



Esophageal cancer as initial presentation of Fanconi anemia in patients with a hypomorphic *FANCA* variant

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Abstract Fanconi anemia (FA) is a clinically heterogenous and genetically diverse disease with 22 known complementation groups (FA-A to FA-W), resulting from the inability to repair DNA interstrand cross-links. This rare disorder is characterized by congenital defects, bone marrow failure, and cancer predisposition. *FANCA* is the most commonly mutated gene in FA and a variety of mostly private mutations have been documented, including small and large indels and point and splicing variants. Genotype–phenotype associations in FA are complex, and a relationship between particular *FANCA* variants and the observed cellular phenotype or illness severity remains unclear. In this study, we describe two siblings with compound heterozygous *FANCA* variants (c.3788_3790delTCT and c.4199G > A) who both presented with esophageal squamous cell carcinoma at the age of 51. The proband came to medical attention when he developed pancytopenia after a single cycle of low-dose chemotherapy including platinum-based therapy. Other than a minor thumb abnormality, neither patient had prior findings to suggest FA, including normal blood counts and intact fertility. Patient fibroblasts from both siblings display increased chromosomal breakage and hypersensitivity to interstrand cross-linking agents as seen in typical FA. Based on our functional data demonstrating that the c.4199G > A/p.R1400H variant represents a hypomorphic *FANCA* allele, we conclude that the residual activity of the Fanconi anemia repair pathway accounts for lack of spontaneous bone marrow failure or infertility with the late presentation of malignancy as the initial disease manifestation. This and similar cases of adult-onset esophageal cancer stress the need for chromosome breakage testing in patients with early onset of aerodigestive tract squamous cell carcinomas before platinum-based therapy is initiated.

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INTRODUCTION

Fanconi anemia (FA) is a rare genetic disorder that results in chromosomal instability and is characterized by congenital defects, progressive pancytopenia, and cancer predisposition. FA is the most common genetic bone marrow failure syndrome affecting approximately one in 100,000 births and is caused by germline mutations in one of the 22 known *FANCA* genes (*FANCA-W*) (Wang and Smogorzewska 2015; Niraj et al. 2019). A hallmark of FA is cellular hypersensitivity to DNA-damaging agents, such as diepoxybutane (DEB) and mitomycin C (MMC), because of an inability to remove DNA interstrand cross-links (ICLs) by the Fanconi anemia repair pathway.

Presentation of FA is very heterogeneous. With respect to hematologic function, the majority of patients (90%) exhibit aplastic anemia in the first decade of life by a median age of 7 yr (Rosenberg et al. 2011). Nearly 80% of individuals have at least one or more physical abnormalities, which may include short stature, skeletal defects, microcephaly, microphthalmia, structural defects of the heart or other organ systems, and dermatologic findings such as hypopigmentation and café au lait spots (Fiesco-Roa et al. 2019). Associations with VACTERL birth defects are well reported (Alter and Rosenberg 2013). There is also a high incidence of endocrine dysfunction including growth hormone (GH) deficiency, impaired glucose metabolism, hypothyroidism, hypogonadism, and infertility (Giri et al. 2007; Petryk et al. 2015). Data suggest that both a primary gonadal defect and impairment of the hypothalamus–pituitary axis are responsible for reduced fertility (Petryk et al. 2015).

Individuals with FA are also highly predisposed to malignancies, predominantly acute myelogenous leukemia (AML) and squamous cell carcinomas (SCCs) of the aerodigestive tract. The relative risk for AML for FA patients is increased ~500-fold and the reported cumulative incidence is 13% by 50 yr of age (Rosenberg et al. 2008; Alter et al. 2010; Tamary et al. 2010). They are particularly susceptible to developing tumors of the head and neck, skin, and gastrointestinal and genitourinary tract. In patients with FA, head and neck squamous cell carcinomas (HNSCCs) are the most common solid tumor with a reported 700-fold increased risk. The HNSCC in FA patients often present earlier and more aggressively (Kutler et al. 2003, 2016).

Genotype–phenotype associations in FA are complex. Some of the clinical heterogeneity of the disease can be linked to the genetically distinct subtypes of FA. Biallelic pathogenic variants in *BRCA2/FANCD1* and *PALB2/FANCN* result in a severe subtype of FA with the majority of patients developing embryonal tumors (Wilms, neuroblastoma, medulloblastoma) and AML in early childhood (Howlett et al. 2002; Alter et al. 2007; Xia et al. 2007). However, even within these subtypes, atypical presentations have been reported, suggesting milder phenotypes when some protein functions are preserved (Byrd et al. 2016; Rickman et al. 2020). A study comparing FA-A and FA-G complementation groups found that FA patients with null variants in *FANCA* or pathogenic variants in *FANCG* will manifest with a more severe clinical course, earlier bone marrow failure onset, and hematologic malignancies (Faivre et al. 2000). Similarly, a recent study demonstrated in a cohort of patients with *FANCB* mutations that less severe disease correlated with increased residual *FANCB* function (Jung et al. 2020b).

Moreover, the phenomenon of hematopoietic mosaicism is responsible for some of the varied clinical presentations observed in a small subset of FA patients and can be diagnostically challenging (Rickman et al. 2015; Asur et al. 2018). Hematopoietic mosaicism can result in partial or full rescue of chromosomal breakage levels in the peripheral blood and protect against aplastic anemia through restoration of a functional allele (Gregory et al. 2001; Nicoletti et al. 2020). In suspected cases, cultured fibroblasts are required for definitive diagnosis of FA.

