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ORIGINAL ARTICLE

A novel Leu153Ser mutation of the Fanconi anemia *FANCD2* gene is associated with severe chemotherapy toxicity in a pediatric T-cell acute lymphoblastic leukemia

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Fanconi anemia (FA) is an autosomal recessive disease characterized by pancitopenia, congenital malformations, predisposition to cancers and chromosomal instability. We report the clinical and molecular features of a patient initially identified as a potential FA case only because of chemotherapy toxicity during the treatment of a T-lineage acute lymphoblastic leukemia (ALL). Cells from this patient showed a moderate chromosomal instability, increasing sensitivity to DNA cross-linking agents but normal response to ionizing radiation. The analysis of FA proteins demonstrated a marked reduction of FANCD2 (>95%), but normal levels of FANCA or FANCG. Interestingly, this defect was associated with a homozygous missense mutation of *FANCD2*, resulting in a novel amino-acid substitution (Leu153Ser) at residue Leu153, which is highly conserved through evolution. The FANCD2^{L153S} protein, whose reduced expression was not due to impaired transcription, was detected also in its monoubiquitinated form in the nucleus, suggesting that the mutation does not affect post-translation modifications or subcellular localization but rather the stability of FANCD2. Therefore, the hypomorphic Leu153Ser mutation represents the first example of a FANCD2 defect that might promote clonal progression of tumors, such as T-ALL, and severe chemotherapy toxicity in patients without any clinical manifestations typical of FA.
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Introduction

Fanconi anemia (FA) is an autosomal recessive disease characterized by a variety of clinical symptoms, including congenital malformations, progressive bone marrow failure and high predisposition to develop cancers. FA cells show spontaneous chromosomal instability and an elevated sensitivity to crosslinking agents, such as diepoxybutane (DEB) and mitomycin C (MMC).[1]

Although the function of the eleven cloned FA genes is largely unknown, the proteins participate in a common pathway known as FA/BRCA.[1] Briefly, the majority, FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL and FANCM, interact in a nuclear complex required for monoubiquitination of the downstream FANCD2 protein.[2–5] The *FANCL* gene, also a component of the FA complex, is likely to function as a putative E3 ubiquitin ligase for the FANCD2 post-translational modification.[6] Monoubiquitinated FANCD2 is targeted to nuclear foci, where it colocalizes and interacts with several components of the DNA repair machinery, including breast cancer susceptibility 1 (BRCA1), DNA repair protein RAD51 homolog 1 and nibrin (NBS1) of the Nijmegen breakage syndrome.[1] Moreover, biallelic disruption of the *BRCA2* gene also causes FA, as detected in patients of the FA-D1 complementation group.[7] BRCA2, which functions further downstream of the FA pathway, is implicated in DNA repair by homologous recombination directly binding the DNA repair protein RAD51.[8] Even the most recently identified gene *FANCJ*, a DNA-dependent ATPase and 5′-to-3′ DNA helicase that directly binds to BRCA, is not required for FANCD2 monoubiquitination.[9–11]

A critical role of FA proteins in carcinogenesis has been reported in literature. First, FA patients are highly susceptible to hematological malignancies (especially acute myelogenous leukemia (AML)) and solid tumors of the head and neck, gynecological system and other organs.[12] Second, FANCD2-deficient mice have an increased incidence of epithelial cancers (i.e. breast, ovarian and liver tumors).[13] Finally, defects of the FA genes may contribute to cancer progression. For instance, in 18% of primary ovarian epithelial cancers, the *FANCF* gene is methylated and silenced.[14] Germline *FANCC* or *FANCG* mutations have also been found in patients with inherited pancreatic cancers.[15] Moreover, acquired dysfunction of FANCA may promote cytogenetic instability and clonal progression in patients with AML.[16–18]

Acute leukemias, mostly AML and rarely acute lymphoblastic leukemia (ALL), and myelodisplastic syndromes may follow clinically overt aplastic anemia or develop without a detectable antecedent.[19] Moreover, patients can be clinically suspected to be affected by FA – even failing the typical malformations – when they manifest an inexplicable marked sensitivity to chemotherapeutic agents with devastating and unpredictable clinical consequences that can affect both short- and long-term prognosis.[13,20]

In this paper, we report the case of a patient affected by ALL without the clinical features specific for FA. Because of severe chemotherapy toxicity, occurring with several episodes of marrow aplasia leading to life-threatening infections, a suspicion of FA diagnosis led us to perform the DEB test, which was

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found to be positive. We tested the cells for the presence of FA proteins. Although the non-monoubiquitinated and monoubiquinated FANCD2 isoforms were detected, they were both markedly reduced. A homozygous novel missense mutation, Leu153Ser, was identified, suggesting, for the first time, that a *FANCD2* mutation might lead to leukemia progression.

