Fanconi anemia with sun-sensitivity caused by a Xeroderma pigmentosum-associated missense mutation in XPF

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Abstract

Background: Fanconi anemia (FA) is an inherited genomic instability disorder with congenital and developmental abnormalities, bone marrow failure and predisposition to cancer early in life, and cellular sensitivity to DNA interstrand crosslinks.

Case presentation: A fifty-one-year old female patient, initially diagnosed with FA in childhood on the basis of classic features and increased chromosomal breakage, and remarkable sun-sensitivity is described. She only ever had mild hematological abnormalities and no history of malignancy. To identify and characterise the genetic defect in this lady, who is one of the oldest reported FA patients, we used whole-exome sequencing for identification of causative mutations, and functionally characterized the cellular phenotype. Detection of the novel splice site mutation c.793-2A > G and the previously described missense mutation c.1765C > T (p.Arg589Trp) in XPF/ERCC4/FANCQ assign her as the third individual of complementation group FA-Q. Ectopic expression of wildtype, but not mutant, XPF/ERCC4/FANCQ, in patient-derived fibroblasts rescued cellular resistance to DNA interstrand-crosslinking agents. Patient derived FA-Q cells showed impaired nuclear excision repair capacity. However, mutated XPF/ERCC4/FANCQ protein in our patient’s cells, as in the two other patients with FA-Q, was detectable on chromatin, in contrast to XP-F cells, where missense-mutant protein failed to properly translocate to the nucleus.

Conclusions: Patients with FA characteristics and UV sensitivity should be tested for mutations in XPF/ERCC4/FANCQ. The missense mutation p.Arg589Trp was previously detected in patients diagnosed with Xeroderma pigmentosum or Cockayne syndrome. Hence, phenotypic manifestations associated with this XPF/ERCC4/FANCQ mutation are highly variable.

Keywords: Fanconi anemia, UV sensitivity, XPF, ERCC4, FANCQ, DNA repair

Background

Fanconi anemia (FA) is a rare inherited genomic instability disorder with remarkable clinical and genetic heterogeneity. Whilst variable, typical features include developmental anomalies and malformations, most commonly growth retardation, cutaneous pigment displacement and radial ray defects. FA features also comprise early-onset bone marrow failure and cancer predisposition, specifically for acute myelogenous leukemia and head and neck squamous cell carcinoma [1, 2]. Causative mutations in any one of 22 FA genes (FANCA, -B, -C, -D1, -D2, -E, -F, -G, -I, -J, -L, -M, -N, -O, -P, -Q, -R, -S, -T, -U, -V-and W) have been reported, whose corresponding proteins function together in a replication-dependent DNA interstrand crosslink (ICL) repair pathway [3, 4].

XPF, alias ERCC4 or FANCQ is the catalytic subunit of a heterodimer with ERCC1 that forms a structure-specific DNA repair endonuclease on the SLX4/FANCQ
Peripheral blood lymphoblasts were grown in RPMI 1640 medium supplemented with GlutaMAX (Gibco) and 15% fetal bovine serum (FBS). Primary fibroblasts of the patient concerned were cultured in Amniopan medium (PAN Biotech), while SV40 large T antigen-immortalized fibroblasts were maintained in MEM with GlutaMAX and 10% FBS. For retrovirally transduced fibroblasts 10% Tet System Approved FBS (Clontech Laboratories) was used. All cultures were maintained in incubators with 5% CO₂. Cells were exposed to 40 ng/μl mitomycin C (MMC) for 16 h prior to immunofluorescence analysis of RAD51 foci, immunoblotting or cell fractionation studies. Cell lines from disease and normal controls were maintained as previously reported [9].