Pathogenic variants in *FANCA*, the most commonly mutated FA gene, are responsible for disease in two-thirds of FA patients (Wang and Smogorzewska 2015; Jung et al. 2020b). The spectrum of pathogenic variants consists of point mutations, small insertions and deletions, splicing mutations, and large deletions (Castella et al. 2011; Flynn et al. 2014; Kimble et al. 2018). The high frequency and diverse spectrum of pathogenic variants in *FANCA* are owed in part to the large size of the gene (79 kb) with an open reading frame of 4.3 kb consisting of 43 exons. *FANCA* is susceptible to *Alu*–*Alu* nonallelic homologous recombination because of a large number of small interspersed nuclear elements (SINE) within the gene locus, accounting for a large number the identified indels (Flynn et al. 2014; Kimble et al. 2018). Many pathogenic *FANCA* variants are private mutations; however, common founder variants have been identified in the South African Afrikaners, Spanish Gypsies, and Tunisian populations (Castella et al. 2011; Flynn et al. 2014).

Previous reports have shown that there is not a clear correlation between specific *FANCA* mutations (i.e., homozygous null variants versus hypomorphic gene product) and the severity of the clinical phenotype with respect to congenital abnormalities and age at which hematologic abnormalities arise (Castella et al. 2011). Hematological progression significantly correlated between siblings harboring the same mutations in a recent study (Jung et al. 2020a). However, siblings may also present with widely dissimilar phenotypes (Faivre et al. 2000), suggesting that other, lesser understood factors, such as environmental exposures or modifying genetic variants, may also play a role in disease manifestation.

Our study further highlights the heterogeneous nature of FA by describing an atypical clinical presentation of two brothers diagnosed with FA in their sixth decade of life. The sibling pair initially presented with esophageal malignancies and were subsequently found to have FA by chromosomal breakage and molecular testing. We show that their atypical and delayed presentation, with lack of hematologic failure, is most likely secondary to the mitigating effects of a rare missense hypomorphic *FANCA* allele, c.4199G > A/p.R1400H.

RESULTS

Clinical Presentation, Family History, and Treatment Outcomes

The subjects of this study are two brothers diagnosed with Fanconi anemia in adulthood (see Table 1 for clinical summary). The proband was a previously healthy man who presented at the age of 51 after a 9-mo history of progressive dysphagia and a 20-pound weight loss and was found to have invasive SCC of the distal esophagus and proximal stomach. Other than male gender and social alcohol consumption, no additional risk factors such as race, tobacco, chronic gastroesophageal reflux, or Barrett's esophagus were present. Chemotherapy was initiated, and after a single cycle of low-dose carboplatin and paclitaxel, the proband developed pancytopenia (WBC $3.3 \times 10^3/\mu\text{L}$, Hgb 9.6 g/dL, platelet $13 \times 10^3/\mu\text{L}$) requiring granulocyte-colony stimulating factor (G-CSF) and platelet transfusion. Because of the unusual hypersensitivity to chemotherapy, the proband was referred to a hematologist for further evaluation.

The proband's physical exam was notable for a single café au lait spot on his left flank and minor thumb anomalies including a small left thumb with flexion contraction of the phalangophalangeal joint and a right thumb that was proximally implanted. Bone marrow biopsy revealed hypoplastic marrow (40% cellularity), decreased myeloid and erythroid precursors, megaloblastoid changes, and an inappropriately low megakaryocyte count, which raised concern for FA. Subsequent chromosomal breakage studies with DEB testing revealed elevated breakage (2.22 breaks/cell at a dose of 0.1 $\mu\text{g}/\text{mL}$). The proband was found to be compound heterozygous for *FANCA* variants, c.3788_3790delTCT and c.4199G > A (Table 2).

Table 1. Summary of patient history, physical exam findings, clinical presentation, and diagnosis

	Proband	Affected sibling
Age at FA diagnosis	51 yr	51 yr
PMH	Hyperlipidemia No hematologic concerns Intact fertility	History of melanoma Hyperlipidemia No hematologic concerns Intact fertility
Physical exam	Minor thumb anomalies Single café au lait spot	Minor thumb anomalies
Clinical presentation	Invasive SCC of the distal esophagus and proximal stomach Severe pancytopenia after initial round of chemotherapy	Invasive well-differentiated distal esophagus SCC (T2 N0) Laryngeal SCC (T2 N0 M0) Invasive moderately differentiated anal SCC
Treatment	Surgical: esophagectomy with gastric pull through Chemotherapy	Surgical: esophagectomy with gastric pull through and local resection for anal cancer Radiation for laryngeal and anal cancer
DEB/MMC testing	Elevated chromosomal breakage upon clinical testing (2.22 breaks/cell at 0.1 µg/mL DEB)	Elevated chromosomal breakage in research testing (4.48 breaks/cell at 50 nM MMC)
Molecular testing	Heterozygous for FANCA variant c.4199G > A Heterozygous for FANCA variant c.3788_3790delTCT	Heterozygous for FANCA variant c.4199G > A Heterozygous for FANCA variant c.3788_3790delTCT
Family history	Father pancreatic cancer (death at 63) Paternal uncle melanoma (death at 72) Paternal grandfather melanoma (death in 70s)	

(FA) Fanconi anemia, (PMH) past medical history, (SCC) squamous cell carcinoma, (DEB) diepoxybutane, (MMC) mitomycin C.