Patient and methods

Patient clinical history

In February 2002, a 9-year-old boy was admitted in the Hematology Unit in poor clinical conditions. His parents were first cousins, and familiar history was positive for cancers (mother with a parotid cancer and a paternal uncle died of pheocromocitoma). He had a 3-month history of laterocervical lymphonode enlargement and fever unresponsive to several antibiotic treatments. White blood cell's count was 6100/μl (80% lymphoblasts), hemoglobin was 13.1 g/dl and platelet count was 243.000/μl. A bone marrow aspirate with immunophenotype was performed and a diagnosis of T-ALL with coexpression of CD13 and CD33 markers was made. Induction chemotherapy protocol, including vincristine, anthraciclines, steroids, L-asparaginase and cyclophosphamide, was administered. The patient achieved a complete remission but the clinical outcome was characterized by extremely severe chemotherapy toxicity, with several episodes of post-chemotherapy pancytopenia, leading to life-threatening infections. Although there was no evidence of congenital malformations or aplastic anemia before the onset of T-ALL, we performed a DEB test that was positive. Consequently, a therapeutic protocol was tailored in order to maintain remission without lethal complications, whereas a search for related or unrelated bone marrow donor was activated.

Cell cultures and chromosome breakage analysis

A lymphoblastoid cell line named LFB was established by Epstein–Barr virus transformation of the patient's blood mononuclear cells. After 2 weeks, the immortalized cells were grown in 25 cm^2 tissue culture flasks in the RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA, Life Technologies, MD, USA) supplemented with 20% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in an atmosphere of 5% CO_2 in a humidified incubator.

Both peripheral lymphocytes and lymphoblastoid cells were used to test chromosomal instability. Primary lymphocytes (1×10^7) were cultured for 2 days with 2 μg/ml of phytohemagglutinin (PHA), a treatment also required for the expression of the FANCD2, FANCA and FANCG proteins in these cells.[21] The DEB test was performed as reported previously,[22] using two different concentrations of DEB (0.01 and 0.02 μg/ml). From each cell culture, 100 metaphases were analyzed by Giemsa standard staining method in order to verify the frequency of chromosome breakages, rearrangements and endoreduplications.

Cell fractionation and Western blot analysis

The cells were harvested when semiconfluent. Whole-cell extracts were obtained as reported.[23] Nuclear and cytosol extracts were prepared using Ne-Per nuclear and cytoplasmic extraction reagents following the manufacturer's instructions (Pierce Biotechnology, Rockford, IL, USA).

For the immunoblotting experiments, pellets were resuspended at 0.5–1 $\times 10^8$ cells/ml density in the lysis buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1% Triton X100, 0.57 mM phenylmethylsulfonyl fluoride, 0.27 mM *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 21 mM leupeptin, 0.83 mg/ml chymostatin, 10 g/ml soybean trypsin inhibitor, 0.5 mM dithiothreitol, 16 mM *p*-nitrophenyl phosphate, 1 mM Na_3VO_4, 40 mM NaF and 1 mM sodium pyrophosphate) and incubated for 1 h at 4°C. Cellular extracts were then centrifuged at 15 000 *g* for 20 min and the supernatants were harvested and stored at −80°C if not used immediately. Protein concentration was determined using the 'Protein assay kit' by Bio-Rad Laboratories (Richmond, CA, USA). Immunoblotting analyses were performed as reported previously.[23,24] Total protein extracts (5–80 μg) were separated by vertical electrophoresis on denaturing polyacrylamide gel (sodium dodecylsulfate/polyacrylamide gel electrophoresis).[25] After electrophoresis, proteins were electroblotted on a nitrocellulose membrane (Hybond-C extra, Amersham Life Science) in a Trans-Blot system apparatus (Bio-Rad) for 4 h at 4°C, using as buffer, 20 mM Tris-HCl, pH 8.3, 0.19 M glycine, 20% methanol.[26] The nitrocellulose membrane was incubated initially with 2.5% non-fat dry milk (Santa Cruz Inc., Santa Cruz, CA, USA) solution as blocking agent and, successively, with the specific antibodies against FANCA, FANCG and FANCD2 (diluted in 1 mg/ml bovine serum albumin solution) for 2 h at room temperature.[27] After incubation with secondary anti-rabbit horse radish peroxidase-conjugated antibodies, the immunoreactive bands were visualized by Enhanced ChemiLuminescence detection system (Amersham Life Science).

