 showed that the ring originated from a chromosome 7. With the Williams Syndrome probe set, which hybridizes to bands 7q11 and 7q31, we demonstrated that most of the long arm was present in the ring. The short arm appeared to be missing.

Surface Marker Analysis. Cell surface markers of FA-AML1 cells were analyzed and compared with primary leukemic cells from the bone marrow (Table 3). Both the primary leukemic cells and FA-AML1 cells were positive for CD11b, CD13, CD33, CD34, and CD38, suggesting that the cells were presumably derived from colony-forming unit, granulocyte-macrophage myelomonocytic stem cells. All of the erythroid-, platelet-, and lymphoid-associated antigens were negative. Most of the surface antigens present in the primary cells were also present in the cell line, except for Ia (HLA-DR), which

Table 3 *Surface antigens of primary leukemic cells and the derived cell line FA-AML1*

Circular dichroism	Monoclonal antibody	Antibody specificity	Precultured leukemia cells[a]	FA-AML cells cultured with	
				GM-CSF[a]	IL-3[a]
CD3	Leu 4	Mature T cells	3[a]	<1	<1
CD4	Leu 3a	Helper/inducer T cells	ND[b]	4	5
CD5	Leu 1	Mature T cells/activated B cells	4	<1	<1
CD7	Leu 9	Pan T cells	ND	1	1
CD8	Leu 2a	Killer/suppressor T cells	ND	<1	<1
CD10	CALLA	Common acute lymphocytic leukemia antigen	5	1	1
CD19	Leu 12	Pan B cells	6	<1	<1
CD20	Leu 16	Pan B cells	5	<1	<1
CD41	TP-80	Platelet GP IIb	ND	2	1
Ia (DR)	HLA-DR	Human class II antigen	63	<1	<1
CD11b	Leu 15	Myeloid/natural killer cells	ND	97	99
CD13	WM-47	Myeloid	92	87	95
CD14	Leu M3	Monocytes/macrophages/Langerhans cells	4	22	16
CD33	WM-54	Myeloid/monocytes	33	90	97
CD34	HPCA-2	Hematopoietic cells	90	99	>99
CD38	TÜK4	Hematopoietic cells, activated marker	78	98	99

[a] Numbers indicate percentage positive cells.
[b] ND, not determined.

was strongly positive in the primary cells but absent from the cell line. The adhesion molecule CD11b was strongly positive in FA-AML1 cells but not determined in the primary leukemic sample. These results indicated a myeloid origin of the leukemic cells with a fair degree of preservation of surface antigen expression in the established cell line.

Effect of Hemopoietic Growth Factors. Over a 5-day period, FA-AML1 cells clearly responded to growth stimulation by GM-CSF, IL-3, and stem cell factor in a dose-dependent manner (Fig. 1*A*), whereas in parallel experiments M-CSF, granulocyte colony-stimulating factor, TPO, and TPA failed to support a sustained proliferative response. When tested in a [^{3}H]thymidine incorporation assay over a 24-h time period, both GM-CSF and IL-3 induced a significant incorporation of label, whereas in the same experiment EPO was ineffective (Fig. 1*B*). Very similar responses were noted for the B2 clonal cell line derived from FA-AML1 cells (results not shown). GM-CSF or IL-3 were necessary but not sufficient for supporting the growth in the media used, because without the FCS or human AB serum supplement, the cells failed to proliferate.

To examine the possibility of inducing differentiation in FA-AML1 cells, we tested the differentiation-inducing agents TPA (5 nM), DMSO (1.3%), all-*trans* retinoic acid (1 μM), and 1α,25-dihydroxy vitamin D_3 (100 nM) during a 7-day culturing period in RPMI 1640 with 10% FCS. None of the agents appeared to have a detectable effect on cell number or cellular morphology, except for TPA, which induced a spindle shape in a minority of the cells. In addition, TPA at 5 nM supported a weak proliferative response, comparable with TPO and M-CSF (Fig. 1*A*).