Table 2. Summary of FANCA variants

Gene	Genomic location	HGVS DNA reference	HGVS protein reference	Variant type	Predicted effect	Observed effect	dbSNP/dbVar ID	Parent of origin
FANCA	16q24.3	NG_011706.1: g.80814_80816delTCT NM_000135.4: c.3788_3790delTCT	NP_000126.2: p.F1263del	Deletion	Pathogenic	Pathogenic	397507553/41003	Unknown
FANCA	16q24.3	NG_011706.1: g.82715G > A NM_000135.4: c.4199G > A	NP_000126.2: p.R1400H	Substitution (missense mutation)	Uncertain significance	Likely pathogenic; hypomorphic	149851163/408175	Unknown

Prevention Genetics Fanconi Anemia Nextgen sequencing panel tested full coding regions plus ~20 bases of noncoding DNA flanking each exon of these genes: BRIP1, FANCM, PALB2, FANCL, FANCA, FANCC, FANCG, FANCE, FANCF, FANCB, FANCI, SLX4, ERCC4, FANCD2 isoform A and B, RAD51C.

Further treatment for the proband was largely surgical including a subsequent esophagectomy with gastric pull-through for esophageal SCC. The proband was found to have a mediastinal mass consistent with tumor reoccurrence within 6 mo. Treatment with capecitabine was initiated but was later changed to oxaliplatin and 5-fluorouracil. These agents were ultimately discontinued because of complications of diarrhea and acute pericarditis. The proband developed disseminated esophageal cancer and died at the age of 52.

A year after the proband's diagnosis of FA, his younger brother presented with a well to moderately differentiated SCC of the upper esophagus on screening endoscopy, also at the age of 51. Targeted FANCA testing was performed and he was found to also carry the familial FANCA variants, c.3788_3790delTCT and c.4199G > A. His past medical history was relevant for melanoma of his left upper extremity, which had been resected 2 yr prior. His physical exam was significant for an underdeveloped thenar eminence, but no hematological abnormalities. He underwent esophagectomy with gastric pull-through for the esophageal cancer. Approximately 18 mo later the patient was diagnosed with synchronous primary laryngeal and anal SCC, which were both treated with radiation (5400 cGy to the pelvis and 6900cGy to the laryngeal and hypopharyngeal regions). The patient then developed reoccurrence of the esophageal cancer and died at the age of 55.

As is typical with FANCA etiology, the family history was negative for FA prior to the proband's diagnosis. However, the father died of pancreatic cancer and the extended family history was notable for a paternal uncle and paternal grandfather who both had melanoma. Neither parent was reported to have abnormal cell counts. Both proband and younger brother had healthy biological children.

Identification of FANCA Variants

Clinical testing by Prevention Genetics was performed on peripheral blood from the proband. A next-generation sequencing panel for Fanconi anemia identified the heterozygous FANCA variants c.3788_3790delTCT and c.4199G > A (Table 2). A CGH array analysis of the FANCA gene revealed no evidence of a genomic deletion. The affected sibling was also found heterozygous for these same two variants by clinical targeted gene sequencing of FANCA at Prevention Genetics the following year. CancerNext-Expanded panel (Ambry Genetics) identified no other pathogenic variants in 43 genes associated with hereditary cancer (Supplemental Table S1). The presence of the two FANCA variants was confirmed by research-based Sanger sequencing on blood of the proband (Supplemental Fig. S1) and complementary DNA (cDNA) from fibroblasts derived from both siblings (Supplemental Fig. S2). The c.3788_3790delTCT variant is a known pathogenic variant that produces an in frame deletion in exon 38 (p.F1263del) and has an allele frequency of 0.0001 (ExAC database) (Levrán et al. 2005; Castella et al. 2011). The second variant (c.4199G > A) is a point mutation in exon 42 and is a rare variant (allele frequency of 0.00004, ExAC database) that results in p.R1400H FANCA. It has previously been documented in two FA patients (Ameziane et al. 2008; Kimble et al. 2018). Clinical testing identified the presence of the same FANCA variants in the proband's brother. These findings were confirmed by research-based Sanger sequencing on blood of the proband (Supplemental Fig. S1) and cDNA from fibroblasts derived from both siblings (Supplemental Fig. S2).

Functional Analyses

Cells Derived from the Proband and Brother Display Characteristics Typical of Fanconi Anemia

Consistent with a diagnosis of FA, fibroblasts derived from both brothers (proband RA3518, younger sibling RA3590) show increased chromosomal fragility when treated with DNA interstrand cross-linking agents including DEB and MMC (Fig. 1A,B). Chromosomal breakage