Screening for FANCD2 *mutations*

Total RNA was prepared from LFBs using a standard protocol with TRIzol Reagent (Invitrogen). One microgram of RNA was retrotranscribed with random hexamers using SuperScriptTM First Strand System for reverse transcriptase-polymerase chain reaction (RT-PCR) (Invitrogen) in a volume of 20 μl. One microliter of the cDNA reaction was amplified in 50 μl, containing 1 U of PFU DNA polymerase (Promega, Madison, WI, USA), 5 μl of 10 × buffer with $MgSO_4$, and 10 pmol of the following five pairs of oligonucleotides: D2F1 (5′-GTGCACAA GACATTGGTC-3′) and D2R1 (5′-AGCGGTCAGAGCTGTATT -3′), D2F2 (5′-GAGCTTCGGGAGAAGTTG-3′) and D_2R2 (5′-CATGAAAAGCTGGATCCA-3′), D2F3 (5′-AGTTTGACCCAA GAGAGA-3′) and D2R3 (5′-CTTGTGACGAAGTTCATC-3′), D2F4 (5′-AGAGGCCAGCTAAACAAG-3′) and D2R4 (5′-ATT-GAGCCTGGCACAGCA-3′), D2F5 (5′-GAGGAGATTGCTGGT GTT-3′) and D2R5 (5′-GACTCTGATTAGACCCCA-3′). Initial denaturation was for 3 min at 94°C, followed by amplification for 30 cycles, each with denaturation for 30 s at 94°C, annealing for 30 s at the appropriate temperature, and extension for 1 min at 72°C. PCR products were purified and used for direct sequencing (Big Dye Terminator Cycle Sequencing Kit; Applied Biosystems Inc., Foster City, CA, USA). The electrophoresis of cycle-sequencing products was carried out in an ABI3100 automated sequencer and data were analyzed using a specific ABI sequencing analysis software (Applied Biosystems Inc.).

To confirm the mutation at the DNA level, genomic DNA was amplified using the following pairs of primers: D2mutF (5′-CCAAGTAGCTGGGATTATAC-3′) and D2mutR (5′-TATTCTG GCAACTTCTCACAT-3′). In the D2mutR primer, the underlined C nucleotide substitutes the wild-type A nucleotide in order to create a restriction enzyme site for *Nla*III in the presence of the 458T > C substitution. PCR was performed as described above in 50 μl, containing 50 ng of genomic DNA, 10 pmol of each primer, 4 μl of dNTP 2.5 mM, 2 U of Taq polymerase and 5 μl of

10 × buffer (Applied Biosystems Inc.). The PCR products from the patient's DNA and 100 healthy controls were digested with the *Nla*III restriction enzyme.

Quantitative real-time RT-PCR

Quantitative real-time RT-PCR (QRT-PCR) was performed in a total volume of 20 μl using 9 μl of the cDNA synthesis mixture (1:10 dilution) as a template, 0.62 μl of 300 nM of forward and reverse primers and 10.25 μl of 2 × SYBR green PCR Master Mix (Applied Biosystems Inc.). The universal thermal cycling parameters were used as recommended (10 min activation at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C). The primers for QRT-PCR, designed using the Primer Express software (Applied Biosystems Inc.), were D2F (5′-TGACATACT GCAGCCTGCCA-3′) and D2R (5′-CGAGGTATGTTGATTTCATC ACTGTT-3′). As an internal control, the *GAPDH* gene was amplified using oligonucleotides GAPDHF (5′-GAAGGTGAAG GTCGGAGTC-3′) and GAPDHR (5′-GAAGATGGTGATGGGA TTTC-3′). Each reaction was run in triplicate. Results were calculated using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems Inc.). Relative quantification of gene expression was performed using the comparative CT method.