Absence of the FA Phenotype in FA-AML1 Cells. To assess whether FA-AML1 cells expressed the FA phenotype, we determined their sensitivity to growth inhibition by MMC. As shown in Table 4, IC_{50}s for the various sublines of FA-AML1 cells were variable and ranged between 10.1 and 36.5 nM MMC, with an average for all observations of 24.1 nM MMC (n = 14). This value was lower than that for SKNO-1 (non-FA AML) cells but higher than typically observed for EBV-immortalized lymphoblasts from FA patients (Table 4), suggesting that FA-AML1 cells were not as sensitive to MMC as might be expected for FA cells. We then tested their sensitivity to chromosomal breakage by MMC. Table 5 shows the clastogenic effect of MMC in the patient's T lymphocyte cultures, B lymphoblasts, and AML cells. In contrast to the patient's T lymphocytes and B lymphoblasts, which exhibited a FA-like hypersensitivity to MMC, the AML cells responded as normal. We repeated the assay by comparing FA-AML1 cells with SKNO-1 cells and lymphoblasts from an established FA group D1 patient (HSC62), which confirmed that FA-AML1 cells responded as non-FA (Fig. 2). These results indicated that the FA-AML1 cell line did not express an FA phenotype, suggesting that during the development of AML the FA phenotype had reverted to wild type.

Genetic Subtyping. Because stably growing MMC-sensitive cell lines were not available the patient could not be classified by functional complementation assays. Therefore, mutation screening of the known FA genes was carried out. Previous studies had failed to reveal sequence

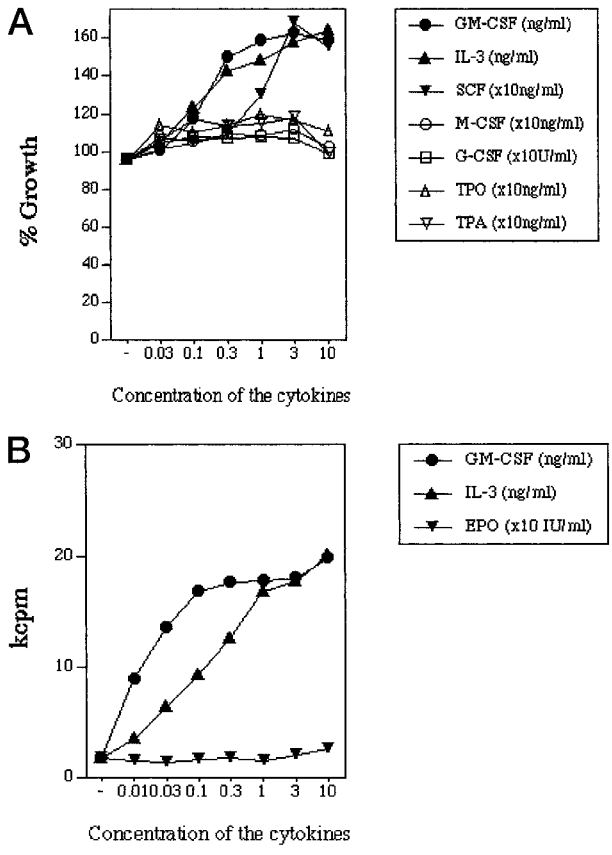

Fig. 1. Growth factor requirement of FA-AML1 cells. *A*, FA-AML1 cells (20,000) were incubated in the presence or absence of cytokine. On day 5, fresh 20,000 were set at 100%. Ten μl of WST-1 were added to each well of a 24-well plate and $A_{650\,nm}$ was measured after 2 h. *B*, FA-AML1 cells (10,000) were cultured for 24 h in the presence of the indicated concentrations of GM-CSFF, IL-3, or EPO. One μCi of [^{3}H]thymidine was added, and incorporation of label was measured after 22 h. Results are expressed as mean cpm from triplicate cultures.