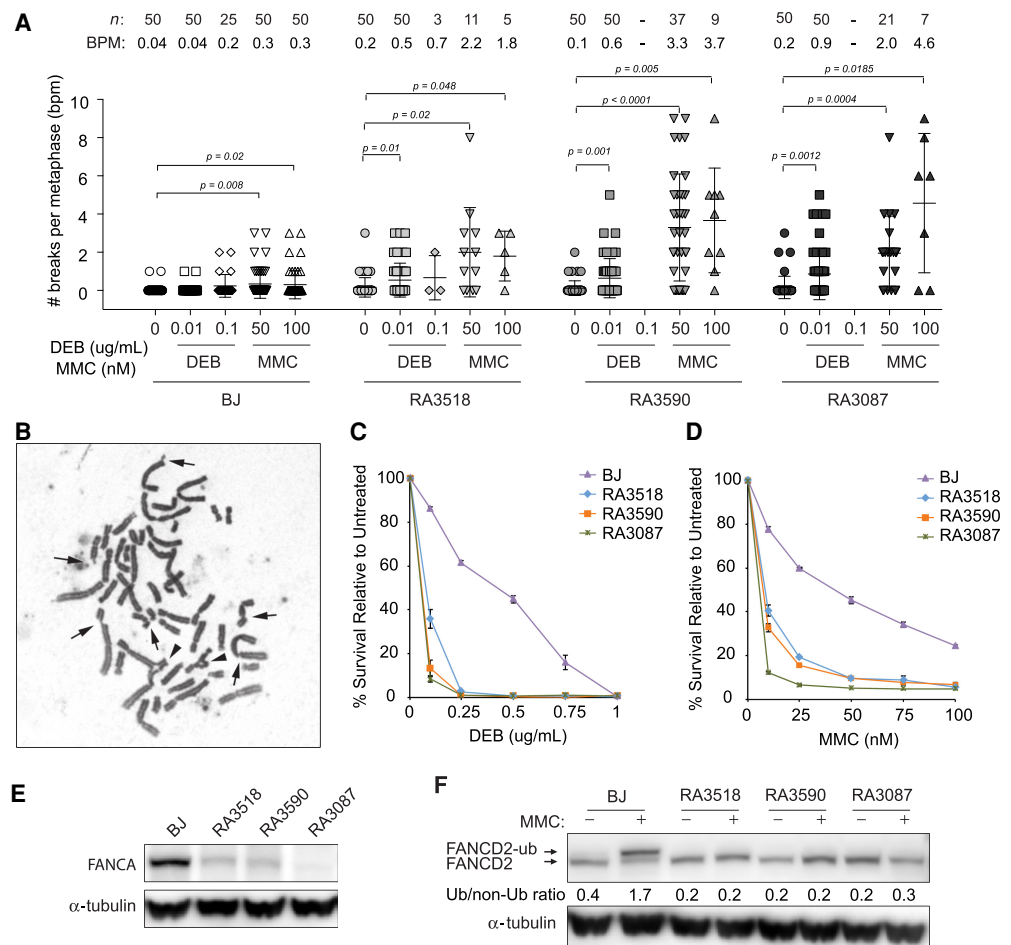


Figure 1. Characterization of fibroblasts from FA siblings. (A) Quantification of chromosome breaks of diepoxybutane (DEB)-treated and MMC-treated primary fibroblasts from proband (RA3518) and affected sibling (RA3590) in comparison to BJ wild-type and FANCA-deficient primary fibroblasts (RA3087). Number of metaphases analyzed (*n*) and breaks per metaphase (BPM) are indicated. Welch's unpaired two-tailed parametric *t*-test was used to determine statistical significance between untreated and treated cells as indicated. (B) Example of a metaphase spread from the proband's primary fibroblasts following 24-h treatment with 50 nM mitomycin C (MMC). Arrows indicate chromosome breaks and the arrowheads highlight radial figures. (C,D) Cellular sensitivity assays of patients' E6E7 transformed/hTERT immortalized (EH) fibroblasts in comparison to BJ wild-type and FANCA-null EH fibroblasts after DEB or MMC treatment. Cells were treated in triplicate with increasing drug concentrations. Cells were then counted after 7 d and normalized to untreated control to determine percent survival. Error bars represent standard deviation. (E) Western blot for endogenous FANCA in untreated BJ, affected individuals (RA3518 and RA3590), and RA3087 FANCA-deficient primary fibroblasts. (F) Western blot assessing FANCD2 monoubiquitination in BJ, affected individuals (RA3518 and RA3590), and RA3087 FANCA-deficient primary fibroblasts. Cells were either untreated or cultured with 1 μ M MMC for 24 h. Slower migrating band represents monoubiquitinated FANCD2. Relative ratio of monoubiquitinated to nonubiquitinated FANCD2 was measured for each variant.

is elevated after DEB and MMC to levels similar to those observed in fibroblasts from typical FA-A patients (RA3087). RA3087 cells are deficient for FANCA because of homozygous deletion at the *FANCA* locus (g.88311999_88394999del83001(hg18), delEx9_43) (Kim et al. 2013). Fibroblasts derived from both siblings also show hypersensitivity to these cross-linking agents in cell survival assays (Fig. 1C,D). Cellular sensitivity of RA3518 and RA3590 fibroblast to DEB-treated cells is similar to that seen in RA3087 FA-A patient fibroblast, whereas

they are slightly less sensitive to MMC. Analysis of FANCA levels by western blot show that fibroblasts from the siblings (RA3518 and RA3590) have lower levels of endogenous FANCA in comparison to wild-type cells (Fig. 1E).

A key step in the FA repair pathway is monoubiquitination of the FANCI and FANCD2 proteins by FANCL, part of the multiprotein core complex composed of eight FA proteins including FANCA. Fibroblasts from the siblings (RA3518 and RA3590) were found to be deficient for FANCD2 monoubiquitination (Fig. 1F). Taken together these findings confirm that both subjects share a classic FA cellular phenotype, have reduced FANCA levels, and have defective FA pathway function.