**Chromosome studies**

Cytogenetic assays were performed on whole blood cultures to assess spontaneous and MMC-induced breakage rates. MMC at indicated final concentrations was added to blood samples as a G₀ pulse before culture initiation. After 1 h the cells were washed and transferred to fresh complete RPMI 1640 medium. Lymphocytes were stimulated with phytohemagglutinin (PHA). The cultures were incubated at 37 °C and harvested after 72 h. Chromosome preparations were made by the air drying method [12]. Solid (Giemsa)-stained metaphases were examined for chromatid and chromosome type damage, and the results were compared to age and sex-matched normal controls.

**Cell cycle analysis**

PHA-stimulated lymphocytes or primary fibroblasts were cultured untreated for 72 h or continuously exposed to MMC. Mono- or bivariate (BrdU-Hoechst 33,258/Ethidium bromide) cell cycle analysis was performed on a triple-laser-equipped flow cytometer (LSRII, BD Biosciences). Data were analyzed using the MPLUS AV software package (Phoenix Flow Systems) [13].

**Nucleic acid isolation and cDNA synthesis**

A modified salting-out technique [14] or the GeneJET Genomic DNA Purification Kit (Thermo Fisher) were used for isolation of genomic DNA (gDNA). For total RNA isolation we employed the High Pure RNA Isolation Kit (Roche) while transcription into cDNA was performed by SuperScript Reverse Transcriptase (Invitrogen).

**Quantitative PCR**

For relative quantification of mRNA expression levels, allele-specific primers were designed (WT for: 5’-GAC GCAGAGCTAACCTTTGTT T; c.1765C > T MUT for: 5’- GAGCGAGCTAACCTTTGTTT-3’; WT/ MUT rev: 5’- GTTCCTCAGTTGACCTCCTGA-3’).
Fig. 1 (See legend on next page.)
For the detection of exon 5 skipping due to c.793-2A > G primer sequences were as follows: WT for: 5′-TCTGGAATCTCTGAGAGCAACG-3′, WT rev: 5′-AACATCGAGGTGCTGGAGTC-3′, MUT for: 5′-ATAACCCATCGCTTGAAGTGGA-3′, MUT rev: 5′-CAAGAAACAGCCAACCTTGTCA-3′. The PCR reaction was performed with HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis BioDyne) on an ABI ViiaTM 7 System (Applied BiosystemsTM). Evaluation of melting curves and amplification plots, and relative quantification (RQ) was done with the ViiaTM 7 System (Applied BiosystemsTM) using the ΔΔCt method. Each sample was analyzed in technical triplets.

Table 1 Clinical manifestation of affected systems and clinical management in the FA-Q patient

| Affected System                  | Clinical manifestations                                                                 | Management                                  |
|---------------------------------|----------------------------------------------------------------------------------------|---------------------------------------------|
| Growth                          | Low birthweight 2010 g; Short stature; Microcephaly                                       | Pollicizations at age 3 and 4               |
| Skeletal abnormalities           | Right thumb absent; Left thumb hypoplastic; Hip dislocation; Tibia torsion               | Conservative; Internal rotation osteotomy age 25 |
| Gastrointestinal                | Ectopic anus; Fatty liver with persistently elevated liver enzymes                      | Monitoring                                  |
| Urogenital                      | Crossed kidney fusion ectopia, both kidneys on the right                                 | Surveillance of kidney function only        |
| Haematological                  | Thrombocytopenia with macrocytosis; Mild bone marrow hypoplasia without evidence of clonal chromosomal aberrations | Surveillance only                           |
| Endocrine and reproductive health | Diabetes Type 1, age 42; No children; Menopause age 29                                 | Low dose insulin (~0.2 U/kg/ day) Hormone replacement therapy since age 32 |
| Skin                            | Café-au-lait spots; Lifelong sun sensitivity with severe sunburn and blistering after minimal sun exposure | Meticulous sun avoidance and skin protection |
| Hearing                         | Sensorineural hearing loss increasingly relevant age 45                                  | Hearing aid                                 |
| Brain                           | Cerebral and cerebellar atrophy; Small pituitary gland Microangiopathic white matter signal changes |                                             |

Retroviral complementation

Transduction with different retroviral vectors containing XPF/ERCC4/FANCQ cDNA constructs or mock was performed according to standard protocols [15, 16]. Transduced immortalized fibroblasts were analyzed for their sensitivity. Aliquots of 20,000 cells per well were seeded in 6-well culture plates and grown at the indicated concentrations of MMC. After eight days cell viability was determined by image cytometry using
VitaBright-48 and propidium iodide staining on a NucleoCounter NC-250 instrument (ChemoMetec A/S).