Table 4 *Growth inhibition of cell lines by MMC*[a]

Cell line/growth factor	IC_{50} MMC ± S.D. (range, *n*)
AML cell lines	
FA-AML1/GM-CSF	36.5 ± 5.2 (30–42, *n* = 4)
FA-AML1/IL-3	36 ± 0 (*n* = 1)
FA-AML1A/GM-CSF	10.1 ± 7.9 (3.5–22, *n* = 6)
FA-AML1C/IL-3	31.7 ± 18.8 (10.2–45, *n* = 3)
FA-AML1 (all sublines)	24.1 ± 15.7 (3.5–45, *n* = 14)
SKNO-1	62 ± 22.6 (46–78, *n* = 2)
LCLs	
HSC93 (wild type)	49.7 ± 28.7 (35–80, *n* = 6)
HSC72 (FA-A)	10.9 ± 4.5 (6–18, *n* = 5)
HSC62 (FA-D1)	11.8 ± 2.8 (9.5–15, *n* = 3)

[a] IC_{50}s indicate the concentration of MMC causing 50% inhibition of growth in a standard proliferation assay (17).

alterations in the *FANCA* gene (13). Similarly, we failed to find mutations in *FANCC*, *FANCE*, *FANCF*, and *FANCG* by sequencing of exons amplified from blood cell-derived genomic DNA (results not shown). A search for mutations in *BRCA2*, the gene recently identified to have biallelic mutations in B and D1 patients, revealed two mutations, 8415G>T (K2729N) in exon 18 and 8732C>A (S2835STOP) in exon 20 (7), which were also detected in a parent and a healthy sibling, respectively (Fig. 3, A and B). Thus, these inherited mutations were most likely the underlying cause of the disease in the present FA patient.

Reversion of the cellular FA phenotype to wild type, which apparently occurred in the leukemic cells, has also been reported in lymphocytes from FA patients with somatic mosaicism. In these cases, a spontaneous genetic correction of a mutant FA gene allele, resulting from intragenic mitotic recombination or gene conversion (19, 20) or from secondary sequence alterations *in cis* (21), restored a functionally active gene (21). We therefore examined the mutations in FA-AML1 cells and found that the S2835STOP codon was changed into a coding triplet (2835E, resulting from 8731T>G), thus restoring the open-reading frame of the gene. This finding strongly indicated that the absence of a cellular FA phenotype as observed in FA-AML1 cells was attributable to this corrective sequence alteration within the disease gene.

To determine whether the secondary (correcting) mutation had occurred relatively early in the genesis of the leukemia, we tested frozen peripheral blood leukocytes collected at diagnosis, *i.e.*, before the bone marrow transplant, which had a blast count of 45%. As shown in Fig. 3*B*, the secondary mutation was not detectable in these cells, although the mutation was readily detectable in samples that contained 50% of FA-AML1 DNA, which harbors this mutation. This result indicated that the secondary mutation had occurred during a relatively late stage in the progression of the disease. The possibility that the secondary mutation had originated *in vitro* was considered highly unlikely because the secondary mutation was present in three independently established AML cell lines (FA-AML1, FA-AML1A, and FA-AML1C; see Fig. 3*C*). Interestingly, FA-AML1A cells appeared to be a mixed population of cells that contained either the original mutation or the reverted allele with the secondary mutation. This is in agreement with the relatively low IC_{50}s observed for this cell line (Table 4).

DISCUSSION

We document here the first AML cell line established from a patient with FA, a disorder associated with a high predisposition to this type of leukemia. The critical feature that allowed permanent growth of FA-AML1 cells *in vitro* was an apparently stable response of the cells to the growth factors GM-CSF and IL-3. This characteristic was not observed until the patient was in second relapse, suggesting that the acquisition of additional genetic alterations, including the expression of certain cytokine receptors, may have added to the growth potential of the leukemic cells.

