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Diagnosis of Fanconi Anaemia by ionising radiation- or mitomycin C-induced micronuclei

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ABSTRACT

Fanconi Anaemia (FA) is an autosomal recessive disorder characterised by defects in DNA repair, associated with chromosomal instability and cellular hypersensitivity to DNA cross-linking agents such as mitomycin C (MMC). The FA repair pathway involves complex DNA repair mechanisms crucial for genomic stability. Deficiencies in DNA repair genes give rise to chromosomal radiosensitivity. FA patients have shown increased clinical radiosensitivity by exhibiting adverse normal tissue side-effects. The study aimed to investigate chromosomal radiosensitivity of homozygous and heterozygous carriers of FA mutations using three micronucleus (MN) assays. The G0 and S/G2 MN assays are cytogenetic assays to evaluate DNA damage induced by ionising radiation in different phases of the cell cycle. The MMC MN assay detects DNA damage induced by a crosslinking agent in the G0 phase. Patients with a clinical diagnosis of FA and their parents were screened for the complete coding region of 20 FA genes. Blood samples of all FA patients and parents were exposed to ionising radiation of 2 and 4 Gy. Chromosomal radiosensitivity was evaluated in the G0 and S/G2 phase. Most of our patients were homozygous for the founder mutation *FANCG* c.637_643delTACCGCC; p.(Tyr213Lysfs*6) while one patient was compound heterozygous for *FANCG* c.637_643delTACCGCC and *FANCG* c.1379G > A, p.(Gly460Asp), a novel missense mutation. Another patient was compound heterozygous for two deleterious *FANCA* mutations. In FA patients, the G0- and S/G2-MN assays show significantly increased chromosomal radiosensitivity and genomic instability. Moreover, chromosomal damage was significantly elevated in MMC treated FA cells. We also observed an increase in chromosomal radiosensitivity and genomic instability in the parents using 3 assays. The effect was significant using the MMC MN assay. The MMC MN assay is advantageous as it is less labour intense, time effective and has potential as a reliable alternative method for detecting FA patients from parents and controls.

1. Introduction

Fanconi Anaemia (FA) is primarily an autosomal recessive disorder that is clinically characterized by congenital malformations, progressive development of hypoplastic anaemia and cancer predisposition that often results in haematological malignancies such as acute

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myelogenous leukaemia (AML) or myelodysplasia (MDS) as well as various solid tumours, especially head and neck squamous carcinoma. Recent determination of the carrier frequency gave an estimate of more than 1/200 [1], with an expected prevalence at birth of at least 1/160 000. In certain populations, the carrier frequency is much higher, due to founder mutations. In South Africa, with mixed ethnicity in the population, the prevalence of FA ranges between 1/22 000 for the white Afrikaners to 1/40 000 in the black South Africans [2,3].

Twenty-one different FANC genes have been reported in literature *FANCA*, *FANCB*, *FANCC*, *FANCD1* (also known as *BRCA2*), *FANCD2*, *FANCE*, *FANCF*, *FANCG*, *FANCI*, *FANCJ* (also known as *BRIP1* or *BACH1*), *FANCL*, *FANCM*, *FANCN* (also known as *PALB2*), *FANCO* (also known as *RAD51C*), *FANCP* (also known as *SLX4*), *FANCQ* (also known as *XPF* or *ERCC4*), *FANCR* (also known as *RAD51*), *FANCS* (also known as *BRCA1*), *FANCT* (also known as *UBE2T*), *FANCU* (also known as *XRCC2*) [4,5], and very recently *REV7* (also known as *MAD2L2* or *FANCV*) was identified as a novel FA gene [6]. FA is most often inherited in an autosomal recessive manner, however, also autosomal dominant (*FANCR/RAD51*) and X-linked forms (*FANCB*) have been reported [7]. Next generation sequencing (NGS) allows efficient identification of causal mutations in this genetically heterogeneous disease. Deleterious mutations in *FANCA*, *FANCC* and *FANCG* are identified in 80–90% of the FA cases [8].

All currently known FA genes encode for a cluster of proteins responsible for repair of stalled DNA replication forks by unhooking DNA interstrand cross-links (ICL) and promoting homologous recombination (HR). The FA/BRCA repair pathway is pivotal in maintaining genomic stability. The FANC genes are sub grouped into 3 main categories known as (i) the core complex (ii) ID2 complex and (iii) the downstream effectors. In response to damage caused by DNA crosslinking agents or ionising radiation (IR), the activated core complex formed by 8 FA genes (A, B, C, E, F, G, M, and L) activates the ID2 complex, comprised of *FANCD2/FANCI* gene, by mono-ubiquitination and phosphorylation. The mono-ubiquitination of ID2 is catalysed by the E2 ubiquitin-conjugating enzyme *FANCT/UBE2T* [9]. The ID2 complex plays a critical role in the pathway by translocating to the damage sites which triggers the recruitment of downstream effectors (D1, J, N, O, P, Q, R, S and U) in the S/G2 phase. The damage is subsequently repaired by HR. Following the repair, the ID2 complex undergoes deubiquitination and the core complex genes are unassembled [10,4,11]. The latest identified FA gene, *REV7/FANCV*, a subunit of DNA polymerase ζ involved in translesion DNA synthesis (TLS) seems to act as a necessary downstream effector of the FA-BRCA pathway, most likely functioning in the TLS step of ICL repair [12].

In FA patients' cancer treatment by chemotherapy or radiotherapy is complicated because of the possibility of side effects due to the underlying defect in DNA damage response. Sensitivity to chemotherapy and radiotherapy in FA patients has previously been documented [13,14]. Radiosensitivity is the susceptibility of cells to the DNA damaging effects of IR. Exposure to IR causes a variety of DNA damage of which the double strand breaks are the most important [15]. Double strand breaks (dsb) are repaired predominantly by two main DNA repair pathways: i) HR which requires a homologous DNA strand for repair and ii) non-homologous end-joining (NHEJ). The cell is selective on which DNA repair pathway to use depending on the phase of the cell cycle. In the S/G2 phase where a homologous strand is present, the HR repair pathway is preferred over NHEJ. The NHEJ repairs double strand breaks is predominant in the remaining phases of the cell cycle [16].

Some older studies suggested that FA patients, similar to patients with ataxia telangiectasia or Nijmegen breakage syndrome, are characterised by an increased *in vitro* chromosomal radiosensitivity [17,18], while others report the contrary [19]. However, literature on chromosomal radiosensitivity of FA patients is very limited.