**Lymphoblast survival**
LCLs were grown in T25 cell culture flasks at concentrations of 0–1000 nM MMC for eight days. Cell viability was analyzed by propidium iodide exclusion and assayed by flow cytometry.

**Immunofluorescence**
Nuclear RAD51 focus formation was analyzed in fibroblasts grown on glass chamber slides (Sarstedt). Cells were washed with PBS and subsequently fixed in 4% (vol/vol) paraformaldehyde in PBS for 15 min at room temperature. Thereafter they were exposed to ice-cold 100% methanol and kept on ice for 30 min. Blocking of non-specific antibody binding sites was accomplished with 20% (vol/vol) FBS in PBS for 30 min at room temperature. Rabbit anti-RAD51 (1:800; ab63801, Abcam) served as primary antibody, to be detected by Alexa 594-conjugated anti-rabbit secondary antibody (1:2000; A11037, Molecular Probes/Life Technologies). ProLong Gold Antifade Mountant with DAPI (Thermo Fisher) was used for counter-staining and as mounting medium. Foci-positive cells (>5 foci/nucleus) were scored by fluorescence microscopy (Axiovert 40C, Zeiss).

**Immunoblot analysis**
Aliquots of 40 μg whole protein extracts from cultured cells were loaded on NuPAGE Novex 7% Tris-Acetate protein gels (Invitrogen). Electrophoresis was carried out overnight at 70 V with constant cooling. Proteins were transferred using a dry blotting system (iBlot 2, Life Technologies). For XPF, YY1, histone H3 immunodetection we employed Anti-XPF (1:200; ab17798, Abcam), Anti-Tubulin (1:1000; ab44928, Abcam), Anti-YY1 (1:5000; ab199998, Abcam) and Anti-Histone H3 (1:800; ab1791, Abcam) antibodies. Secondary antibodies were as above.

**UV-C survival**
Aliquots of 5000 fibroblasts per well were seeded in 6-well plates. After two days cells were washed with PBS and irradiated at the indicated doses in quadruplicate (0 J/m²) or triplicate (others) using an UV-C germicidal lamp (254 nm; Philips). An UVX Digital Radiometer (Serial No. E27846, UVP) was used to quantify the exact UV dose. Control cells were UV irradiated simultaneously to serve as an internal control. After another five days, before the non-irradiated cultures reached confluency, cells were pulse-labeled with [methyl-³H]-thymidine (40–60 Ci/mmol; 5 μCi/ml; Amersham Biosciences)-containing medium for 3 h, washed with PBS and chased for 15 min in medium without ³H–thymidine. Finally cells were lysed in 0.25 M NaOH. Each lysate was counted with 7.5 ml Hionic Fluor scintillation fluid in a liquid scintillation counter (Packard) for 10 min. The results obtained from
irradiated plates were expressed as percentages of non-irradiated plates (set 100%) and plotted [17].