Chromosomal analysis revealed a ring 7 in which the short arm appeared to be missing. This was the sole abnormality in about one-third of the cells analyzed in the first sample, and this primary change persisted in all of the abnormal cells in all samples investigated subsequently. One of the most frequent numerical aberrations myelodysplastic syndrome found in patients with and AML is monosomy 7, which is not specific for M2 or any other subtype. The frequent involvement of chromosome 7 in clonal abnormalities in AML in patients with FA has been summarized in the literature (22). In most FA cases, the whole of one chromosome 7 is absent, but cases in which the short arm of chromosome 7 is missing have also been described [deletion 7p (23); isochromosome 7q (24)].

Given the strong predisposition of FA patients to AML, the question arises as to whether AML cells occurring in FA are in any way different from those occurring in non-FA leukemia patients. The morphological, immunological, or cytogenetical characteristics of FA-AML1 cells have failed to reveal any distinct features. Remarkably, the hypersensitivity to MMC that is characteristic for FA cells was not expressed in the FA-AML1 cells in terms of both drug-induced growth inhibition and chromosomal breakage rates or cell cycle arrest. Absence of the FA trait (G_2 arrest) has been reported in blood samples from 3 of 4 FA patients who presented with overt leukemia (25). In addition, progressive disappearance of initially observed G_2 arrest was observed in a FA patient during development of AML (26). These observations suggest that loss of the FA trait may be a common feature of AML developing in FA patients.

Loss of the cellular FA phenotype is a well-known phenomenon occurring in primary lymphocytes from patients with somatic mosaicism. Such mosaicism occurs in ~20–30% of FA cases and is caused by acquisition of a functional allele at the disease locus because of secondary mutations or mitotic recombination, which apparently renders these cells a proliferative advantage over nonreverted cells (19, 21). We have found

Table 5 *Clastogenic responses to mitomycin C*[a]

Cells	% aberrant cells	No. of aberrations/ aberrant cell
Patient		
T-lymphocytes		
Untreated	43	1.9
+ MMC	95	8.8
Healthy sibling		
T lymphocytes		
Untreated	5	1.0
+ MMC	7	1.0
Patient		
B-lymphoblasts[b]		
Untreated	16	1.6
+ MMC	98	7.6
Healthy sibling		
B lymphoblasts		
Untreated	7	1.0
+ MMC	9	1.3
Patient		
AML-B2		
Untreated	3	1.0
+ MMC	7	1.0
Patient		
FA-AML1/GM-CSF		
Untreated	5	1.0
+ MMC	5	1.0
Patient		
FA-AML1/IL3		
Untreated	6	1.0
+ MMC	6	1.0

[a] Exposure to MMC was at 60 nM for 48 h. Chromatid-type aberrations were included only; results are based on 100 cells scored/culture.

[b] The EBV-immortalized cell line grew only for a limited number of passages and subsequently stopped growing, so that it was not suitable for additional analysis in functional studies.

