

Heterozygote *FANCD2* mutations associated with childhood T Cell ALL and testicular seminoma

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Published online: 25 July 2012
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Abstract Fanconi anaemia (FA) is an inherited disease with congenital and developmental abnormalities characterised by cellular cross linker hypersensitivity. FA is caused by mutations in any of so far 15 identified *FANCD2* genes, which encode proteins that interact in a common DNA damage response (DDR) pathway. Individuals with FA have a high risk of developing acute myeloid leukaemia (AML) and squamous cell carcinoma. An increased cancer

risk has been firmly established for carriers of mutations in *FANCD1/BRCA2*, *FANCI/BRIP1*, *FANCD3/PALB2*, *RAD51C/FANCO* and link the FA pathway to inherited breast and ovarian cancer. We describe a pedigree with *FANCD2* mutations *c.458T > C* (p.Leu153Ser) and *c.2715 + 1G > A* (p.Glu906LeufsX4) with mild phenotype FA in the index case, T cell ALL in the Leu153Ser heterozygous brother and testicular seminoma in the p.Glu906LeufsX4 heterozygous father. Both *FANCD2* alleles were present in the T Cell ALL and the seminoma. This links specific *FANCD2* mutations to T cell ALL and seminoma without evidence of allelic loss in the tumour tissue.

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Keywords Fanconi anaemia · *FANCD2* · T Cell ALL ·
Leukaemia · Seminoma · Systemic Lupus erythematosus

Introduction

Fanconi anaemia (FA) is an inherited disease with congenital and developmental abnormalities and cancer predisposition. On a cellular level FA is characterised by chromosomal fragility and hypersensitivity to mitomycin C (MMC). FA results from mutations in any of so far 15 identified *FANCD2* genes, which encode proteins of a DNA damage response pathway that requires the interaction of FA, BRCA and FA-associated proteins. Upstream in this pathway the FA-core proteins encoded by *FANCA*, *-B*, *-C*, *-E*, *-F*, *-G*, *-L* and *-M* interact with FA-associated proteins to facilitate the ubiquitination of the *FANCD2* and *FANCI* proteins (DI-complex). Downstream in this pathway operate *FANCD1/BRCA2*, *FANCI*, *FANCO/RAD51C* and *FANCP/SLX4* [1–4]. Individuals affected by FA have an extreme risk of developing leukaemia and solid malignancies, mainly squamous cell carcinomas (SSC) in the

head and neck region, but also CNS tumours and epithelial cancers of the oesophagus and breast have been reported [5]. Importantly, leukaemia associated with FA is characteristically of myeloid lineage (AML) [5–7], while less than ten cases of non-myeloid leukaemia have been reported in FA. Heterozygous mutation carrier status has been firmly established to be associated with an increased risk of breast and/or ovarian cancer for the *FANCD1/BRCA2*, *FANCN/PALB2*, *FANCI/BRIP1* and *FANCO/RAD51C* genes [8–11], and link the FA pathway with inherited breast and ovarian cancer syndromes [12]. Here we report the clinical findings and molecular analysis of a pedigree with *FANCD2* mutations causing FA in the index case, associated with T Cell acute lymphoblastic leukaemia (ALL) and testicular seminoma in the heterozygote sibling and father and discuss clinical and genetic implications.

Materials and methods

Clinical summary

A 9 year-old girl was investigated for developmental delay and hearing problems, growth failure and microcephaly. MMC testing of her peripheral lymphoblasts was diagnostic of FA. She had a 3 years younger brother who presented age 3 years and 6 months with CD7 and CD2 positive T Cell ALL 3 years before the diagnosis of FA was made in his sister. Cytogenetic analysis of his T Cell ALL showed a *t*(11;14) translocation involving the *LMO2* gene, and a *LEF1* deletion on array CGH analysis. He tolerated treatment without unusual side effects or toxicity. Thirty months after completion of treatment he relapsed and a second remission was achieved. A matched unrelated haematopoietic stem cell transplant (HSCT) was carried out. He died of multi-organ failure associated with chronic grade IV GVHD and recurrent septicaemia 13 months after HSCT. The two children were from a non-consanguineous white British couple. The father had type 1 diabetes from the age of 26, and a testicular seminoma stage IIa at the age of 36, which was successfully treated with 35 Gy fractionated radiotherapy. At this time the father was also noted to have a hypoplastic left kidney. The mother was diagnosed with a connective tissue disease in the SLE (systemic lupus erythematosus) spectrum age 24 with sero-positivity for anti-double strand DNA antibodies initially presenting with fever, lymphadenopathy and interphalangeal joint problems.