Sensitivity to MMC and ionizing radiation

MMC sensitivity assays were carried out as reported previously.[28] Briefly, cells were seeded in quadruplicate in 96-well microtiter plates (10^4 cells/well in 200 μl medium) and incubated for 5 days with different concentrations of MMC (0–100 nM) (Sigma, St Louis, MO, USA). The cells were then pulsed for 3 h with 2 mCi [^{3}H]thymidine, harvested and monitored for radioactivity incorporation by scintillation counting. Radiation-induced DNA synthesis (RDS) inhibition, indicative of the S-phase checkpoint function, was evaluated as described previously.[29] Briefly, cells were labeled for 24 h with 10 nCi/ml [^{14}C]thymidine (Amersham Biosciences, UK), washed and incubated for 6 h in non-radioactive medium. The cells were then irradiated and seeded in quadruplicate in 96-well plates and incubated for 60 min, then pulse labeled for 15 min with 2 mCi [^{3}H]thymidine, harvested and radioactivity analyzed by scintillation counting. The resulting ratios of [^{3}H] to [^{14}C] c.p.m. (corrected for those c.p.m. that were the results of channel crossover) were a measure of the DNA synthesis.

Results

Chromosomal instability in patient's cells

A 9-year-old boy born from a consanguineous marriage and affected by T-ALL manifested several episodes of post-chemotherapy pancytopenia that might lead to life-threatening infections. Although there was no evidence of congenital malformations, including short stature and café au lait spots, and aplastic anemia before the onset of T-ALL, the DEB test was performed for a suspected chromosomal instability in primary lymphocytes and in LFB cells established from the patient. Under routine conditions, cells occasionally exhibited chromosomal breakages. On exposure to DEB, lymphocytes and LFB cells showed typical FA chromosomal breakages and rearrangements in 25% of metaphases (Figure 1).

Marked reduction of FANCD2 protein

In an attempt to explain the chromosomal instability, we evaluated the expression of the FANCD2, FANCA and FANCG proteins in LFB. Although the levels of FANCA and FANCG

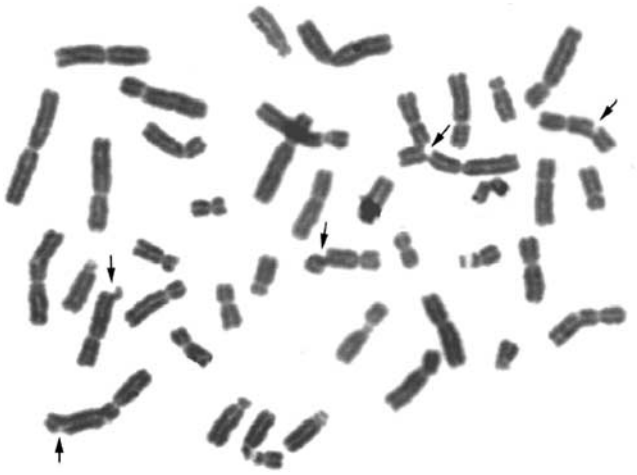

Figure 1 Chromosomal instability detected in the patient's cells. Representative chromosomal anomalies (single chromatid break and complex chromosomal rearrangements; arrows) in the patient's LFB cell line after exposure to DEB.

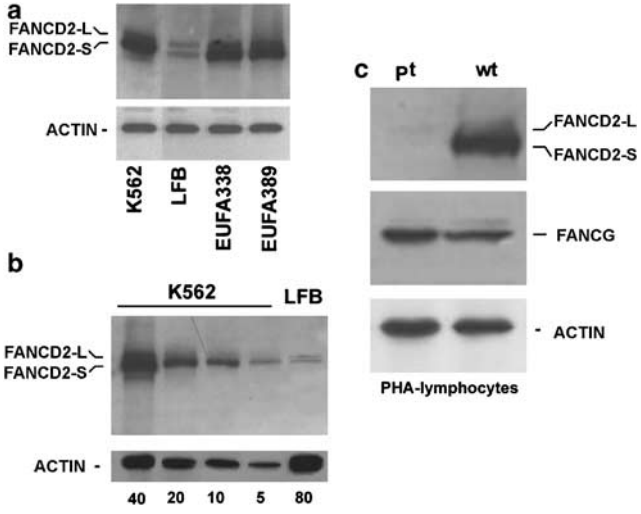

Figure 2 Immunoblot analysis of FANCD2 in the patient's lymphocytes and LFB cell line. (**a**) Equal amounts of protein extract were loaded from LFB, K562 and two normal control EUFA338 and EUFA389 lymphobastoid cell lines. (**b**) Different amounts (40, 20, 10 and 5 μg) of K562 protein extract were loaded to determine the relative amount expressed in 80 μg of LFB protein extract. (**c**) Equal amount of protein extract from PHA-stimulated lymphocytes of patient (pt) and control (wt).