**UV-C-induced UDS and RRS**

Unscheduled DNA synthesis (UDS) and recovery of RNA synthesis (RRS) experiments after UV-C irradiation were done with mixed cell populations [7]. C5RO wild-type (WT) fibroblasts were incubated and preloaded with polystyrene beads of 2 μm diameter for three days, then mixed with the patient (3104 or 1333) fibroblasts and seeded on coverslips. Two days later adherent cells were washed with PBS and UV-C irradiated at 16 J/m². Thereafter they were incubated for 3 h in medium containing 0.1 μM 5-ethynyl-2′-deoxyuridine (EdU; Invitrogen). Afterwards they were washed with PBS, incubated with medium without EdU for 15 min, washed with PBS, fixed with 3.7% formaldehyde in PBS containing 0.5% Triton for 15 min and washed two times with PBS again. The cells were incubated for 30 min with fluorescent dye-coupling buffer containing 10 mM CuSO₄ and Alexa Fluor 594 azide (Qlick-iTTM; Invitrogen). After washing with PBS, cells were mounted in Vectashield Antifade Mounting Medium with DAPI (Vector Laboratories). For RRS studies cells were treated identically as for UDS experiments, but 16 h after irradiation incubated with culture medium containing 0.1 μM 5-ethynyl-uridine (EU) for 2 h.

Visual light distinguished WT cells that contained beads in their cytoplasm from patient cells that did not. On micrographs DAPI is the blue signal that stains nucleoli. Red is the UDS (or RRS) signal; it is much brighter in WT than in patient cells. UDS and RRS levels are expressed as the average fluorescence intensity in the nucleus of patient vs. WT cells (set at 100%).

**Statistics**

An unpaired two-tailed Student’s t test was used to compare the results of qPCR analyses. A p value less than 0.05 was considered significant; *p < 0.05, **p < 0.01, ***p < 0.001.

**Results**

Chromosome breakage studies from peripheral lymphocyte culture of the patient confirmed FA at ages 7, 33 and 49, and demonstrated increased levels of spontaneous damage, and in response to MMC (Additional file 1: Table S1, results of the analysis carried out age 7 are no longer available). In primary fibroblast cultures a spontaneous and MMC-induced G2-phase arrest was observed (Additional file 1: Figure S1A). Also peripheral blood lymphocyte cultures showed G2-phase accumulation within the FA-range, excluding mosaicism in the hematopoietic system (Additional file 1: Figure S1B). Functional analysis of FANCD2 ubiquitination in 3104 fibroblasts demonstrated both FANCD2 isoforms (Additional file 1: Figure S1C), indicating a mutation in an FA gene downstream of those encoding the FA core- and FANCD2/FANCI-complex. RAD51 foci developed normally after exposure to MMC in both 3104 fibroblasts and lymphoblasts (Additional file 1: Figure S1D), excluding the post-FANCD2 groups FA-D1, -N and -O, -R, -S, and -U. WES identified two mutations in the XPF/ERCC4/FANCQ gene, which were confirmed by Sanger sequencing. A single nucleotide substitution located two base pair upstream of exon 5 (c.793-2A > G) was detected on the maternal allele, affecting a canonical splice acceptor (Fig. 1d). cDNA sequencing verified aberrant splicing. A major splice product revealed exon 