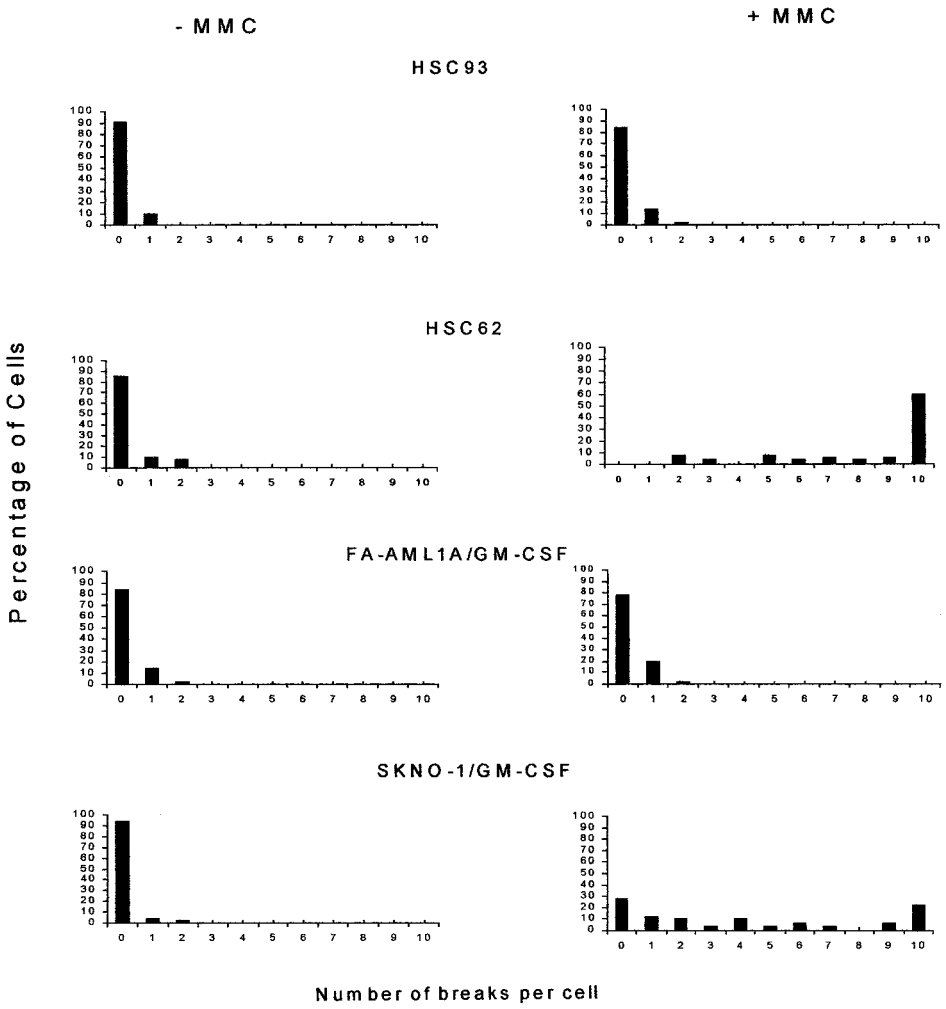

Fig. 2. Clastogenic response of FA-AML1 cells compared with lymphoblasts and non-FA AML cells. Spontaneous (−MMC) and MMC-induced (+MMC at 150 nM during 48 h) chromosomal breakage was determined in FA-AML1A cells, next to SKNO-1 cells (a non-FA AML leukemia cell line of similar classification), and the lymphoblastoid cell lines HSC62 (FA-D1) and HSC93 (wild type). Breakage is expressed as breaks/cell, based on 25 metaphases scored/sample.