were equivalent to those detected in EUFA338, EUFA389 and K562 cell lines used as controls (data not shown), the amount of FANCD2 was strongly reduced (Figure 2a). The FANCD2 expression, evaluated in repeated experiments by comparing its signal in LFB cells with that in control cells, was about 5% of normal (Figure 2b). Although the content of FANCD2 was considerably low, the non-monoubiquitinated (FANCD2-S) and monoubiquitinated (FANCD2-L) forms of FANCD2 were both present. The amount of the two isoforms was almost similar in LFB contrary to that observed in wild-type cells, where FANCD2-S is detected at a higher level than FANCD2-L. Noteworthy, peripheral patient's lymphocytes stimulated with PHA confirmed the low levels of FANCD2 (Figure 2c). Therefore, we hypothesized that the chromosomal instability observed in the patient's cells derived from mutations in *FANCD2* leading to a very limited level of the protein.

Identification of Leu153Ser mutation in the FANCD2 *gene*

To understand the molecular bases for the low FANCD2 levels, we screened the *FANCD2* gene for mutations. We first analyzed *FANCD2* mRNA by RT-PCR and sequence analysis. A novel missense mutation, 458T>C, leading to the substitution of leucine at position 153 with a serine (Leu153Ser) was identified (Figure 3a). This allele was confirmed to be homozygous at the genomic level (data not shown), as expected from a consanguineous marriage. Parents' DNA was not however available to ascertain their carrier status. Although the functional significance of the Leu153Ser mutation remains obscure, it is likely to be pathogenic. The nucleotide substitution was undetectable in a hundred normal controls (data not shown) and the amino-acid alignment of the FANCD2 orthologs from 18 different species showed a complete conservation of the leucine residue, suggesting that it exerts a fundamental role in FANCD2 structure and function (Figure 3b). Of note, whereas the monoubiquitination site Lys561 is missing in *Caenorhabditis elegans* and *briggsae*, leucine at position 153 is the only residue conserved among all species.[30]

To investigate whether the Leu153Ser mutation affected the stability of the *FANCD2* transcripts, a relative quantification of mutant versus wild-type mRNA molecules from LFB and control cell lines was performed by quantitative real-time PCR analysis. The results showed no major differences in the steady-state levels of mRNA among these cells (data not shown), thus excluding a direct effect of the missense mutation on the stability of the *FANCD2* transcripts.

Monoubiquitinated FANCD2 isoform localized in nucleus

In order to determine the potential pathogenetic role of the novel Leu153Ser mutation, we investigated the intracellular localization of the FANCD2, FANCA and FANCG proteins. Because of the low amount of the endogenous FANCD2 protein in LFB, we performed the immunoblotting of cytosolic and nuclear fractions instead of using an immunofluorescence assay. In LFB, as in EUFA338 control cell line, FANCD2-S and FANCD2-L isoforms were both present (Figure 4, whole-cell extract). On the contrary, FANCD2 is not monoubiquitinated in EUFA262, a cell line lacking the FANCA protein.[27] In fact, the post-translational modification of FANCD2, which is thought to be required for normal cellular response to DNA damage, occurs only in cells where all the subunits of the upstream FA multimeric complex are functional. This observation demonstrates the integrity of the FA complex in the patient's LFB cells.

In LFB and wild-type lymphoblasts, FANCD2-S was detected in both nuclear and cytosolic compartments, whereas FANCD2-L was localized only in the nuclear fraction (Figure 4). Absence of nuclear contamination in cytoplasmic preparations was

