On the role of FAN1 in Fanconi anemia

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Abstract

Fanconi anemia (FA) is a rare bone-marrow failure disorder with defective DNA interstrand cross link repair. Still there are FA patients without mutations in any of the fifteen genes individually underlying the disease. A candidate protein for those patients, FA nuclease 1 (FAN1), whose gene is located at chromosome 15q13.3, is recruited to stalled replication forks by binding to monoubiquitinated FANCD2, and is required for interstrand cross link repair, suggesting that mutation of FAN1 may cause FA. Here we studied clinical, cellular and genetic features in four patients carrying a homozygous 15q13.3 microdeletion including FAN1 and six additional genes. Biallelic deletion of the entire FAN1 gene was confirmed by failure of 3’- and 5’-PCR amplification. Western blot analysis failed to show FAN1 protein in the patients’ cell lines. Chromosome fragility was normal in all four FAN1-deficient patients while their cells showed mild sensitivity to MMC in terms of cell survival and G2 phase arrest, dissimilar in degree to FA cells. Clinically, there were no symptoms pointing the way to FA. Our results suggest that FAN1 has a minor role in interstrand cross link repair when compared to true FA genes and exclude FAN1 as a novel FA gene.
Introduction

Fanconi anemia (FA) is characterized by chromosome breakage, congenital malformations, pancytopenia and cancer susceptibility\(^1\). FA is a rare disease with a carrier frequency of 1:65–1:209\(^2,3\). FA cells are hypersensitive to DNA interstrand cross linking (ICL) drugs such as mitomycin C (MMC) and diepoxybutane (DEB), and the diagnostics relies on an excess chromosome fragility after \textit{in vitro} exposing patients’ cells to these agents. There are at least 15 independent FA subtypes, each due to mutation of a distinct FA gene\(^4-7\). However, a minority of FA patients remain unassigned suggesting the existence of additional FA genes. Recently, four groups reported that FA nuclease 1 (FAN1) is a good candidate for a novel FA gene\(^8-11\). Reason is that FAN1 is recruited to stalled replication forks by binding to monoubiquitinated FANCD2, and its nuclease activity is required for ICL repair. Transient depletion of FAN1 in human transformed fibroblasts led to increased MMC-induced chromosome breakage rates. Consequently, all four groups suggested that \textit{FAN1} mutations may cause FA\(^8-11\).

\textit{FAN1} maps to 15q13.3. Heterozygous 15q13.3 microdeletion has been associated with a variety of symptoms including mental retardation, epilepsy, psychiatric disease, autism spectrum disorders, muscular hypotonia, and dysmorphic facial features. Penetrance of the microdeletion disorder is variable and encompasses severely affected patients to normal individuals\(^12\). Apart from \textit{FAN1}, six additional genes are located in 15q13.3 (ARHGAP11B, MTMR10, TRPM1, KLF13, OTUD7A and CHRNA7). Here we studied four patients with homozygous 15q13.3 microdeletion\(^12,13\) in order to clarify whether lack of FAN1 may lead to FA.
Methods

Clinical features and blood samples were obtained from four homozygous 15q13.3 microdeletion patients (MD1-MD4) all previously diagnosed by aCGH and qPCR. Two of these patients (MD1 and MD2) have been mentioned before\textsuperscript{12, 13}. Lack of FAN1 was confirmed at the gene level by PCR and at the protein level by Western blotting. The PCR primers used to amplify the 3’ and 5’ flanking regions of FAN1 were ex1F: 5’AGGGTTGTCTCCTCGTTACAGGA3’, ex1R: 5’GCTGAATCACTTTGGCCAGG3’, ex15F: 5’CTTCCTAAAACCTGCTGGAGG3’ and ex15R: 5’AATGTACTGACCGTGTGCTCA3’. PCR, Western Blot analysis, survival assays and chromosome breakage assays were performed as described elsewhere\textsuperscript{3, 14-18}. FAN1-monospecific antibody was kindly provided by Dr. John Rouse (Dundee, UK) and used at 1:500 dilution. 27 genetically unassigned FA cell lines had previously been excluded from belonging to any of the reported 15 FA complementation groups. This study was ethically approved by the Universitat Autònoma de Barcelona Institutional Review Board. Informed consent was obtained from all families in accordance with the Declaration of Helsinki.
Results and discussion

Study of FA candidate genes may enable the final classification of unassigned FA patients. Four recent studies have proposed *FAN1* as a putative FA gene\textsuperscript{8-11}. Here we studied four patients, MD1-MD4, with homozygous 15q13.3 microdeletion to clarify whether FAN1 deficiency leads to features consistent with FA. Two of these patients, MD1 and MD2, have previously been mentioned in unrelated reports\textsuperscript{12, 13} and the other two are newly recognized siblings, detected by aCGH (supplementary Fig S1). Considering that 15q13.3 microdeletion may have three different extensions\textsuperscript{19}, we aimed to corroborate homozygous absence of the *FAN1* gene by failure to PCR amplify its first (exon 1) and last (exon 15) exon from genomic DNA. DNA of two additional lymphoblastoid cell lines (LCL), from a normal individual and from a FANCA-deficient patient, served as controls. As shown in Fig 1A, upper panel, FAN1 PCR products are absent in all MD patients, confirming that all four MD patients have biallelic deletion of the entire *FAN1* gene. Moreover, lack of FAN1 expression was confirmed by FAN1 immunoblotting. Clearly, the FAN1-specific band was missing in the two available LCL from MD patients (MD1 and MD2), while FAN1 was readily detected in the control LCL (Fig 1A, lower panel).