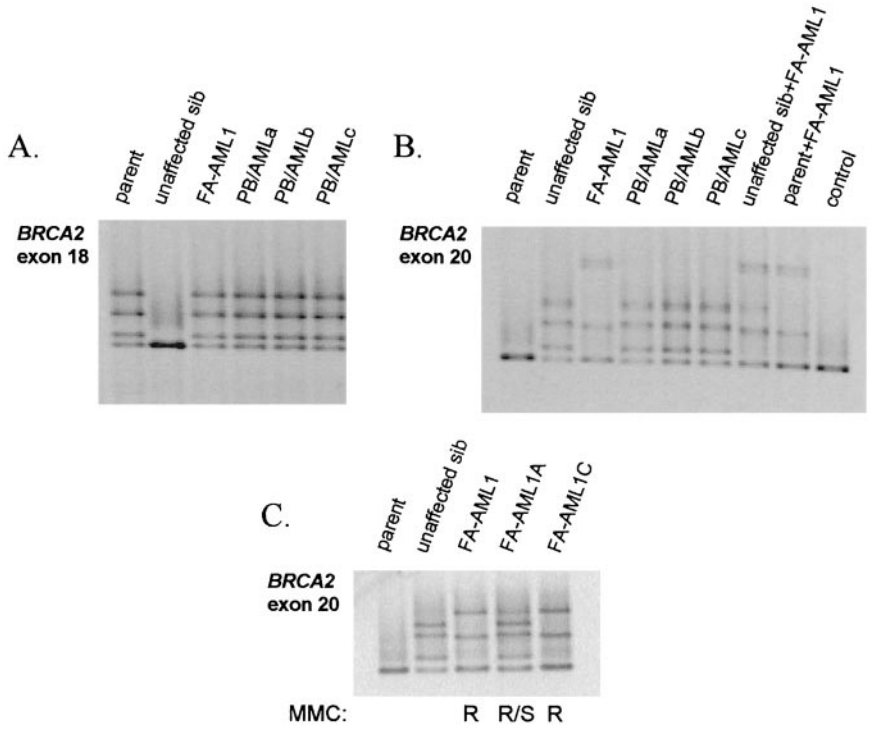

Fig. 3. Mutation detection in exons 18 and 20 of *BRCA2*. Mutations were detected by denaturing gradient electrophoresis in exon 18 (*A*) and exon 20 (*B* and *C*) of *BRCA2*. Exons were amplified from genomic DNA derived from a healthy control, the patient's parent, patient's unaffected sibling, FA-AML1 cell line, and three DNA samples isolated from a peripheral blood sample containing 45% blasts (PB/AMLa-c) taken from the patient before the bone marrow transplant. In addition, genomic DNA from FA-AML1 sample was mixed 1:1 with either DNA from the unaffected sibling or from the parent before PCR amplification. *A*, detection of heterozygous mutation 8415G>T in exon 18 in all samples, except the unaffected sibling and the control. *B*, detection of heterozygous mutation 8732C>A in exon 20 in the unaffected sibling and peripheral blood/AML samples from the patient. Heterozygous pattern associated with the secondary mutation 8731T>G is observed in the FA-AML1 cells only. *C*, detection of heterozygous mutant (8732C>A) and heterozygous-reverted (8731TC>GA) alleles in exon 20. Genomic DNA samples were from the parent, unaffected sibling, and three independently established AML cell lines (FA-AML1, FA-AML1A, and FA-AML1C). In both FA-AML1 and FA-AML1C, only the reverted allele was detected; FA-AML1A has both the mutant and the reverted allele. R, MMC-resistant; R/S, this cell line had an IC_{50} consistent with a mixed population of sensitive and resistant cells.

here that a similar mechanism, functional correction of a mutated *BRCA2* allele by a secondary mutation, accounts for the loss of a cellular FA phenotype in AML cells. Unfortunately, no material was available to determine whether the secondary mutation was present in primary cells. However, the fact that the same secondary mutation was detected in the three independently established AML cell lines strongly suggests that the event had occurred *in vivo*. Because the secondary mutation was not detected in early samples, taken before the bone marrow transplant, this mutational event may represent a relatively late growth-promoting step in the progression of the disease. In addition to providing the leukemic cells with more stable growth characteristics, reversion at the disease locus may be an important contributing factor in the development of drug resistance, particularly against cross-linking agents, in later stages of the disease.

The phenomenon of genetic reversion in leukemia developing in FA patients may be more widespread because flow cytometry of AML-containing blood samples have repeatedly resulted in false-negative FA diagnoses. However, additional leukemic FA patients, preferably with defects in known FA genes, should be studied to test this hypothesis. Because the reverted AML cells in our patient were only detected after several chemotherapy treatments, treatment may have played a role, either by directly generating the mutation *de novo* or by positive selection of reverted cells that preexisted at a low level that escaped detection.

Our findings may have important consequences for the treatment of AML in FA patients where the use of cross-linking cytostatic agents such as cyclophosphamide may be considered a risk factor. Because genetic correction of the FA defect in (pre)leukemic cells in FA patients presumably will promote their growth potential and drug resistance, gene therapy trials intended to correct the marrow failure in FA patients may hold a leukemogenic risk in cases where preexisting (pre)leukemic cells may become targets of the therapeutic vector. Finally, our results highlight a potentially general mechanism of carcinogenesis in which an initial defect in a caretaker gene is spontaneously corrected after the necessary genomic alterations have been accumulated.

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