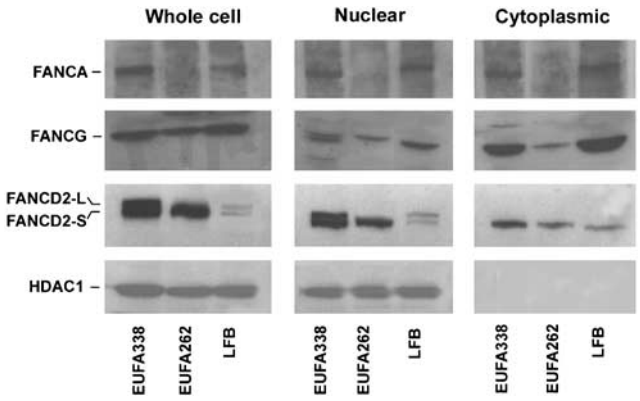

Figure 4 Cellular localization of the FANCD2^{L153S} protein. Protein extracts from whole cells, nuclear and cytoplasmic fractions were immunoblotted using antibodies against FANCA, FANCG and FANCD2. The HDAC1 antibody was used to reveal nuclear contamination in cytoplasmic extracts. LFB and EUFA338 are lymphoblastoid cell line from patient and normal individual, respectively, whereas EUFA262 is a FANCA-deficient lymphoblastoid cell line.

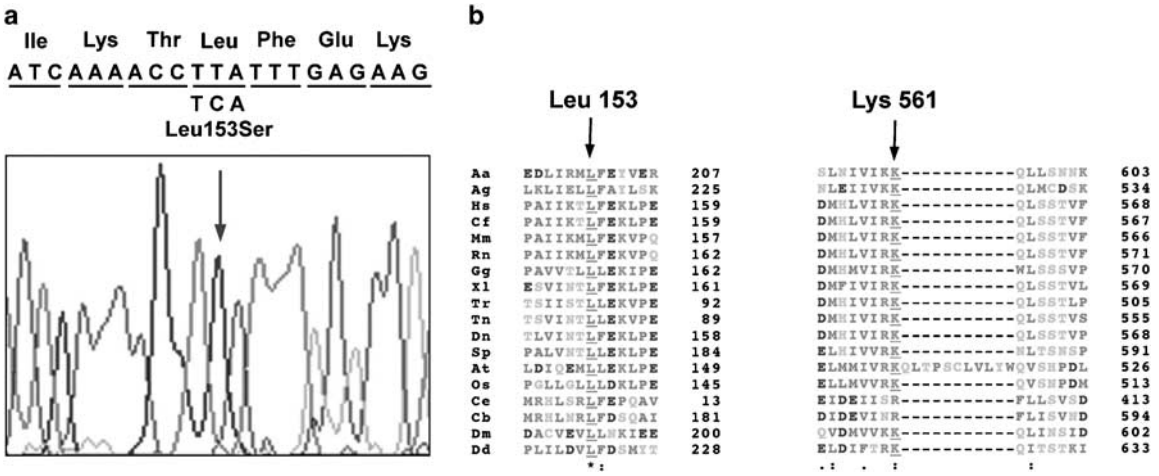

Figure 3 Identification of the Leu153Ser mutation. (**a**) Sequencing analysis of RT-PCR product with D2F1 and D2R1 primers. The single homozygous T>C at position 458 transition in exon 7 causes the Leu153Ser substitution. (**b**) ClustalW alignment (http://www.ebi.ac.uk/clustalw/) of known and predicted amino-acid sequences of FANCD2 orthologs from different species, showing complete conservation of the Leu153 residue (underlined) even in *Caenorhabditis elegans* (Ce), where the monoubiquitination site Lys561 (underlined) is missing.[30] Ae, *Aedes aegypti* (EAT36440); Ag, *Anopheles gambiae* (XP_320741); Hs, *Homo sapiens* (NM_033084); Cf, *Canis familiaris* (XM_541774); Mm, *Mus musculus* (XM_132796); Rn, *Rattus norvegicus* (NM_001001719); Gg, *Gallus gallus* (NM_001039261); Xl, *Xenopus laevis* (AY633665); Tr, *Takifugu rubripes* (SINFRUP00000141543, Ensembl database); Tn, *Tetraodon nigroviridis* (CAG06801); Dr, *Danio rerio* (NM_201341); Sp, *Strongylocentrotus purpuratus* (XP_798439); At, *Arabidopsis thaliana* (NP_193233); Os, *Oryza sativa* (BAD31595); Ce, *Caenorhabditis elegans* (NM_070413); Cb, *Caenorhabditis briggsae* (CAE68125); Dm, *Drosophila melanogaster* (AJ459772); Dd, *Dictyostelium discoideum* (XP_647652).

confirmed by reprobing stripped filters with antibodies against histone deacetylase 1 (HDAC1), which is uniquely localized in the nucleus. Therefore, the Leu153Ser mutation does not affect the nuclear localization of FANCD2 and does not prevent the monoubiquitination of FANCD2, supporting the hypothesis that chromosomal instability is due to a low level of FANCD2 as a consequence of defective translation or protein degradation.