It is well established that FA cells are hypersensitive to DNA crosslinking agents such as mitomycin C (MMC) and diepoxybutane (DEB) [20]. Chromosome fragility is pathognomonic in the diagnosis: FA is classically diagnosed by treating lymphocytes of patients with MMC or DEB and subsequent quantification of all types of chromosomal breakages and radial forms [21]. The major challenge with this assay is the low quality slides and the need of highly experienced personnel to analyse chromosomal breakages; furthermore, it is labour intense and time consuming. We propose here a more robust assay to identify FA patients by detecting micronuclei (MNi) and evaluate if this can be used to distinguish heterozygous carriers from non-carriers. The cytokinesis-block micronucleus assay (CBMN) in human lymphocytes is a well-established assay for measuring chromosomal aberrations after *in vivo* or *in vitro* exposure to genotoxic agents. Here, we propose a novel protocol where MMC is applied to induce MN. Furthermore, we evaluated two MN protocols using ionising radiation as genotoxic agent: the G0 MN assay, a well-known cytogenetic assay where cells are irradiated in the G0 phase of the cell cycle [22,23], and a S/G2 MN assay, in which the damage is induced in the S/G2 phase [24,25]. In the S/G2 MN assay, cells were also subjected to caffeine treatment. The addition of caffeine to cells abrogates the G2/M cell cycle checkpoint. The abrogation of the checkpoint permits the progression of damaged cells into mitosis [26]. In this way the efficiency of the repair pathway activated when cells are irradiated in the S/G2 phase can be evaluated.

2. Methods and material

2.1. Study population

Thirteen patients with clinical manifestation of FA and their parents were recruited from the Paediatric Oncology department at Charlotte Maxeke Johannesburg Academic hospital and Chris Hani Baragwanath hospital in South Africa. In addition, one patient and parent was recruited from Cliniques Universitaires Saint-Luc, Belgium. In total, fourteen FA patients and 14 parents were enrolled in the study and heparin blood samples were collected. Blood samples from 14 healthy donors were also collected from student and staff members from the university and hospital. The mean age of the patients is 11 years. The youngest patient enrolled in the study was 5 years and the oldest being 17 years. The mean age of the parents enrolled in the study was 40 years and healthy individuals had a mean age of 30 years. Informed consents were obtained from all donors. Ethics for the study was approved by the Wits Human Research Ethics Committee (Medical) (clearance number M141031).

2.2. Mutation analysis

2.2.1. DNA extraction

Genomic DNA was extracted from peripheral blood using the automated Tecan Freedom EVO®-HSM Workstation (Promega). DNA extraction for 14 patients with clinical manifestations of FA and 14 parent samples was carried out as per manufacturer's instructions.

2.2.2. Target enrichment, library preparation and sequencing

Singleplex PCR reactions were performed for the coding region of all 20 known FA genes. Primers for the coding regions and splice site regions of all the genes were designed using PrimerXL (www.pxlence.com). The PCR conditions and the modified version of the Nextera XT (Illumina) library preparation protocol utilised in this study were conducted as previously described [27]. Subsequently sequencing was performed on the Miseq instrument (Illumina).

2.2.3. Sanger sequencing

All pathogenic mutations and variants classified as likely pathogenic were confirmed by Sanger sequencing on the ABI3730XL (ThermoFisher) instrument in the patients. Carriership of a heterozygous mutation was also confirmed in DNA of the parents available.

2.2.4. MLPA

In order to detect large exon spanning deletions/duplications, MLPA was conducted using the commercially available MLPA kits (MRC-Holland) for some FA genes (MLPA P031-*FANCA* mix 1 and P032-*FANCA* mix 2 (version: B2-0116), P057-*FANCD2-PALB2* (version: B2-0415) and P260-*PALB2-RAD50-RAD51C-RAD51D* (version: B1-1114)). Fragment separation is achieved by capillary electrophoresis using the ABI model 3730XL sequencer (ThermoFisher).

2.2.5. Data analysis

Sequencing data obtained from the Miseq run was mapped using CLC Bio Genomics Workbench v7 software (CLC bio Inc.). The sequencing analysis was also conducted using other in-house scripts as previously described [27]. Sanger sequencing data were analysed using the SeqPilot software v4.1.2 build 512. Coffalyer.NET (MRC-Holland) software was used for MLPA data analysis.

2.3. The cytokinesis-block micronucleus (CBMN) assay to assess radiation induced DNA damage

2.3.1. G0 CBMN assay

To quantify the chromosomal radiosensitivity of FA patients and heterozygous carriers, the MN assay was performed as previously described. Heparinised blood (0.5 ml) was added to 4.5 ml of pre-warmed RPMI-1640 supplemented with L-glutamine (Bio-Whittaker, USA), 13% of foetal bovine serum (FBS; Gibco-Invitrogen, USA) and antibiotics (50 U/ml penicillin and 50 mg/ml streptomycin; Gibco-Invitrogen, USA). The cells were irradiated in the G0 phase of the cell cycle with doses of 2 and 4 Gy 6 MV X-rays at a dose rate of 1.33 Gy/min using a linear accelerator (Siemens Healthcare, Germany). A sham irradiated control (0 Gy) culture was also set up to detect spontaneously occurring MNi. After irradiation, cell division was stimulated in lymphocytes by the addition 100 µl of phytohaemagglutanin (PHA − stock solution 1 mg/ml; Sigma-Aldrich, USA), and cytokinesis was blocked after 23hr by adding 20 µl cytochalasin B (Cyto B − stock solution 1.5 mg/ml; Sigma-Aldrich, USA). Seventy hours post PHA-stimulation, cells were harvested using a cold hypotonic shock of 7 ml of KCL (0.075 M; Merck, Germany) and fixed in a methanol: acetic acid: ringer solution (4:1:5) (Merck, Germany). After overnight storage at 4 °C, the cells were further fixed 3 times by using a methanol: acetic acid solution (4:1). A suspension of cells (40 µl) was dropped onto slides and stained with acridine orange stain (10 µg/ml) (Sigma-Aldrich, USA). Duplicate slides were made of each sample, coded and 500 BN cells per slide were scored using the Zeiss Axioskop fluorescent microscope (Carl Zeiss, Gottingen, Germany). To obtain radiation-induced MN values, the spontaneous MN were deducted from the MN values of irradiated samples.

2.3.2. S/G2 CBMN assay

Compared to the G0 MN assay, this assay is modified to analyse chromosomal radiosensitivity when cells are irradiated in the S/G2 phase of the cell cycle as previously described [25]. In short, heparinised blood (0.5 ml) in culture medium was set up and lymphocyte stimulation was immediately achieved by addition of 100 µl PHA (stock solution 1 mg/ml; Sigma-Aldrich, USA). Following stimulation, the cultures were incubated at 37 °C and 5% CO_2. The lymphocytes were irradiated after 72 h to doses of 2 and 4 Gy. To detect spontaneous MNi in the S/G2 phase of the cell cycle, a control (not irradiated) culture was also started. Cytokinesis was immediately inhibited after irradiation by the addition of 20 µl Cyto B (stock solution 1.5 mg/ml; Sigma-Aldrich, USA) and 200 µl caffeine (stock solution 100 mM; Sigma-Aldrich, USA) was added to a part of the cultures. The cells were harvested 8 h post irradiation and fixed as described above. Duplicate slides were stained, coded and 500 BN per slide were scored. Also here, radiation-induced MN values were obtained by deduction of spontaneous MN from the MN values of irradiated samples.