5 skipping (Fig. 1e). This event is predicted to result in premature termination of translation (pThr265Valfs*13). The second change in the XPF/ERCC4/FANCQ sequence is the missense mutation c.1765C > T in exon 8 (Fig. 1f) on the paternal allele, which is listed on the ExAC-Browser with a minor allele frequency of 0.0066%. It substitutes a highly conserved amino acid residue (p.Arg589Trp) in the SF2 helicase-like domain. This mutation has previously been reported in two XP-F patients (XP24BR and XP32BR) [18], one patient with XP with neurodegeneration (AS871) [5], and an individual showing an intermediate XP/CS phenotype and features of FA (XPCSICD) [10]. Expression of both mutations in mRNA was re-confirmed by qPCR (Additional file 1: Figures S1E and F). Complementation of 3104 fibroblasts by wildtype, but not Arg589Trp-mutant XPF/ERCC4/FANCQ rescued MMC resistance, providing evidence that the mutations of XPF/ERCC4/FANCQ cause the cellular FA phenotype (Fig. 1g). Premature termination of translation due to exon 5 skipping would result in a truncated protein of 31.4 kDa. However, on XPF/ERCC4/FANCQ immunoblot analysis of 3104 fibroblasts only one low signal intensity band of approximately 100 kDa was detected (Fig. 1h), suggesting the presence of residual mutant XPF/ERCC4/FANCQ protein of normal size but reduced abundance. Dilution studies demonstrated that XPF/ERCC4/FANCQ residual protein with the Arg589Trp mutation is present at approximately 1:15th the level of wildtype XPF/ERCC4/FANCQ protein (Additional file 1: Figure S1G). However, it is difficult to tell the individual contributions of mutant transcript and protein instability to this reduction. Fractionation studies of 3104 fibroblasts showed that residual full-length XPF/ERCC4/FANCQ protein was detectable in the nucleus (Fig. 1i), while in cells from XP-F or XFE patients the mutant XPF/ERCC4/FANCQ appears to be mislocated and not to be part of the XPF-ERCC1 complex, presumably as a consequence of XPF protein misfolding [19].
In addition to ICL repair, NER activity was also impaired in 3104 fibroblasts. Cell survival after UV-C irradiation was reduced (Fig. 2a) (LC_{50} = 2.8 J/m^2), as it was the case in fibroblasts from an XP-F patient with known UV sensitivity and a mild clinical phenotype (LC_{50} = 2.2 J/m^2) [20], and FA-Q 1333 fibroblasts as previously reported [9]. Fibroblasts from the unique XFE patient [8] showed much higher sensitivity (LC_{50} = 1.3 J/m^2). Both global genome NER (GG-NER) and transcription-coupled NER (TC-NER) were affected in cells from patient 3104. Unscheduled DNA synthesis (UDS) and recovery of RNA synthesis (RRS) in 3104 fibroblasts after UV irradiation were more than 80% decreased compared to controls (Fig. 2b-d), to similar levels as for FA1333 fibroblasts was previously reported [9] and is shown here again (Fig. 2c and d). Fibroblasts from an XP-F patient with mild clinical UV-light sensitivity (XP42RO) [7] also exhibited comparable reduction of UDS and RRS rates, whereas fibroblasts from the XPE patient (XP51RO) had even lower activity (Fig. 2c and d). In contrast, 3104 cells were more MMC-sensitive than 1333 cells (Fig. 2e).