Cellular Complementation with Wild-Type FANCA Rescues Sensitivity to ICL-Inducing Agents

To demonstrate that FANCA deficiency causes the observed cellular defects, cells derived from the proband (RA3518) were complemented with wild-type FANCA cDNA. Overexpression of wild-type FANCA rescued hypersensitivity and FANCD2 monoubiquitination after treatment with MMC (Fig. 2A,B). Surprisingly, overexpression of FANCA^{c.4199G>A} (p.R1400H) also rescued MMC hypersensitivity and FANCD2 monoubiquitination (Fig. 2A, B). Monoubiquitination of FANCD2 is required for its retention on chromatin and participation in the repair of ICLs, so pathway function can also be assayed by immunofluorescence demonstrating FANCD2 foci formation after DNA damage. Overexpression of wild type and R1400H FANCA in RA3518 cells in immunofluorescence studies also rescued FANCD2 foci formation compared to RA3518 cells expressing an empty vector control (Fig. 2C). However, the overexpressed R1400H FANCA protein was found to have cytoplasmic localization compared to the largely nuclear localization of the wild-type protein (Fig. 2C). Nuclear localization of FANCA is critical for proper function and impairment of this process by pathogenic missense variants has been recognized by multiple investigators (Naf et al. 1998; Castella et al. 2011; Kimble et al. 2018). The proband and sibling carry the c.3788_3790delTCT variant that is predicted to express p.F1263del FANCA, a previously reported and well-studied pathogenic mutant (Adachi et al. 2002). Overexpression of F1263del FANCA does not rescue cellular sensitivity of FA-deficient RA3087 patient fibroblasts or FANCD2 monoubiquitination after treatment with MMC (Fig. 2D–F). These data support a hypothesis that p.R1400H FANCA is a hypomorphic allele and p.F1263del is a loss of function allele. Unexpectedly, FANCA R1400H, when overexpressed, provides enough function to restore resistance to cross-linking agents and rescue FANCD2 ubiquitination to wild-type levels.

Genome Engineered Cells Expressing R1400H FANCA from the Endogenous Locus Have Defective ICL Repair

To further support our hypothesis that the R1400H FANCA is responsible for the milder FA phenotype observed in the siblings, the c.4199G > A missense variant was knocked-in at the endogenous FANCA locus of BJ cells to produce R1400H FANCA expressing cells (Fig. 3A; Supplemental Fig. S3). A biallelic c.4199G > A knock-in created a homozygous state. A carboxy-terminal frameshift allele (p.E1394Gfs*31) was engineered in *trans* with c.4199G > A variant creating a compound heterozygous state. In addition, a cell line with biallelic p.L1256Hfs*11 frameshift (FS) was used as a null FANCA control. BJ cells demonstrated a dose effect with homozygous FANCA^{R1400H/R1400H}-expressing cells having a mild sensitivity to MMC, compound heterozygous FANCA^{R1400H/FS}-expressing cells demonstrating a more moderate sensitivity, and FANCA^{FS/FS}-expressing cells demonstrating sensitivity similar to typical FA-A fibroblasts RA3087 (Fig. 3B,C). Analysis of FANCA levels in BJ fibroblast expressing the FANCA variants by western blot show progressively decreased levels in