Patient material, cell lines and tissue culture

MMC breakage analysis and growth inhibition was carried out using standard methodologies on peripheral lymphoblasts and EBV transformed lymphoblastoid cell lines

(LCLs). Bone marrow samples from the boy at initial diagnosis of ALL and in formalin fixed paraffin embedded (FFPE) sections of the testicular tumour from the father were retrieved from archived material. Samples were analysed with ethical approval and informed consent according to the declaration of Helsinki.

FANCD2 western blot and immunohistochemistry

FANCD2 Western blot with lysates of the *FANCF* mutated LCL CV1785 and normal LCL NLB as controls, and immunohistochemistry with target retrieval solution pH 6 (Dako S2369, Dako, Ely, UK) and microwaving for 15 min at 98 °C, was carried using an anti-*FANCD2* antibody (Abcam # ab2187, Cambridge, UK) with standard methodologies.

FANCD2 mutation analysis

FANCD2 mutation analysis was carried out as described previously on genomic DNA from peripheral lymphoblasts, LCLs, and thick tissue sections of the testicular tumour and normal tissue using standard procedures, and cDNA generated using standard procedures from RNA extracted from cell lysates and bone marrow aspirates [13].

Results

Mild phenotype FA with *FANCD2* mutations *c.2715 + 1G > A/p.Glu906LeufsX4* and *c.458T > C/p.Leu153Ser*

A nearly absent *FANCD2* signal on Western blot analysis of the lymphoblastoid cell line CV1665 from the girl with FA suggested that this patient belongs to the FA-D2 group (Fig. 1a). Mutation analysis of *FANCD2* using DNA from peripheral lymphoblasts and parental DNA revealed the missense mutation *c.458T > C* resulting in p.Leu153Ser (Fig. 1b, panel 2–5) in the maternal allele. This mutation has been reported only once before in a homozygous Italian boy with FA, intriguingly presenting with T Cell ALL and excessive toxicity to chemotherapy [14]. In the paternal allele we identified the mutation *c.2715 + 1G > A* resulting in aberrant splicing (p.Glu906LeufsX4) (Fig. 1b, panel 9), which has been described previously in two patients of German or East European origin [15]. Sequence analysis of the *FANCD2* transcript of the LCL CV1665 derived from the girl with FA detected mainly the *c.458T > C* mutated allele (Fig. 1c, panel 2), implying the possibility that the aberrantly spliced transcript from the *c.2715 + 1G > A* resulting in p.Glu906LeufsX4 might not be stable.