Moreover, our data indicate that FANCD2 is imported to the nucleus in the FANCD2-S form and then monoubiquitinated to FANCD2-L. The nuclear localization of FANCD2 in FANCA-deficient cells also indicates that FANCA is not involved in the nuclear-cytoplasmic trafficking of the protein.

Crosslinking and radio sensitivity findings

Chromosomal breakage tests performed on the LFB cell line and peripheral blood lymphocytes showed an increased rate of chromosomal abnormalities. To better assess the sensitivity of LFB to MMC, the proliferation activity was measured by [^{3}H]thymidine uptake assay. LFB cells were more sensitive to MMC compared to normal lymphoblastoid cell line, albeit not as much as the FA-A cell line EUFA262, used as a positive control (Figure 5a).

As FANCD2 defects are also associated with radioresistant DNA synthesis indicative of an S-phase checkpoint defect,[31] we performed RDS measurements to determine the function of the S-phase checkpoint in LFB cells. Following ionizing radiation (IR)-induced DNA damage, the transient decrease in [^{3}H]thymidine in LFB cells was comparable to that of normal cells, in contrast to an ataxia telangiectasia (AT) cell line, which as expected, failed to inhibit the DNA synthesis (Figure 5b). These results indicate that the FANCD2 alteration in LFB does not impair the S-phase checkpoint.

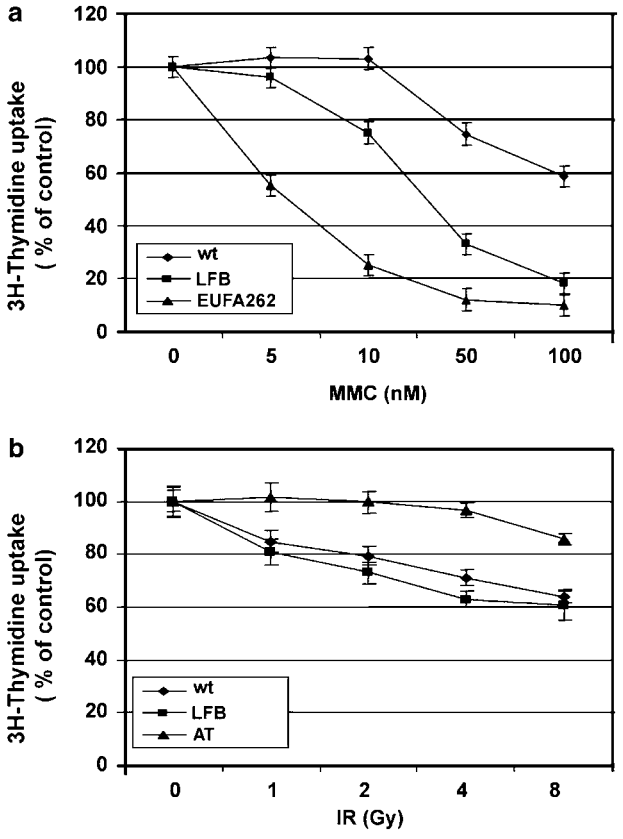

Figure 5 MMC and IR sensitivity in LFB cells. [^{3}H]thymidine uptake assay performed on lymphoblast cells from the patient (LFB), a normal donor (wt), an FA-A (EUFA262) or AT patients. (**a**) Cells were incubated with different concentrations of MMC (0–100 nM). (**b**) Cells were treated with increasing doses of IR (1–8 Gy).

Discussion

Patients with FA manifest a high risk of developing cancer and defects in the FA/BRCA pathway have recently been identified in so-called 'sporadic' tumors, such as cisplatin-sensitive ovarian cancers, pancreatic cancers, AML.[2–4] In addition to that, there might be subjects without the clinical features typical of FA ('atypical' FA) but with FA protein dysfunctions that may contribute to chromosomal instability, toxic response to therapy, and development and/or progression of cancers. Such was the case of a 9-year-old boy affected by T-ALL, who manifested several episodes of post-chemotherapy pancytopenia, leading to life-threatening infections. On these bases, we suspected that the patient could exhibit sensitivity to alkylating agents, a characteristic feature of the FA cellular phenotype. In fact, exposure of the patient's lymphocytes and EBV-immortalized lymphoblasts to DEB demonstrated chromosomal anomalies.