Fig. 2 (See legend on next page.)
UV sensitivity can be a feature of FA-Q, and the patient, which was confirmed on the cellular level. Hence, first case who gave a strong history of UV-light sensitivity describe one of the oldest FA patients reported, and the effects in the DNA-damage response network. Here we relations in rare variants of DNA-repair disorders unlock a Long-term observations and genotype/phenotype correlation. Discussion and conclusion

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UV sensitivity can be a feature of FA-Q patients include XPF/FA-Q, and the patient’s perception might be the clinical diagnostic clue for complementation group assignment and mutation detection in unclassified FA patients. From a hematological perspective she has done remarkably well, particularly as she has radial ray malformations which are often associated with early hematological problems [21].

The Arg589Trp mutation observed in individual 3104 appears to be associated with mislocalization of XPF/FANCQ/ERCC4 protein in XP-F cells, where missense-mutant protein failed to properly translocate to the nucleus [19]. Therefore, the DNA repair defect in XP with this mutation is at least in part likely due to mislocalized protein that still interacts with ERCC1, irrespective of its subcellular localization [19]. We performed co-immunoprecipitation experiments using anti-ERCC1 or anti-XPF antibodies. Both ways, we were able to confirm the interaction in 3104 fibroblasts. In contrast to the literature [19], residual mutant XPF/FANCQ/ERCC4 of patient 3104 reported here was detectable with chromatin, as previously described for both other reported FA-Q-associated mutations Leu230Pro and Arg689Ser [9], where an interaction of missense-mutant XPF/FANCQ/ERCC4 proteins with the scaffolding protein SLX4/FANCQ and its dimeric partner ERCC1 was identified. Hence, the loss of mutant protein interactions that is critical for the FA-phenotype in FA-Q patients seems unlikely, as Arg589Trp should have the same effect also in XP-F patients. Additional factors modulating the clinical phenotype, which are relevant for FA-Q patients could include a mechanism allowing a proportion of mutant protein to escape misfolding. It will be important to determine if natural chaperones play a role in rescuing XPF/FANCQ/ERCC4 conformational defects, as in other protein-misfolding diseases, to pave the way clinically from XP or CS to FA [21]. The concept of manipulating XP, CS, or XFE therapeutically by targeting nuclear re-localization of mutant XPF-ERCC1 as previously suggested [19], e.g. by pharmaceutical chaperones, is questioned by our study, as the problem with ICL repair may remain. The reverse, however, targeting interactions or functions of XPF/FANCQ/ERCC4, a key protein at the intersection of DNA repair pathways, could disable NER and ICL repair functions at the same time and might represent a promising approach to overcome chemo-resistance in malignant disease [22].

In the two other reported FA-Q patients FA104 and 1333 both second XPF/FANCQ/ERCC4 mutations are null alleles [9]. In XPCS1CD the missense mutation p.Cys236Arg confers the XPCS phenotype by conveying insufficient NER activity, but fails to provide endonuclease activity for ICL removal, being a functional null allele in this respect [10]. In the FA patient presented here, the second mutation affects a canonical splice acceptor site (c.793-2A > G) resulting in exon 5 skipping, frameshift and predicted protein truncation. We show that the mutant transcript is relatively unstable and can be stabilized with cycloheximide (Additional file 1: Figure S1F). Moreover, we demonstrate that residual abridged XPF/FANCQ/ERCC4 protein is absent (Fig. 1h) such that this splice site mutation can be regarded as a null allele. In addition there is no cryptic splice acceptor nearby that could restore the reading frame. Pathogenicity of the splice mutation is underscored by the fact that there is neither a listed transcript isoform lacking exon 5 sequence nor a reported alternative
Additional file

Additional file 1: Figure S1. and Table S1. (DOCX 4108 kb)

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Availability of data and materials

The material (cell lines, primer sequences) and data generated are available from the corresponding authors on reasonable request.

Web resources

dbSNP: https://www.ncbi.nlm.nih.gov/projects/SNP/
Ensembl Genome Browser: http://www.ensembl.org/
Exome Aggregation Consortium browser: http://exac.broadinstitute.org/
MutationTaster: http://www.mutationtaster.org/
PolyPhen-2: http://genetics.bwh.harvard.edu/pph2/
SIFT: http://sift.jcvi.org/
UCSC: https://genome.ucsc.edu/

Authors’ contributions

IP designed, performed and analyzed experiments, prepared figures and wrote the draft. MP, NT, SS, DGE, KC, KM, AC, CH and LH performed clinical investigations. AR and AFT analyzed UDS, RRS and UV sensitivity. SM contributed to final manuscript preparation. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Institutional Review Board of the Faculty of Medicine at the University of Wurzburg. The patient provided written informed consent to studies and publication of clinical and personal data and photographs without eye bars. A copy of the consent form is available to the editor.

Consent for publication

The patient gave written informed consent to studies and publication of clinical and personal data and photographs without eye bars. A copy of the consent form is available to the editor.

Competing interests

The authors declare that they have no competing interests.

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ERCC4/XPF/FANCQ splicing product that involves skipping of exon 5. Our findings imply that the common basis of all FA-Q cases to date is compound heterozygosity for one null and one hypomorphic missense mutation in XPF/FANCQ/ERCC4, which facilitates the retention of residual mutant protein capable of chromatin relocation and the provision of NER activity [9], possibly providing the genetic basis for long-term survival of certain FA-Q patients.

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