2.4. Cytokinesis-block micronucleus assay to assess MMC induced DNA damage

Mitomycin C Micronucleus assay: This assay was optimized to analyse the extent of chromosomal breakage induced by MMC in FA patients and parents (heterozygous carriers). Cultures were initiated by the addition of 0.5 ml heparinized blood in 4.5 ml culture medium. To optimize this technique, we used 2 different concentrations of MMC that can induce detectable damage. Concentrations of 0.02 µg/ml or 0.1 µg/ml MMC (stock solution 0.5 mg/ml; Sigma-Aldrich, USA) were added and lymphocytes were immediately stimulated with PHA (Sigma-Aldrich, USA). The cultures were incubated at 37 °C and supplemented with 5% CO_2. Similar to the G0 MN assay, 23 h later the cytokinesis was blocked by the addition of Cyto B (Sigma-Aldrich, USA). Cells were harvested 70 h post stimulation. The staining and scoring was conducted as described above. MMC-induced MN values were obtained by deducting spontaneous MN from the MN values in the samples treated with MMC.

2.5. Nuclear division index

The nuclear division index (NDI) is a measure of cell proliferation where poor nuclear division is indicated by a low NDI value and a higher NDI value suggests good proliferative capacity of the cells. The NDI was calculated for each of the assays by evaluating the number of mononucleate (N1), binucleate (N2), trinucleate (N3) and polynucleate (N4) cells. A total (Ntotal) of 500 cells per slide was scored. The formula used to calculate NDI is: NDI = (N1 + 2N2 + 3N3 + 4N4)/Ntotal.

2.6. Statistical analysis

GraphPad Prism 7 was used to analyse the statistical significance of the study. The comparison of the MN scores between the groups was conducted using the non-parametric Mann-Whitney. The significance level was set at < 0.05.

3. Results

3.1. Germline mutations

As expected in the 13 black South African patients the large majority (12/13) was found to be homozygous for the *FANCG* founder mutation c.637_643delTACCGCC; p. (Tyr213Lysfs*6). One patient was heterozygous for the *FANCG* c.637_643delTACCGCC mutation, and a novel heterozygous *FANCG* c.1379G > A, p. (Gly460Asp) missense variant, which has not been previously described. This variant is not reported in large databases like ExAC or gnomAD. By this substitution a highly conserved amino acid (Gly460) is changed into an amino acid (Asp) with different physicochemical properties (Grantham distance: 94 [0–215]). Prediction programs like SIFT, Polyphen and MutationTaster all support a deleterious effect for this variant. The clinical symptoms manifested in this patient included microcephaly, hypopigmentation, short stature, flattened thenar eminence and the bone marrow aspirate showed aplasia strongly supporting a FA diagnosis. No deleterious mutation was identified in the other FA genes screened.

The patient recruited in Belgium, of Cameroonian origin, was found to be compound heterozygote for two *FANCA* mutations: c.987_990delTCAC, p. (His330Alafs*4) and a large deletion spanning exons 22–28 (c.1901-?_2778 + ?del).

In all 14 parents, from whom DNA was available, we confirmed heterozygous carriership of the relevant *FANCG* or *FANCA* mutation.

3.2. G0 Micronucleus assay

Table 1 shows the data for the 3 endpoints scored in the G0 MN assay. The spontaneously occurring mean MNi values (non-irradiated

Table 1
G0 MN assay: Comparison of mean ± SD spontaneous (0 Gy) and radiation-induced (at 2 and 4 Gy) MNi values in 1000 binucleated cells of FA patients, parents and healthy controls.

	0 Gy	2 Gy	4 Gy
[a]Patients	27.93 ± 9.94*	356.6 ± 70.67**	915.0 ± 160.5**
[a]Parents	23.21 ± 9.80	286.5 ± 78.26	776.2 ± 185.3
[a]Controls	18.29 ± 8.73	255.4 ± 58.48	706.6 ± 179.6

[a] Mean and SD of 14 patients, 14 parents and 14 controls.
* Significantly different from controls ($p < 0.05$).
** Significantly different from parents and controls ($p < 0.05$).

cells) of the FA patients are significantly higher compared to the control group ($p = 0.01$; see Supplementary tables for p values) indicating genomic instability in the FA patients. The mean MNi values observed in BN cells irradiated with 2 Gy and 4 Gy are significantly higher in the patient group when compared to the control group (2 Gy: $p = 0.0003$; 4 Gy: $p = 0.0025$); however, MN frequencies between patients and controls overlap at an individual level. Significant differences in radiation-induced MNi are also observed between FA homozygotes and FA heterozygous carriers (2 Gy: $p = 0.02119$; 4 Gy: $p = 0.0444$). Although the MN yields were higher in the heterozygous carriers compared to controls, no significant differences in either the spontaneous or irradiated MNi values were demonstrated. In the 2 and 4 Gy irradiated samples respectively, a 1.5 and 1.3 fold increase of MNi was observed in the FA patients compared to controls. The parents showed a 1.1 fold increase both by 2 and 4 Gy radiations. The mean NDI values of the G0 cultures were lower in patients when compared to parents and controls. This difference was significant in irradiated cultures ($p < 0.05$).

3.3. S/G2 Micronucleus assay

Table 2 represents the response to DNA damage induction in the S/G2 phase of the cell cycle, the MNi values of cells irradiated in the S/G2 phase, in the presence of caffeine were compared between the 3 groups. In this phase of the cell cycle, spontaneously occurring MNi values in the FA patients and interestingly also in the heterozygous carriers were significantly higher compared to the control group. The differences were more pronounced than in the G0 phase. FA patients showed significant higher radiation-induced MN values than the heterozygous carriers and controls. Also, when adding caffeine we noticed significant differences in MNi between the FA patients and the control group after 2 Gy and 4 Gy. Similar to the G0 MN assay results, significant differences were observed at a group level. When comparing the FA heterozygotes with the control group significant differences were seen after irradiation with 4 Gy and addition of caffeine. In response to IR and caffeine, proliferative capacity of FA cells in the G2 phase was lower when compared to the controls ($p < 0.05$). For radiation-induced samples without caffeine, FA patients had a 1.9 and 2.1 fold increase in MNi values for the 2 and 4 Gy respectively, as compared to the controls. The fold increase in FA heterozygote carriers was 1.2 for both the 2 and 4 Gy radiation-induced samples. MNi values of radiation-induced samples in the presence of caffeine for both FA patients and parents were similar to the fold increase as those without caffeine.

Table 3
MMC MN assay: Comparison of mean ± SD spontaneous (0 μg/ml) and MMC-induced (at 0.02 and 0.1 μg/ml) MNi values in 1000 binucleated cells of FA patients, parents and healthy controls.