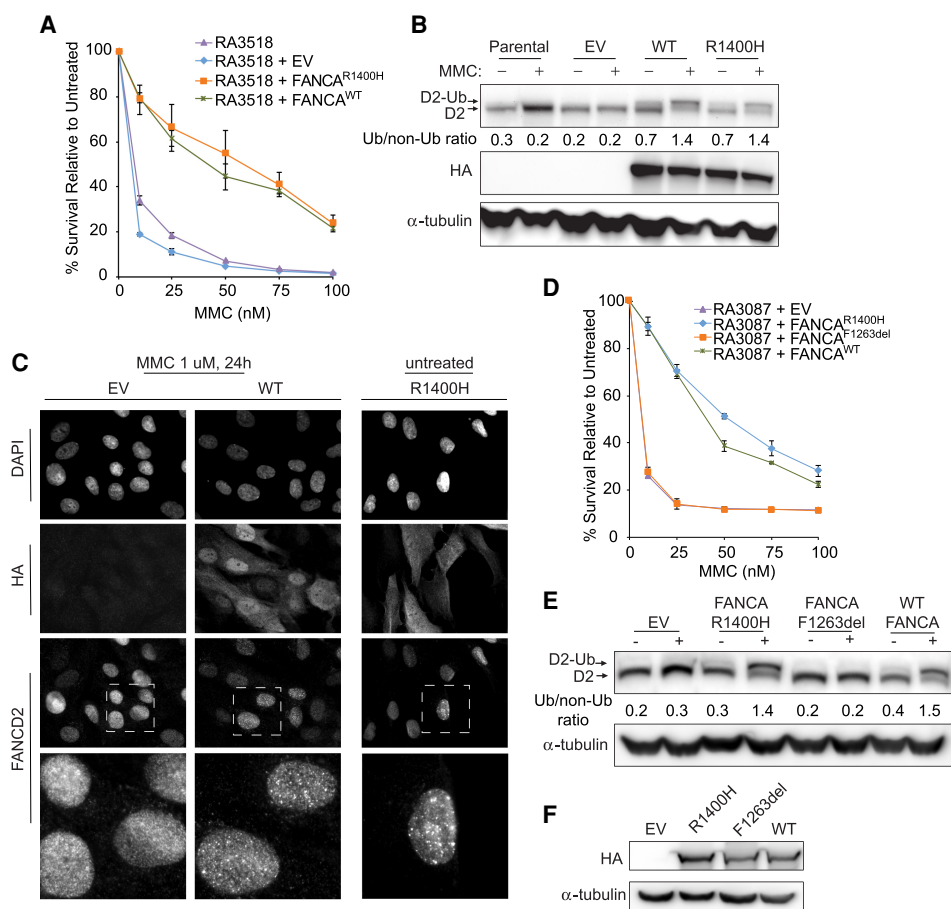


Figure 2. Overexpression of FANCA R1400H rescues hypersensitivity to mitomycin C (MMC), FANCD2 ubiquitination, and localization of FANCD2 to sites of damage. (A) MMC cellular sensitivity assay of proband RA3518 fibroblasts with no vector expression (parental), expressing empty vector (EV), or overexpression of FANCA mutant (FANCA^{R1400H}) or wild-type FANCA (FANCA^{WT}). (B) Western blot assessing FANCD2 mono-ubiquitination and level of HA tagged FANCA expression in parental (no vector) and EV, FANCA^{R1400H}, and FANCA^{WT} expressing RA3518 fibroblasts. Cells were either untreated or cultured with 1 μ M MMC for 24 h. (C) Immunofluorescence images with either anti-HA to detect overexpressed FANCA or anti-FANCD2 to detect FANCD2 localization to chromatin after treatment with 1 μ M MMC for 24 h in RA3518 fibroblasts expressing EV, or overexpression of wild-type FANCA (FANCA^{WT}) or FANCA mutant (FANCA^{R1400H}). (D) MMC cellular sensitivity assay of FANCA-deficient RA3087 fibroblasts either expressing EV, a FANCA mutant (FANCA^{R1400H} or FANCA^{F1263del}), or wild-type FANCA (FANCA^{WT}). (E) Western blot assessing FANCD2 monoubiquitination in FANCA deficient RA3087 fibroblasts overexpressing p.F1263del FANCA. Cells were either untreated or cultured with 1 μ M MMC for 24 h. Relative ratio of monoubiquitinated to nonubiquitinated FANCD2 was measured for each variant. (F) Western blot assessing level of HA-tagged FANCA expression in RA3087 cells.

FANCA^{R1400H/R1400H} and FANCA^{R1400H/FS}-expressing cells and absence of protein in FANCA^{FS/FS}-expressing cells (Fig. 3D). Consistent with the partially cytoplasmic localization of the overexpressed R1400H FANCA protein (Fig. 2C), greater portion of the endogenously expressed R1400H FANCA is present in the cytoplasmic than in the nuclear fraction (Fig. 3E). BJ cells also demonstrated a dose effect with regard to FANCD2 monoubiquitination. A mild decrease in the slower migrating monoubiquitinated FANCD2 band is observed in the homozygous FANCA^{R1400H/R1400H}-expressing cells, a more pronounced decrease in the