To check whether FAN1 deficiency leads to DEB-induced chromosome fragility\textsuperscript{20, 21}, we performed DEB tests on a LCL from patient MD1 and on blood T cells from patients MD2 to MD4. Chromosome breakage rates were quantified with the recently described chromosome fragility index (CFI)\textsuperscript{17} and the results compared with our historical database\textsuperscript{17}. Clearly, the CFI of all MD patients fell into the range of the non-FA group (Fig 1B). Similar results were obtained with MMC (data not shown).
We next tested the survival of the two available MD LCL in response to MMC. MD1, MD2, a wild type and a FANCA LCL were challenged with 0 to 100 ng/ml of MMC. Based on LD50 values, the MD cell lines showed mild sensitivity to MMC: while FANCA-deficient cells were >30-fold more sensitive to MMC than WT cells, MD1 and MD2 cells were, on an average, 5-fold more sensitive to MMC than WT cells (Fig 1C). Silencing of the FAN1 gene by siRNA was previously shown to impair ICL repair, leading to hypersensitivity of cells to ICL. However, this hypersensitivity was also intermediate when compared with mRNA depletion of authentic FA genes, such as FANCA, FANCD2 or FANCJ. This set of data suggests that the cellular response of FAN1-deficient cells to MMC is not fully functional but not impaired like in FA.

To further study the FA pathway in the absence of FAN1, cell cycle distributions of FAN1-deficient cell lines were analyzed by flow cytometry. Exposure to increasing concentrations of MMC for 72 hours resulted in G2 arrest at low MMC concentrations in FA-A LCL, while G2 arrest was very mild in the MD samples (Fig 1D), compatible with the mild sensitivity to MMC shown before. These results are consistent with a recent report on ΔFAN1-DT40 cells showing that FAN1 protects cells against ICL agents in a pathway which is not epistatic with the FA pathway and that FAN1 assumes in the processing of ICL only a secondary role or functions independently of the FA pathway. We finally analyzed FAN1 protein expression levels in 27 cell line from unassigned FA patients by Western blotting. All of the unassigned FA cell lines expressed FAN1 protein at control levels, suggesting that none of these patients had major deficiency of this protein (supplementary Fig S2).
In order to assess the hematological impact of FAN1 deficiency, we obtained clinical data and hemograms of MD2, MD3 and MD4. Normal hematology had earlier been reported for MD112. As shown in Table 1, MD patients do not present with anemia, bone marrow failure, skin pigmentation anomalies or FA-typical malformations such as skeletal abnormalities of the upper limbs. Three of the MD patients (MD1, MD3 and MD4) showed microsomy and microcephaly which is often seen in FA patients but also in other syndromes with defective processing of stalled replication forks such as Seckle and Bloom syndromes and can be regarded as common symptoms of patients with DNA repair defects24. Yet we cannot conclude for certain that microcephaly and microsomy found in MD patients are caused by FAN1 deficiency since six additional genes are included in the 15q13.3 region. However, it is tempting to speculate that this is the case as FAN1 directly interacts with FANCD2 and 90% of patients with FANCD2 mutations have microcephaly15.

Even though LCL with total FAN1 deficiency reveal mild sensitivity to MMC on some assays, normal expression of FAN1 in 27 unassigned FA cell lines, the lack of DEB- or MMC-induced chromosome fragility and the absence of hematological defects or FA-archetypal malformations exclude FAN1 as being an FA gene.
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Authorship Section

JPT, LBM, MB, RP and BS performed experiments and helped in writing the manuscript. JA and MH provided essential research materials and clinical data and performed experiments. DS designed experiments and provided essential research materials. JS coordinated and supervised the study, designed experiments and wrote the paper with the help of LBM.

The authors declare that there is no conflict of interest.
References


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<th>Patient</th>
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**Figure Legend**

**Figure 1. Absence of FAN1 and FA cellular phenotype in MD patients.** (A) Fragments amplified by PCR corresponding to exons 1 and 15 of the *FAN1* gene were observed using DNA templates from a healthy individual (WT) and a FANCA-deficient FA patient included as controls, whereas they were absent when templates from microdeletion patients (MD1 to MD4) were used, confirming the biallelic deletion of *FAN1* in the MD patients (upper panel). Immunoblotting against FAN1 protein revealed a FAN1-specific band in WT and FA (FA1 and FA2) LCL that failed to be detected in MD1 and MD2. (lower panel). (B) Dot plot of CFI (Chromosome Fragility Index) showing individual values and average (solid line) of DEB-induced chromosome breakage from non-FA (n=56), FA (n=90, excluding mosaics) and MD (n=4) individuals. The CFI values of all MD patients ranged within the non-FA population. (C) Mild sensitivity of MD LCL to MMC on survival assay. The graph shows intermediate sensitivity to MMC of MD1 and MD2 compared with the highly sensitive FA (FANCA) cell line. (D) Near-normal sensitivity of MD LCL to MMC on cell cycle analysis. The graph plots the percentage of cells in G2/M phase after exposure to increasing concentrations of MMC for 72h. A WT and an FANCA cell lines were included as controls.
Figure 1

(A) Western blot analysis of FAN1 and Vinculin proteins in different cell lines.
(B) Box plot showing CFI in different conditions: no FA, FA, and MD.
(C) Graph showing survival of different cell lines in response to MMC (ng/ml).
(D) Bar graph showing the percentage of cells in G2/M phase for different cell lines at different MMC concentrations (nM).
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