	0 μg/ml	0.02 μg/ml	0.1 μg/ml
[a]Patients.	30.77 ± 10.87**	466.30 ± 218.40**	[b]979.00 ± 348.10**
[a]Parents	21.42 ± 8.88	42.45 ± 21.54	[b]256.80 ± 90.09*
[a]Controls	17.69 ± 8.21	31.54 ± 17.76	101.10 ± 34.45

[a] Mean and SD of 13 FA patients, 12 parents and 13 controls.
[b] Mean and SD of 6 FA patients and 9 parents.
* Significantly different from controls ($p < 0.05$).
** Significantly different from parents and controls ($p < 0.05$).

3.4. Mitomycin C micronucleus assay

In order to quantify the sensitivity to DNA crosslinking agents, cells were subjected to MMC. This assay detects MMC-induced damage during the G0 phase of the cell cycle. The results of this assay are shown in Table 3. Spontaneous MNi values of patients obtained in the sham treated cultures set up together with the radiation and MMC treated cultures are similar as expected (Table 1 and Table 3). Both concentrations of MMC induced significantly higher MNi in the FA patients compared to the FA heterozygotes and control group. The higher concentration of MMC even resulted in significant differences in MNi between the FA heterozygotes and the controls ($p < 0.0001$). On average, a 9.7 fold increase of MNi was observed in the FA homozygotes compared to the control group and a 2.5 fold increase of MNi in the parents versus the control group was seen after treatment with the higher concentration of MMC (0.1 μg/ml).

Fig. 1 demonstrates the individual fold increase in FA patients and parents versus the mean of the control population for 0.02 μg/ml and 0.1 μg/ml MMC concentrations; also illustrating similar results in *FANCG* and *FANCA* patients. The horizontal lines represent one standard deviation (1SD) and two standard deviations (2SD) of the mean. As presented in Fig. 1, the fold increase can be utilised to identify FA patients successfully with MMC treatment of 0.02 μg/ml as all patients have a fold increase above the 2SD. A better discrimination between parents and controls at the individual level was demonstrated when 0.1 μg/ml MMC concentration was used and parents show a fold increase above 1SD. However, with the higher dose of 0.1 μg/ml MMC, more experimental failures were observed in the patient group. FA patients also showed significantly lower NDI values following MMC treatment ($p < 0.05$); the NDI values were further decreased when FA cells were treated with larger concentrations of MMC ($p < 0.05$).

4. Discussion

In this study we applied different MN assays in patients diagnosed with Fanconi Anaemia and their parents. We compared the results of these two groups with a healthy control group. The genotypes in all patients were determined and showed biallelic inactivation of *FANCG* in the South African patients or *FANCA* in a patient of Cameroonian descent. Carriership testing confirmed heterozygosity for a deleterious *FANCG/FANCA* mutation in the parents. Our study corroborates the

Table 2
G2 MN assay: Comparison of mean ± SD of spontaneous (0 Gy) and radiation-induced (at 2 and 4 Gy) MNi values in 1000 binucleated cells of FA patients, parents and healthy controls.

	0 Gy	2 Gy	4 Gy	2 Gy C+	4 Gy C+
[a]Patients	27.14 ± 9.16**	91.00 ± 40.67**	198.80 ± 73.18**	262.80 ± 121.10**	494.60 ± 257.30*
[a]Parents	18.36 ± 8.57*	57.21 ± 19.54	117.80 ± 31.58	147.40 ± 66.12	346.80 ± 144.70*
[a]Controls	11.50 ± 6.01	48.36 ± 14.51	95.43 ± 32.42	112.20 ± 40.67	248.20 ± 89.61

[a] Mean and SD of 14 patients, 14 parents and 14 controls.
* Significantly different from controls ($p < 0.05$).
** Significantly different from parents and controls ($p < 0.05$).

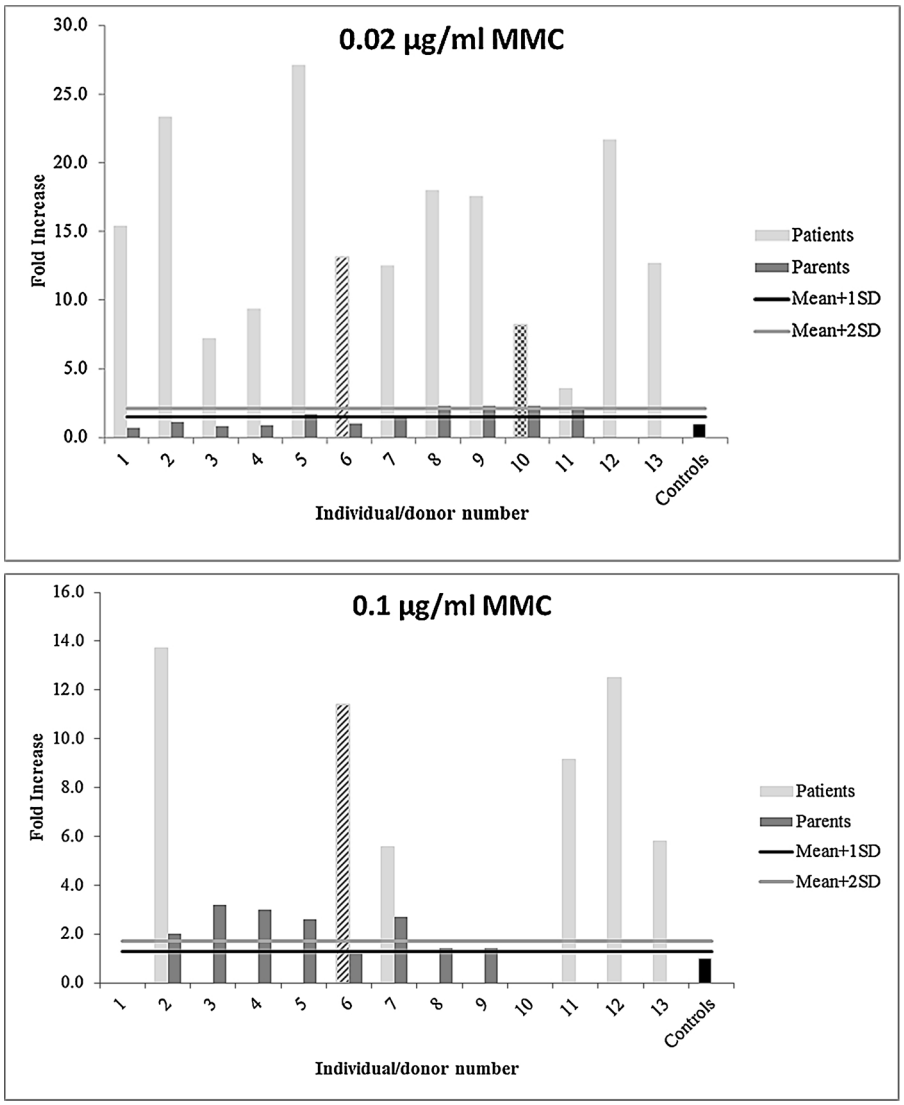

Fig. 1. Fold increase of MNi following MMC treatment. The individual fold increase of FA patients and parents versus mean of controls for concentrations of 0.02 µg/ml and 0.1 µg/ml MMC is presented as bars. The horizontal lines represent one standard deviation (1SD) and two standard deviations (2SD) of the mean of controls. Bar with diagonal lines (donor 6): *FANCG* compound heterozygote patient; bar with checked blocks (donor 10): *FANCA* compound heterozygote patient. All other patients are homozygous for *FANCG*.