In order to identify which step of the FA/BRCA pathway was altered, we analyzed FANCA, FANCG and FANCD2 protein levels. A significant reduction (>95%) of the FANCD2 expression was detected in the peripheral lymphocytes and a lymphoblastoid cell line established from the patient. However, both non-monoubiquinated FANCD2-S and monoubiquitinated FANCD2-L isoforms were detected, although at a low level, suggesting that the molecular basis of chromosomal instability was not associated with alterations of the FA complex function but rather with a strong decrease of FANCD2 cellular content.

The screening for mutations of the *FANCD2* gene allowed us to identify a homozygous Leu153Ser amino-acid substitution. It is unlikely for this mutation to represent a polymorphism, as it was not detected in more than a hundred healthy controls. Moreover, it affects only one residue out of 1471 evolutionary conserved among all species analyzed, suggesting that it is functionally important. Although the *FANCD2* gene is the only FA gene to be conserved through evolution, it does not bear domain homologies either to other known proteins or to other FA products. Therefore, how the Leu153Ser mutation leads to FANCD2 dysfunction is not predictable on the basis of bioinformatics analysis.

In order to understand how the mutation affects the FA/BRCA pathway, we first studied the intracellular localization of the mutated FANCD2 protein. As in normal controls, the protein was detected in both cytoplasmic and cellular compartments of the patient's LFB cells, indicating that the missense mutation does not prevent FANCD2 from being translocated to the nucleus, where it is monoubiquitinated. Given that FA-A cells also showed FANCD2 within the nucleus even if only in the non-monoubiquitinated form, we can conclude that nuclear localization is independent of the assembly of FANCA in the FA complex.

To investigate the mechanism implicated in the low content of FANCD2, we performed real-time PCR analyses that showed a similar level of FANCD2 transcripts in LFB as in control cells. Thus, we ruled out a downregulation of transcription or RNA instability of the FANCD2 gene as the cause of the extremely low level of the protein, suggesting that the Leu153Ser mutation alters the conformational structure and consequent removal of the protein. Protein degradation is likely to occur promptly after

synthesis, as the low level of FANCD2 is also detected in the cytosolic compartment.

Multifunctional activities of FA proteins have been proposed and specifically FANCD2 seems to play at least a dual role.[31] It mediates the resistance to chemical crosslinking agents by monoubiquitination at residue K561 and migration into nuclear foci where it colocalizes with BRCA1 and other proteins implicated in the DNA repair mechanisms.[31] FANCD2 is also phosphorylated at serine 222 by ataxia telangiectasia mutaded (ATM), in response to IRs activating the S-phase checkpoint.[31] Therefore, we examined the patient's LFB cells for the activation of the S-phase checkpoint following IR exposure, and found a normal response. Nevertheless, these cells displayed a partial sensitivity to the crosslinking agents MMC, a finding consistent with the results on chromosomal instability.

The patient reported in this paper represents the first 'atypical' FA patient with *FANCD2* mutations. Three are the cases of 'classical' FA with mutations in this gene, all associated with a lack of the FANCD2 protein expression.[32] Moreover, the only patient we have characterized with typical signs of the disease and *FANCD2* mutations has no detectable protein (unpublished data). Conversely, the data reported in the present study suggest that hypomorphic alleles, such as $FANCD2^{L153S}$ might protect patients against FA symptoms but not protect against clonal selection, cancer progression and chemotherapy complications. Similarly to our case, mutations of *BRCA2/FANCD1* were identified in patients initially studied because of solid tumors (medulloblastoma and Wilms tumor) and T-ALL.[33]

In brief, we must underline the critical importance of the functional and molecular characterization of the FA/BRCA pathway in patients, who, even without FA clinical manifestations, show severe complications as consequence of chemotherapy. Indeed, we should consider the occurrence of cases with a residual activity of the pathway efficient enough for basal DNA repair activity but ineffective during a prolonged chemotherapy. Moreover, the hypothesis that alteration(s) of FA-dependent mechanisms of DNA repair might be responsible for the development of 'sporadic' cancer should also be taken into consideration. Although this view is at present difficult to assess, our observation suggests the possible existence of apparently mild alterations of FANCD2 (or other components of the FA pathway) that could favor development and evolution of cancer.

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