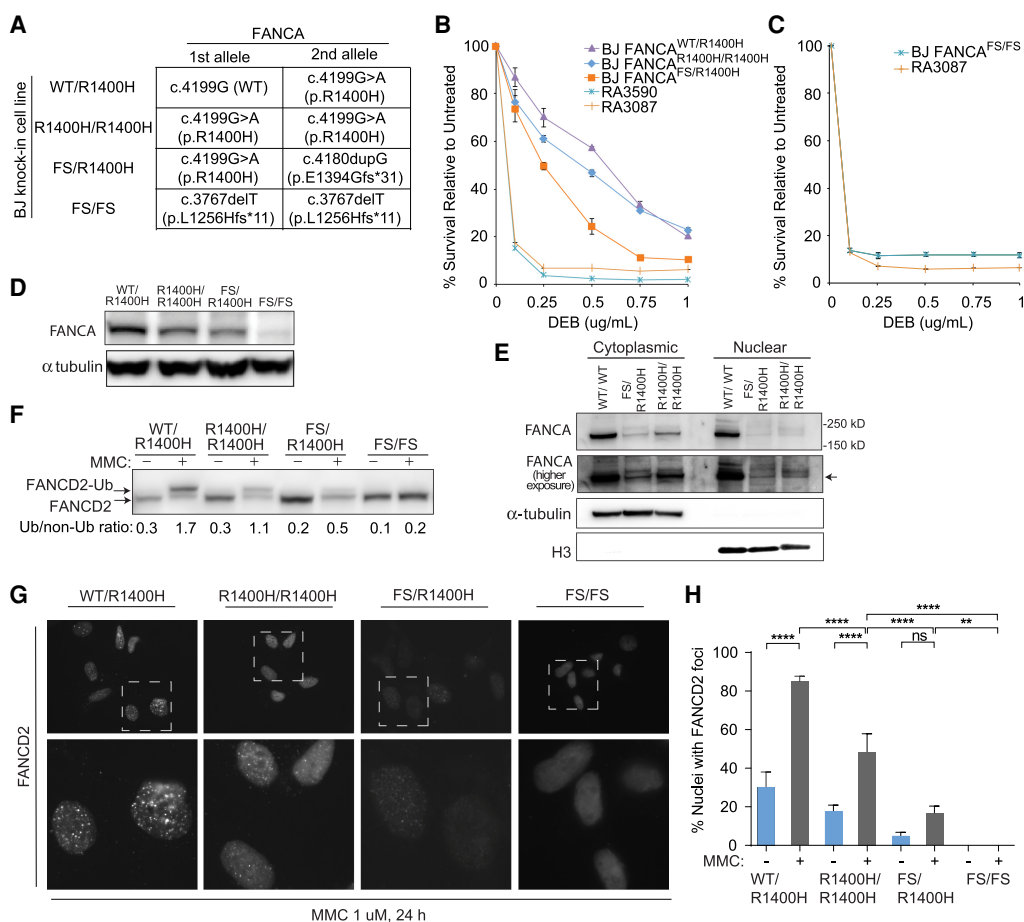


Figure 3. Genome editing of wild-type fibroblasts to express p.R1400H FANCA endogenously results in decreased FANCA levels and impaired FANCD2 ubiquitination and foci formation upon MMC-induced damage. (A) Genotypes and predicted effects of CRISPR-mediated FANCA variant knock-in clones including heterozygous (WT/R1400H), homozygous (R1400H/R1400H), compound heterozygous frameshift (FS/R1400H), and homozygous frameshift (FS/FS) were generated in BJ fibroblasts. (B,C) DEB cellular sensitivity assays showing survival of patient cell lines in comparison to FANCA variant knock-in clones. (D) Western blot assessing endogenous FANCA expression in untreated BJ fibroblast FANCA clones. (E) Western blot assessing endogenous FANCA upon cellular fractionation of untreated FANCA variant knock-in clones. (F) Western blot assessing FANCD2 monoubiquitination in BJ fibroblast FANCA clones. Cells were either untreated or cultured with 1 μ M MMC for 24 h. Relative ratio of monoubiquitinated to nonubiquitinated FANCD2 was measured for each variant. (G) Images of immunofluorescence of FANCD2 foci in BJ fibroblast FANCA clones. Lower panel images are magnified to demonstrate differences in foci formation. Cells were preextracted prior to fixation. (H) Quantification of cells observed to have FANCD2 foci in BJ fibroblast FANCA clones by immunofluorescence. Cells were either untreated or cultured with 1 μ M MMC for 24 h. Statistical significance determined by ordinary one-way ANOVA with multiple comparisons. (****) $P < 0.0001$; (**) $0.0021 < P < 0.0002$.

compound heterozygous FANCA^{R1400H/FS}-expressing cells, and absence in the FANCA^{FS/FS}-expressing cells (Fig. 3F). These results correlated well with the observed defect in FANCD2 foci formation in the BJ fibroblasts expressing the FANCA variants (FANCA^{R1400H/R1400H} < FANCA^{R1400H/FS} < FANCA^{FS/FS}) (Fig. 3G–H). In summary, CRISPR–Cas9 mediated genome engineering of the FANCA c.4199G > A (p.R1400H) variant into an alternate wild-type human fibroblast cell line demonstrates it is a pathogenic hypomorphic allele and that ICL repair is sensitive to the amount of hypomorphic allele available in the cell.

DISCUSSION

Here we have presented a family with two affected brothers both diagnosed with esophageal cancer at the age of 51 yr as their initial presentation of FA. Prior to entering the sixth decade of life, both brothers were relatively healthy. Neither had exhibited any of the characteristic hematologic findings of FA in terms of bone marrow failure or myelodysplasia. Notably, they had very subtle stigmata of FA in the form of minor thumb-related anomalies with otherwise unremarkable physical exams including normal height, weight, and BMI. Additionally, both patients were able to father biological children, in stark contrast to the majority of FA male patients who have hypogonadism and impaired fertility.

This atypical and mild clinical presentation of FA later in life is consistent with the hypothesis that although the p.F1263del allele likely provides no residual FANCA function, the second hypomorphic p.R1400H allele provides partial function. p.R1400H FANCA likely supports enough FA pathway function to protect germ cell development and the bone marrow under non-stress conditions. However, the excessive toxicity experienced by the proband upon exposure to chemotherapy shows that the hematologic compartment remains vulnerable to effects of DNA damage under conditions of stress. Cellular sensitivity assays with DEB and MMC confirm hypersensitivity of both patient-derived cell lines to these agents, which is consistent with classic FA findings.

The c.4199G > A/p.R1400H variant has been previously described in two other individuals with Fanconi anemia, but determining pathogenicity was difficult because, similar to our findings, it was reported that overexpression of the mutant allele resulted in complementation comparable to that obtained with the wild-type cDNA (Ameziane et al. 2008). This suggests that overexpression of the mutant protein is able to compensate for protein instability and/or defects in FANCA protein function. Here the additional findings of the c.4199G > A/p.R1400H variant in another FA family and our functional data support the variant as a most likely pathogenic allele. We observe increased mislocalization of mutant protein to the cytoplasm, decreased FANCD2 ubiquitination and foci formation, and cellular sensitivity to cross-linking agents. Examining the variant by CRISPR-Cas9-mediated genome editing in an alternate cell line was key to demonstrating the defects of the R1400H mutant, which were further exacerbated when present in *trans* to a loss-of-function allele indicating a dose effect of a mutant protein.

Although the proband and sibling did not develop bone marrow failure, a classic presentation of FA, the diagnosis of SCC in both siblings demonstrates that the tumor-suppressor function of the FANCA hypomorphic allele is incomplete. This raises the question of whether there are other unidentified factors, either environmental or genetic, that account for increased susceptibility to malignancy. The underlying pathophysiology of SCC is not well-understood, but it is possible that the threshold for FA pathway function is tissue-specific, with the epithelial mucosa being in need of higher levels of function than the bone marrow stem cells over a long period of time. It is remarkable, although not unusual, for Fanconi anemia patients to develop multiple SCCs in their lifetime. In the case described here, the younger sibling developed three mucosal SCCs in the span of 2 yr, emphasizing the need for the fully functional FA pathway of DNA repair in the protection against cancer.

This study highlights the significance of recognizing hypomorphic variants in cases of adult-onset FA. Subtle clinical findings underscore why clinicians must have a low threshold for testing for FA in unusually young patients with SCC as highlighted here and in patients with other cancers including AML, breast cancer, liver, and cholangiocarcinoma (Huck et al. 2006; Stevens et al. 2016; Engel et al. 2019). Malignancy was a major cause of morbidity and mortality for these patients, and FA-specific treatment regimens need to be selected given the extreme hypersensitivity to chemotherapy.