high prevalence of the *FANCG* c.637_643delTACCGCC founder mutation in black FA patients [2,28,29]. However, a portion of black FA individuals present with a heterozygous mutation [30]; this requires additional screening of the *FANCG* gene in first instance and other FA genes, if no second deleterious mutation in *FANCG* can be identified. We report here a novel *FANCG* missense variant c.1379G > A, p.(Gly460Asp) which is likely pathogenic, in a black South African patient heterozygous for *FANCG* c.637_643delTACCGCC. The patient had a clear clinical diagnosis of FA and the applied assays showed similar aberrant values like in individuals homozygous for *FANCG* c.637_643delTACCGCC, indicative for a deleterious effect of this variant. Carriership testing in the mother confirmed heterozygosity for the *FANCG* c.637_643delTACCGCC mutation and absence of c.1379G > A, p.(Gly460Asp); DNA of the father was not available. This observation supports the hypothesis that both variants are most likely *in trans* in the child. Our study confirms that besides the founder mutation *FANCG* c.637_643delTACCGCC a second common *FANCG* mutation is unlikely in the black South African population [31].

We evaluated three MN assays to identify FA patients, as these may be more robust than the classically applied chromosome-breakage analysis on Giemsa-stained metaphases of lymphocytes/fibroblasts after addition of MMC or DEB. We induced MNi by addition of MMC in G0 cells or by irradiation of cells in G0 or S/G2 phase of the cell cycle. NHEJ is the preferentially used DNA repair pathway for dsb in the G0 phase while in S/G2 phase HR plays an important role. The FA/BRCA pathway is involved in DNA dsb repair by HR and in the processing of DNA damage induced by MMC.

In first instance, we evaluated spontaneously occurring MNi in two different phases of the cell cycle. The FA patients' exhibit, on average, significantly higher spontaneous MNi frequencies in the G0 phase compared to controls and heterozygous parents. This confirms the report of Camelo et al. and suggests genomic instability in FA patients [32]. With the S/G2 MN assay we also observed on average significantly more spontaneous MNi in both patients and heterozygous parents compared to controls. This could be attributed to homozygous or heterozygous defects in a FA gene, involved in the HR pathway which is preferentially used during the S/G2 phase of the cell cycle. However, due to the heterogeneity in spontaneous MNi it was not possible to consistently demonstrate higher spontaneous MNi values in each individual FA patient/parent compared to controls.

Subsequently, we scored MNi in irradiated lymphocytes of FA patients, FA parents and controls. Our results show significantly higher mean MNi frequencies in irradiated lymphocytes of patients in both phases of the cell cycle when compared to heterozygotes and controls. Loss-of-function in the FA genes causes disruptions in cell cycle checkpoints which could explain the enhanced sensitivity in irradiated FA cells. Increased radiosensitivity in FA cells was also observed in older and much smaller studies performed on an average of 4 FA

patients [18,33,17]. (missing a reference here; reference is mentioned in query notes).

Overall, the mean differences are larger in S/G2 MNi frequencies than the mean differences in G0 MNi frequencies between FA patients, FA heterozygous carriers and controls. A previous study has also shown a similar trend in chromosomal radiosensitivity in the G2 phase [34]. This could be due to the impaired repair pathways and checkpoints and accumulation of unrepaired DNA damage in the G2 phase in FA cells. Upon exposure to exogenous or endogenous damage, the number of FA cells in the G2 phase increases and is attributed to the S-phase checkpoint inefficiency [35]. This is reflected in our observation by the lower NDI values in FA patients when compared to controls that is also in agreement with previous studies describing poorer proliferative capacity and a prolonged G2 phase in FA lymphocytes [19]. In a normal cell cycle, the cells with unrepaired damage are blocked by G2/M checkpoint in order to repair the damage prior to division. In G2, the damaged cells are repaired by HR [36]. This characteristic is absent in the FA cells as they are thought to override the G2/M checkpoint and a significant quantity of unrepaired DNA is carried through to mitosis. Therefore FA cells have accumulated a greater number of damaged DNA and are highly prone to cancer [37].

The understanding of the FA pathway is still evolving and new components continue to be identified. The FA core complex is normally formed during S/G2 phases and cells preferentially use HR during this phase of the cell cycle. However, recent studies described cells derived from patients with FA mutations in the FA core complex that were not severely defective in HR repair of dsb and FA core complex-deficient cells that had impaired HR pathway and thereby favoured NHEJ [38,39]. These controversial findings evoke further investigation of the connections between the FA pathway and the DNA repair pathways.

The ability of caffeine to induce DNA damage and G2/M cell cycle checkpoint abrogation was used in combination with the S/G2 MN assay in our study. The higher number of MNi in all three groups after treatment of the cells with IR and caffeine confirmed this effect of caffeine on the induction of MNi. The results obtained with the S/G2 MN assay in combination with caffeine also showed higher MN values in the FA homozygotes compared to heterozygotes and controls, supporting the study of [40] where it was stated that cells deficient in *FANCD2* developed higher levels of γ-H2AX foci when exposed to caffeine and where was suggested that patients with germline or somatic *FANCD2* mutations may be hypersensitive to cytotoxicity of coffee [40]. Although our FA patients are carriers of *FANCG/FANCA* mutations, we also noticed elevated radiation-induced MNi values. Inactivation of *FANCG* attributes to a functional collapse of the FA core complex and reduces DNA damage repair capacity. The MN values in heterozygous carriers are higher but not significantly different from the values obtained in the control group after 2 Gy irradiation in combination with caffeine. However, after a 4 Gy dose of IR and caffeine we found significantly higher levels of residual DNA damage suggesting that less damage is repaired after exposure to caffeine and IR even when a functional allele of the FA gene is available.

As a group, FA patients showed significantly elevated radiosensitivity with the G0 and S/G2 MN assays compared to parents and controls. However, the assays are not suitable as a biomarker for detecting individual FA patients. Since the risk of cancer is elevated in FA patients, radiosensitivity information in this patient group is relevant and may be taken into consideration prior to start treatment using IR.

The MMC MN assay is very promising as an individual biomarker (Table 3 and Fig. 1). Even though cells were treated with low MMC concentrations, a clear distinction in MNi frequencies between FA homozygotes, FA heterozygotes and controls was observed. However, only a low number of binucleated cells were detectable in cells of some FA patients treated with the highest 0.1 μg/ml concentration of MMC. This is attributed to the toxic nature of MMC that induces unrepairable interstrand- crosslinks which lowers cell proliferation of FA cells as reflected in the NDI values. Despite the poor cell proliferation, we were able to produce conclusive results to identify FA patients. The test is more robust when applying only 0.02 μg/ml and is optimal for distinguishing individual FA patients from individual controls and parents. Evaluating metaphases in chromosomal-breakage analysis of MMC or DEB requires a trained eye and can be labour intense. In comparison, scoring MNi using the MMC MN assay is amenable to automation, it is relatively straightforward and time efficient. Therefore, we think that our MMC MN assay has the potential for a reliable alternative for the classic chromosome breakage assay.