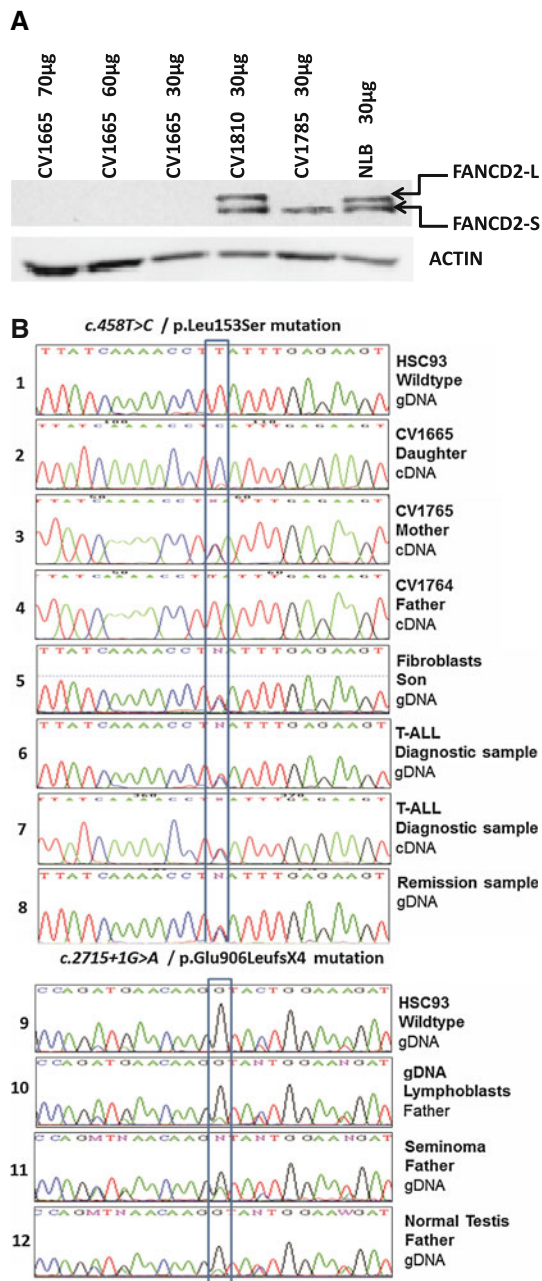


Fig. 1 a Absence of detectable *FANCD2* by Western analysis blot of cell lysates from lymphoblastoid cell line CV1665 derived from the FA patient, but not the heterozygous sibling with T Cell ALL (CV1810). *FANCF* disrupted cell line CV1785 and normal NLB cells as controls. b Sequence analysis of *FANCD2* gDNA showing WT in control and father (panel 1 and 4), and c.458T > C in the cDNA from the lymphoblasts of the FA-affected daughter and mother, fibroblasts of the boy and gDNA from T Cell ALL diagnostic and remission sample, and cDNA from diagnostic sample (2, 3, 5–9). The c.458T > C transcript is the main transcript expressed in the lymphoblastoid cell line also carrying the c.2715 + 1G > A from the girl with FA (2), but heterozygosity for WT/c.458T > C cells from mother (3). Heterozygosity for c.2715 + 1G > A on gDNA analysis of the father's lymphoblast (10), but also presence of both alleles in seminoma and normal testis (11 and 12)

FANCD2 c.458T > C/p.Leu153Ser heterozygosity in T Cell ALL

Given the diagnosis of T Cell ALL in the brother, we determined his mutational status. He carried the c.458T > C/p.Leu153Ser mutation, with a normal paternal allele (Fig. 1a, panel 5). In order to explore the possibility of allelic loss in the T Cell leukaemia, we next sequenced *FANCD2* in gDNA and cDNA from the diagnostic leukaemia sample with more than 99 % blasts. We detected heterozygosity for C/T at c.2715 in the diagnostic and remission sample from the boy's leukaemia (Fig. 1b, panels 6 and 7), implying that both alleles of *FANCD2* at 3p were present in the leukaemic blasts.

Absence of nuclear *FANCD2* in testicular seminoma

To explore the link of the *FANCD2* mutation with the father's seminoma, we carried out *FANCD2* immunohistochemistry in the FFPE tumour tissue. We detected *FANCD2* protein by nuclear staining in normal spermatogenic cells in the presence of unstained spermatozoae (Fig. 2b; left). However, in the same tissue section nuclei of the seminoma cells were *FANCD2* negative (Fig. 2b, right). As loss of heterozygosity of the 3p region has been described in seminoma and could include the *FANCD2* locus [16], we sequenced *FANCD2* in DNA extracted from FFPE of tumour tissue and normal testis. Sequencing of partly degraded DNA in FFPE tissue clearly showed presence of both *FANCD2* alleles in both preparations, making loss of the wild type allele in the seminoma unlikely (Fig. 1b, panel 10 and 11).