METHODS

Cell Culture and Viral Transfection/Transduction

Fibroblasts were grown in DMEM supplemented with 15% [v/v] FBS, 100 units of penicillin per milliliter and 0.1 mg of streptomycin per milliliter, nonessential amino acids, and 1× GlutaMAX (Invitrogen). Fibroblasts were incubated at 3% oxygen. Cell lines were immortalized with a catalytic subunit of human telomerase (hTERT) and were transformed by HPV E6 and E7 protein, unless otherwise stated in the text. cDNAs were delivered using lentiviral transduction after packaging in HEK293T cells according to manufacturer's protocol (Mirus) and selected in puromycin.

Sequencing

Sanger sequencing was used to confirm presence of variants identified through clinical studies. The variants were amplified from the patients' genomic DNA by polymerase chain reaction (PCR) and the products were sequenced directly. First-strand cDNA synthesis (Superscript III, Invitrogen), reverse-transcription (RT)-PCR, and Topo cloning (Thermo Fisher Scientific) allowed for the segregation of the variant alleles. PCR and sequencing primers are indicated in Supplemental Table S2.

Chromosomal Breakage and Sensitivity Assays

Analysis of chromosomal breakage was performed following treatment with DNA damaging agents as described previously (Kim et al. 2011). For sensitivity assays, fibroblasts were seeded overnight and treated the following day with DEB and MMC at the indicated concentrations. Cells were grown for 3–4 d, passaged at appropriate ratios, and counted using Z2 Coulter counter (Beckman Coulter) upon reaching 90% confluency.

Cellular Fractionation

Cellular fractions were prepared by suspending cells in fractionation buffer (20 mM HEPES pH 7.4, 2 mM KCl, 2 mM MgCl₂, 1 mM EDTA, 1 mM EGTA) and incubating on ice for 20 min. Following centrifugation, the supernatant was reserved as the cytoplasmic fraction. The remaining nuclear pellet was washed once in fractionation buffer and resuspended in TBS with 0.1% SDS.

Western Blot and Antibodies

Whole-cell lysates were prepared by lysing in Laemmli buffer (Bio-Rad) and sonication. Samples were boiled and separated on 3%–8% gradient gels (Invitrogen) by SDS-PAGE. Immunoblotting was performed using the following antibodies: FANCA (Bethyl Antibody, A301-980A), FANCD2 (Novus NB100-182), H3 (Abcam, ab1791), and HA (Covance MMS-101R).

Immunofluorescence

Cells were fixed with 3.7% formaldehyde, permeabilized with 0.5% NP-40 in PBS, blocked in 5% [v/v] FBS in PBS, and incubated with indicated antibodies (1:1000) in blocking buffer. Cells were incubated with Alexa Fluor secondary antibodies to visualize FANCD2 foci (488) and HA-FLAG tagged plasmid expression (594). The cells were washed and the coverslips were mounted with DAPI Fluoromount-G (SouthernBiotech). When preextraction was used, cells were treated with 0.5% TritonX-100 in PBS for 5 min at room temperature before fixation with 3.7% formaldehyde.

Plasmids

The wild-type (WT) FANCA cDNA was expressed from a CMV promoter and had an amino-terminal HA-FLAG tag. The microdeletion mutant (c.3788_3790delTCT) and the missense mutant (c.4199G>A) were generated with the QuikChange II XL-Site Directed Mutagenesis kit (Agilent Technologies) using a template of WT FANCA cDNA cloned in pENTR plasmid. Mutant constructs were then moved into a lentiviral vector via LR reaction (Gateway system). Sanger gene sequencing verified presence of variants.

CRISPR–Cas9 Genome Editing

Genome editing was performed by electroporation of Cas9/gRNA ribonucleotide complexes as previously described (Rickman et al. 2020). DirectPCR lysis reagent was utilized per manufacturer's protocol to generate lysates from BJ fibroblast clones and used for PCR screening. PCR was performed using Taq DNA polymerase (QIAGEN) per the manufacturer's protocol (for primers see Supplemental Table S2). Amplified PCR products were purified using ExoSAP-IT protocol (ThermoFisher Scientific) and Sanger sequencing was performed to confirm variants.

ADDITIONAL INFORMATION

Data Deposition and Access

Fibroblasts RA3518 and RA3590 are available from the International Fanconi Anemia Registry. The variant c.4199G>A/p.R1400H is in ClinVar (<https://www.ncbi.nlm.nih.gov/clinvar/>) with accession number VCV000408175.3. The c.3788_3790delTCT/ p.F1263del has ClinVar accession number VCV000041003.13.

Ethics Statement

Both subjects were enrolled in the International Fanconi Anemia Registry (IFAR), under a protocol approved by the Institutional Review Board of Rockefeller University. Medical information and cells were collected after obtaining informed written consent from the subjects. Additional medical information about the subjects was obtained with written consent from the next of kin after subjects' passing.

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Author Contributions

F.P.L., S.S., K.A.R., P.D.R., R.J.N., S.C.C., and A.S. designed and performed experiments and interpreted data. A.S. is IFAR's PI. J.A.K. enrolled the family in the study. K.B.H. and M.D.D. provided clinical data pertaining to the subjects. S.S., K.A.R., and A.S. wrote the manuscript with input from other authors.

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Competing Interest Statement

The authors have declared no competing interest.

Referees

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