The majority of our FA patients are homozygous for the *FANCG* founder mutation. Our cohort also includes two patients that are compound heterozygotes; one patient with two mutations in *FANCG* and the other with two mutations in *FANCA*. The phenotype observed in the *FANCG* homozygous patients are also demonstrated in the *FANCG* and *FANCA* compound heterozygous patients. The observation suggests that the phenotype is valid for both the FANC genes that form part of the FA core complex. Although, further validations in larger patient and parent groups with different FANC genotypes are required prior to implementation as a standard test in clinical setting.

Interestingly, the application of higher MMC concentrations in our MMC MN assay revealed significant differences between the group of FA parents and the control group. This distinction could not be made with a MMC-based chromosome breakage assay [41]. Similarly, identifying FA parents from controls using the DEB test has shown to be unsuccessful with chromosomal breaks of parents overlapping with controls [42,43].

Identification of heterozygous mutation carriers may be clinically relevant in the context of breast cancer prevention as heterozygous mutations in several FA genes were shown to be associated with an increased risk for breast cancer [44–49]. The best known breast cancer gene, *BRCA1 (=FANCS)* has been shown to interact directly with *FANCD2* and *FANCA* in response to DNA damage [50]. Similar to our study, the damaging effects of IR and MMC were evaluated in lymphocytes from heterozygous *BRCA1/FANCS* and *BRCA2/FANCD1* mutation carriers (breast cancer patients and healthy carriers). In response to IR, the heterozygous carriers of both genes did not show elevated MNi values, but upon exposure to MMC enhanced chromosomal sensitivity was detected in lymphocytes of individuals with heterozygous *BRCA2* mutations [51]. It is clear that larger studies are warranted to validate our findings but the MMC MN assay may be promising as an individual biomarker for functional deficiencies in at least some of the FA genes.

5. Conclusions

In conclusion, this study compared 3 different MN assays to characterize FA patients with biallelic inactivated *FANCA/FANCG* alleles and to compare the results with heterozygous carriers of *FANCA* and *FANCG* mutations and controls. Chromosomal radiosensitivity of the FA patients was demonstrated with the G0 and S/G2 MN assay. The MN assay utilising IR showed on average an increased number of MNi in FA patients and heterozygous carriers compared to controls, but cannot be used to identify individual patients. Using the MMC MN assay, we were able to distinguish FA patients from heterozygous carriers and controls. Interestingly, with this assay, higher MNi scores were obtained in the heterozygous parents compared to controls. Further studies are warranted to evaluate the sensitivity and specificity of this test in FA patients and parents with other genotypes/mutated FANC genes. The big advantage of the MMC MN assay is that is less labour intense, more time effective and less subjective compared to the classic chromosomal breakage assay. Future research can indicate if this assay can differentiate FANC heterozygote genotypes from healthy controls and can be used as a functional assay identifying individuals with a heterozygous defect in genes related to the FA/BRCA pathway.

Conflict of interest statement

The authors declare there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.dnarep.2017.11.001.