Discussion

FA presented in this 9 year-old girl with short stature and microcephaly. The FA-phenotype in this girl is mild, in particular as most FA patients with mutations in *FANCD2* have early onset haematological complications [15]. The mutation p.Leu153Ser on the maternal allele has previously been reported without congenital and developmental abnormalities, but with T Cell ALL and severe chemosensitivity [14]. This allele appears to be the dominant transcript in cultured lymphoblasts. Her brother with T Cell ALL carried the *FANCD2* mutation p.Leu153Ser. This is to our knowledge the first case in which a heterozygous sibling of an FA-patient develops a malignancy before the index case with FA. Importantly, the only other T Cell ALL associated with a *FANCD2* mutation has been described in a male FA-patient homozygous for the same p.Leu153Ser *FANCD2* mutation [14]. Only five other cases of T Cell ALL have been described with FA, three of them

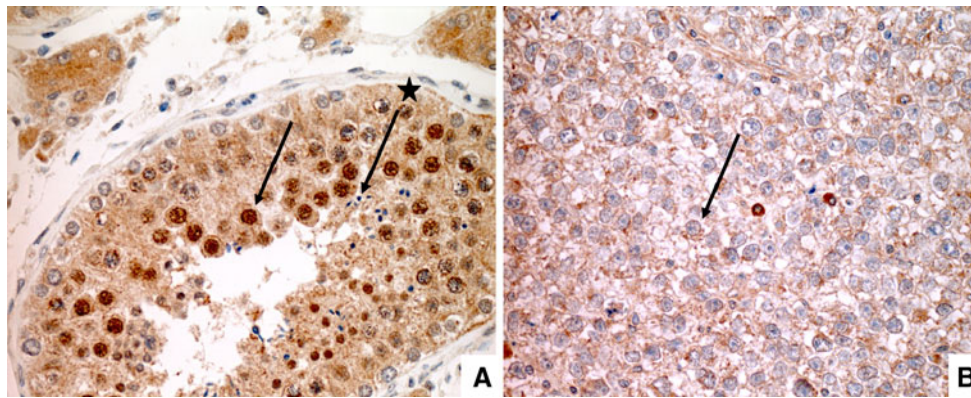


Fig. 2 Immunohistochemistry of normal testicular tissue (a) on the same tissue section as seminoma (b) using a rabbit anti-FANCD2 antibody. Nuclear staining of FANCD2 in the more luminal normal

spermatogenic cells (*arrow*) in the presence of unstained spermatozoae (*arrow**), while nuclei of the seminoma cells in the same tissue section were FANCD2 negative (*arrows*)

in FA-D1 patients with bi-allelic *FANCD1/BRCA2* mutations [17] and two siblings in one family associated with heterozygosity of a *FANCC* mutation [18]. As the c.458T > C mutation is found again associated with a T Cell ALL, this implies an important role of *FANCD2*, and in particular this mutation, for malignant transformation of T cell progenitors. Intriguingly, the sister, who has no normal *FANCD2* allele and mainly expresses the c.458T > C allele in LCL culture, and the mother, heterozygous for the same allele, did not develop leukaemia. Given that the other patient with the c.458T > C/p.Leu153Ser mutation and T Cell ALL was also male, this implies the possibility of sex determined modulators of the oncogenic potential of this *FANCD2* mutation. The mother of the individual homozygous for the c.458T > C allele had parotid cancer [14], implying that carrier status of this *FANCD2* mutation might confer a greater cancer risk than carrier status of mutations in other FA genes of core complex components. However, there is no evidence of increased cancer incidence in first degree relatives of other *FANCD2* patients in general [15]. Inherited defects in the FA pathway have not been associated with germ cell tumours or specifically seminoma. In this family we have detected a germ line pathogenic *FANCD2* mutation and absence of nuclear FANCD2 staining in the tumour tissue, but presence of nuclear *FANCD2* staining in normal tissue, in line with previously published data [19]. Presence of both alleles in the tumour DNA does not support the concept of allelic loss in the T cell ALL or the seminoma either. Had it not been for the diagnosis of FA in the sister, a link between *FANCD2* mutations and the T Cell ALL or the seminoma would not have been possible to make. SLE has not previously been described associated with FA or in heterozygote FA-gene mutation carriers. However, sequence variants in other DNA damage response genes such as nibrin (NBN, mutated in Nijmegen breakage

syndrome), and altered DNA damage response have been implicated in the pathogenesis of SLE [20, 21]. In summary, clinical and molecular data of this family implies novel associations between inherited disruption of the FA pathway, specifically mutations in *FANCD2*, with malignant and autoimmune disease.

Acknowledgments We are grateful to Gary Ashton for excellent immunohistochemistry. Grant support: CRUK Clinician Scientist Fellowship to SM. ADW and SM are supported by LLR, UK.

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