References

[1] P.S. Rosenberg, H. Tamary, B.P. Alter, How high are carrier frequencies of rare recessive syndromes? Contemporary estimates for Fanconi anemia in the United States and Israel, Am. J. Med. Genet. A 155A (2011) 1877–1883.
[2] N.V. Morgan, F. Essop, I. Demuth, T. De Ravel, S. Jansen, M. Tischkowitz, C.M. Lewis, L. Wainwright, J. Poole, H. Joenje, M. Digweed, A. Krause, C.G. Mathew, A common Fanconi anemia mutation in black populations of sub-Saharan Africa, Blood 105 (2005) 3542–3544.
[3] A.J. Tipping, T. Pearson, N.V. Morgan, R.A. Gibson, L.P. Kuyt, C. Havenga, E. Gluckman, H. Joenje, T. DE Ravel, S. Jansen, C.G. Mathew, Molecular and genealogical evidence for a founder effect in Fanconi anemia families of the Afrikaner population of South Africa, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 5734–5739.
[4] H. Dong, D.W. Nebert, E.A. Bruford, D.C. Thompson, H. Joenje, V. Vasiliou, Update of the human and mouse Fanconi anemia genes, Human Genomics 9 (2015) 32.
[5] J.Y. Park, E.L. Virts, A. Jankowska, C. Wiek, M. Othman, S.C. Chakraborty, G.H. Vance, F.S. Alkuraya, H. Hanenberg, P.R. Andreassen, Complementation of hypersensitivity to DNA interstrand crosslinking agents demonstrates that XRCC2 is a Fanconi anaemia gene, J. Med. Genet. 53 (2016) 672–680.
[6] D. Bluteau, J. Masliah-Planchon, C. Clairmont, A. Rousseau, R. Ceccaldi, Dubois D'enghien, C. Bluteau, O. Cuccuini, W. Gachet, S. Peffault de latour, R. Leblanc, T. Socie, G. Baruchel, A. Stoppa-Lyonnet, A.D. D'andrea, J. Soulier, Biallelic inactivation of REV7 is associated with Fanconi anemia, J. Clin. Invest. 126 (2016) 3580–3584.
[7] P.A. Mehta, J. Tolar, et al., Fanconi anemia, in: R.A. Pagon, M.P. Adam, H.H. Ardinger (Eds.), GeneReviews® [Internet], University of Washington, Seattle, Seattle, 2002.
[8] J.D. Brooks, S.N. Teraoka, A.S. Reiner, J.M. Satagopan, L. Bernstein, D.C. Thomas, M. Capanu, M. Stovall, S.A. Smith, S. Wei, R.E. Shore, J.D. Boice Jr., C.F. Lynch, L. Mellemkjaer, K.E. Malone, X. Liang, R.W. Haile, P. Concannon, J.L. Bernstein, Variants in activators and downstream targets of ATM, radiation exposure, and contralateral breast cancer risk in the WECARE study, Hum. Mutat. 33 (2012) 158–164.
[9] R. Ceccaldi, P. Sarangi, A.D. D'andrea, The Fanconi anaemia pathway: new players and new functions, Nat. Rev. Mol. Cell Biol. 17 (2016) 337–349.
[10] J.P. De Winter, H. Joenje, The genetic and molecular basis of Fanconi anemia, Mutat. Res. 668 (2009) 11–19.
[11] T. Walsh, S. Casadei, K.H. Coats, E. Swisher, S.M. Stray, J. Higgins, K.C. Roach, J. Mandell, M.K. Lee, S. Ciernikova, L. Foretova, P. Soucek, M.C. King, Spectrum of mutations in BRCA1 BRCA2, CHEK2, and TP53 in families at high risk of breast cancer, JAMA 295 (2006) 1379–1388.
[12] N.E. Mamrak, A. Shimamura, N.G. Howlett, Recent discoveries in the molecular pathogenesis of the inherited bone marrow failure syndrome Fanconi anemia, Blood Rev. 31 (2017) 93–99.
[13] R.E. Goldsby, S.L. Perkins, D.M. Virshup, A.R. Brothman, C.S. Bruggers, Lymphoblastic lymphoma and excessive toxicity from chemotherapy: an unusual presentation for Fanconi anemia, J. Pediatr. Hematol. Oncol. 21 (1999) 240–243.
[14] B.P. Alter, Radiosensitivity in Fanconi's anemia patients, Radiother. Oncol. 62 (2002) 345–347.
[15] K.K. Khanna, S.P. Jackson, DNA double-strand breaks: signaling: repair and the cancer connection, Nat. Genet. 27 (2001) 247–254.
[16] J. Russo, G.A. Balogh, R. Heulings, D.A. Mailo, R. Moral, P.A. Russo, F. Sheriff, J. Vanegas, I.H. Russo, Molecular basis of pregnancy-induced breast cancer protection, Eur. J. Cancer Prev. 15 (2006) 306–342.
[17] J.A. Heddle, C.B. Lue, E.F. Saunders, R.D. Benz, Sensitivity to five mutagens in Fanconi's anemia as measured by the micronucleus method, Cancer Res. 38 (1978) 2983–2988.
[18] M. Higurashi, P.E. Conen, In vitro chromosomal radiosensitivity in chromosomal breakage syndromes, Cancer 32 (1973) 380–383.
[19] G. Duckworth-Rysiecki, A.M. Taylor, Effects of ionizing radiation on cells from Fanconi's anemia patients, Cancer Res. 45 (1985) 416–420.
[20] A.D. D'andrea, M. Grompe, The Fanconi anaemia/BRCA pathway, Nat. Rev. Cancer 3 (2003) 23–34.
[21] A.B. Oostra, A.W. Nieuwint, H. Joenje, J.P. De Winter, Diagnosis of Fanconi anemia: chromosomal breakage analysis, Anemia 2012 (2012) 238731.
[22] A. Baeyens, H. Thierens, K. Claes, B. Poppe, L. Messiaen, L. De Ridder, A. Vral, Chromosomal radiosensitivity in breast cancer patients with a known or putative genetic predisposition, Br. J. Cancer 87 (2002) 1379–1385.
[23] A. Vral, M. Fenech, H. Thierens, The micronucleus assay as a biological dosimeter of in vivo ionising radiation exposure, Mutagenesis 26 (2011) 11–17.
[24] K. Claes, J. Depuydt, A.M. Taylor, J.I. Last, A. Baert, P. Schietecatte, V. Vandersickel, B. Poppe, K. De Leeneer, M. D'hooghe, A. Vral, Variant ataxia telangiectasia: clinical and molecular findings and evaluation of radiosensitive phenotypes in a patient and relatives, Neuromol. Med. 15 (2013) 447–457.
[25] A. Baert, J. Depuydt, T. Van Maerken, B. Poppe, F. Malfait, K. Storm, J. van den Ende, T. Van Damme, S. De Nobele, G. Perletti, K. De Leeneer, K.B. Claes, A. Vral, Increased chromosomal radiosensitivity in asymptomatic carriers of a heterozygous BRCA1 mutation, Breast Cancer Res. 18 (2016) 52.
[26] G. Gargano, V. Agnese, V. Calo, S. Corsale, C. Augello, L. Bruno, L.A.L. Paglia, A. Gullo, L. Ottini, A. Russo, F. Fulfaro, G. Rinaldi, A. Crosta, G. Cicero, O. Majorana, L. Palmeri, C. Cipolla, A. Agrusa, G. Gulotta, V. Morello, D.I.G. Fede, V. Adamo, G. Colucci, R.M. Tomasino, M.R. Valerio, V. Bazan, Detection and quantification of mammaglobin in the blood of breast cancer patients: can it be useful as a potential clinical marker? Preliminary results of a GOIM (Gruppo Oncologico dell'Italia Meridionale) prospective study, Ann. Oncol. 17 (Suppl. 7) (2006) vii41–vii45.
[27] K. De Leeneer, J. Hellemans, W. Steyaert, S. Lefever, I. Vereecke, E. Debals, B. Crombez, M. Baetens, M. Van Heetvelde, F. Coppieters, J. Vandesompele, A. DE Jaegher, E. DE Baere, P. Coucke, K. Claes, Flexible, scalable, and efficient targeted resequencing on a benchtop sequencer for variant detection in clinical practice, Hum. Mutat. 36 (2015) 379–387.
[28] S.N. Teraoka, J.L. Bernstein, A.S. Reiner, R.W. Haile, L. Bernstein, C.F. Lynch, K.E. Malone, M. Stovall, M. Capanu, X. Liang, S.A. Smith, J. Mychaleckyj, X. Hou, L. Mellemkjaer, J.D. Boice Jr., A. Siniard, D. Duggan, D.C. Thomas, P. Concannon, Single nucleotide polymorphisms associated with risk for contralateral breast cancer in the Women's Environment, Cancer, and Radiation Epidemiology (WECARE) Study, Breast Cancer Res. 13 (2011) R114.
[29] K. Ando, N. Masumoto, M. Sakamoto, K. Teraoka, T. Suzuki, T. Kurihara, S. Abe, M. Tozaki, E. Fukuma, K. Hoshi, Parotid gland metastasis of Breast cancer: case report and review of the literature, Breast Care (Basel) 6 (2011) 471–473.
[30] C. Feben, C. Spencer, A. Lochan, N. Laing, K. Fieggen, E. Honey, T. Wainstein, A. Krause, Biallelic BRCA2 mutations in two black South African children with Fanconi anaemia, Fam. Cancer 16 (2017) 441–446.
[31] T. Wainstein, R. Kerr, C.L. Mitchell, S. Madaree, F.B. Essop, E. Vorster, R. Wainwright, J. Poole, A. Krause, Fanconi anaemia in black South African patients heterozygous for the FANCG c. 637–643delTACCGCC founder mutation, S. Afr. Med. J. 103 (2013) 970–973.
[32] R.M. Camelo, F.S. Kehdy, C.E. Salas, M.T. Lopes, Amifostine protection against mitomycin-induced chromosomal breakage in Fanconi anaemia lymphocytes, Molecules 13 (2008) 1759–1772.
[33] M. Higurashi, P.E. Conen, In vitro chromosomal radiosensitivity in Fanconi's anemia, Blood 38 (1971) 336–342.
[34] S.B. Bigelow, J.M. Rary, M.A. Bender, G2 chromosomal radiosensitivity in Fanconi's anemia, Mutat. Res. 63 (1979) 189–199.
[35] M. Sala-Trepat, D. Rouillard, M. Escarceller, A. Laquerbe, E. Moustacchi, D. Papadopoulo, Arrest of S-phase progression is impaired in Fanconi anemia cells, Exp. Cell Res. 260 (2000) 208–215.
[36] K. Nakanishi, Y.G. Yang, A.J. Pierce, T. Taniguchi, M. Digweed, A.D. D'andrea, Z.Q. Wang, M. Jasin, Human Fanconi anemia monoubiquitination pathway promotes homologous DNA repair, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 1110–1115.
[37] A. Rodriguez, L. Torres, U. Juarez, D. Sosa, E. Azpeitia, B. Garcia-De Teresa, E. Cortes, R. Ortiz, A.M. Salazar, P. Ostrosky-Wegman, L. Mendoza, S. Frias, Fanconi anemia cells with unrepaired DNA damage activate components of the checkpoint recovery process, Theor. Biol. Med. Modell. 12 (2015) 19.
[38] K. Nakanishi, F. Cavallo, E. Brunet, M. Jasin, Homologous recombination assay for interstrand cross-link repair, Methods Mol. Biol. 745 (2011) 283–291.
[39] E. Renaud, A. Barascu, F. Rosselli, Impaired TIP60-mediated H4K16 acetylation accounts for the aberrant chromatin accumulation of 53BP1 and RAP80 in Fanconi anemia pathway-deficient cells, Nucleic Acids Res. 44 (2016) 648–656.
[40] E. Burgos-Moron, J.M. Calderon-Montano, M.L. Orta, E. Guillen-Mancina, S. Mateos, M. Lopez-Lazaro, Cells deficient in the fanconi anemia protein FANCD2 are hypersensitive to the cytotoxicity and DNA damage induced by coffee and caffeic acid, Toxins (2016) 8.
[41] M.M. Cohen, S.J. Simpson, G.R. Honig, H.S. Maurer, J.W. Nicklas, A.O. Martin, The identification of fanconi anemia genotypes by clastogenic stress, Am. J. Hum. Genet. 34 (1982) 794–810.

[42] J. Cervenka, B.A. Hirsch, Cytogenetic differentiation of Fanconi anemia, idiopathic aplastic anemia, and Fanconi anemia heterozygotes, Am. J. Med. Genet. 15 (1983) 211–223.
[43] A. Deviren, N. Yalman, S. Hacihanefioglu, Differential diagnosis of Fanconi anemia by nitrogen mustard and diepoxybutane, Ann. Hematol. 82 (2003) 223–227.
[44] J.I. Kiiski, R. Fagerholm, A. Tervasmaki, L.M. Pelttari, S. Khan, M. Jamshidi, T. Mantere, K. Pylkas, J. Bartek, J. Bartkova, A. Mannermaa, M. Tengstrom, V.M. Kosma, R. Winqvist, A. Kallioniemi, K. Aittomaki, C. Blomqvist, H. Nevanlinna, FANCM c.5101C > T mutation associates with breast cancer survival and treatment outcome, Int. J. Cancer 139 (2016) 2760–2770.
[45] P. Peterlongo, I. Catucci, M. Colombo, L. Caleca, E. Mucaki, M. Bogliolo, M. Marin, F. Damiola, L. Bernard, V. Pensotti, S. Volorio, V. D.A.L.L'olio, A. Meindl, C. Bartram, C. Sutter, H. Surowy, V. Sornin, M.G. Dondon, S. Eon-Marchais, D. Stoppa-Lyonnet, N. Andrieu, O.M. Sinilnikova, G. Mitchell, P.A. James, E. Thompson, M. Marchetti, C. Verzeroli, C. Tartari, G.L. Capone, A.L. Putignano, M. Genuardi, V. Medici, I. Marchi, M. Federico, S. Tognazzo, L. Matricardi, S. Agata, R. Dolcetti, L. Della Puppa, G. Cini, V. Gismondi, V. Viassolo, C. Perfumo, M.A. Mencarelli, M. Baldassarri, B. Peissel, G. Roversi, V. Silvestri, P. Rizzolo, F. Spina, C. Vivanet, M.G. Tibiletti, M.A. Caligo, G. Gambino, S. Tommasi, B. Pilato, C. Tondini, C. Corna, B. Bonanni, M. Barile, A. Osorio, J. Benitez, L. Balestrino, L. Ottini, S. Manoukian, M.A. Pierotti, A. Renieri, L. Varesco, F.J. Couch, X. Wang, P. Devilee, F.S. Hilbers, C.J. Van Asperen, A. Viel, M. Montagna, L. Cortesi, O. Diez, J. Balmana, J. Hauke, R.K. Schmutzler, L. Papi, M.A. Pujana, C. Lazaro, A. Falanga, K. Offit, J. Vijai, I. Campbell, B. Burwinkel, A. Kvist, H. Ehrencrona, S. Mazoyer, S. Pizzamiglio, P. Verderio, J. Surralles, P.K. Rogan, P. Radice, FANCM c.5791C > T nonsense mutation (rs144567652) induces exon skipping: affects DNA repair activity and is a familial breast cancer risk factor, Hum. Mol. Genet. 24 (2015) 5345–5355.
[46] M.U. Rashid, N. Muhammad, F.A. Khan, U. Hamann, Absence of the FANCM c. 5101C > T mutation in BRCA1/2-negative triple-negative breast cancer patients from Pakistan, Breast Cancer Res. Treat. 152 (2015) 229–230.
[47] S. Seal, D. Thompson, A. Renwick, A. Elliott, P. Kelly, R. Barfoot, T. Chagtai, H. Jayatilake, M. Ahmed, K. Spanova, B. North, L. Mcguffog, D.G. Evans, D. Eccles, D.F. Easton, M.R. Stratton, N. Rahman, Truncating mutations in the Fanconi anemia J gene BRIP1 are low-penetrance breast cancer susceptibility alleles, Nat. Genet. 38 (2006) 1239–1241.
[48] K. Somyajit, S. Subramanya, G. Nagaraju, RAD51C: a novel cancer susceptibility gene is linked to Fanconi anemia and breast cancer, Carcinogenesis 31 (2010) 2031–2038.
[49] R.M. Tamimi, S.J. Schnitt, G.A. Colditz, L.C. Collins, Luminal B breast tumors are not HER2 positive – author's response, Breast Cancer Res. 10 (2008) 405.
[50] A. Folias, M. Matkovic, D. Bruun, S. Reid, J. Hejna, M. Grompe, A. D'andrea, R. Moses, BRCA1 interacts directly with the Fanconi anemia protein FANCA, Hum. Mol. Genet. 11 (2002) 2591–2597.
[51] S. Gutierrez-Enriquez, Y.C.T. Ramon, C. Alonso, A. Corral, P. Carrasco, M. Cornet, J. Sanz, M. Ribas, M. Baiget, O. Diez, Ionizing radiation or mitomycin-induced micronuclei in lymphocytes of BRCA1 or BRCA2 mutation carriers, Breast Cancer Res. Treat. 127 (2011) 611–622.