UDC 575
DOI: 10.2298/GENSR1302393J
Original scientific paper

ENHANCED FREQUENCY OF SISTER CHROMATID EXCHANGES INDUCED BY DIEPOXYBUTANE IS SPECIFIC CHARACTERISTIC OF FANCONI ANEMIA CELLULAR PHENOTYPE

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Joksić I, S. Petrović, A. Leskovac, J Filipović, M.Guč-Šćekić, D.Vujić and G. Joksić (2013): *Enhanced frequency of sister chromatid exchanges induced by diepoxybutane is specific characteristic of Fanconi anemia cellular phenotype*.Genetika, Vol 45, No. 2, 393-403.

Fanconi anemia (FA) is a rare genetically heterogeneous disease characterized by developmental abnormalities, progressive bone marrow failure, and cancer susceptibility. We examined spontaneous, diepoxybutane (DEB)-induced and radiation-induced sister chromatid exchanges (SCEs) in whole-blood lymphocyte cultures of bone marrow failure (BMF) patients including Fanconi anemia, mothers of affected individuals, and healthy controls. The base-line frequency of SCE in FA cells was similar to that observed in controls. However, in response to DEB SCE frequencies in FA patients and their mothers were significantly increased compared to both non-FA BMF families and healthy controls. In response to ionizing radiation, cells displayed increased frequency of SCE, but no differences between FA patients and non-FA BMF patients were seen. Our data confirm and expand previous findings by showing that SCE induced by DEB can be used as an adjunct diagnostic test not only for FA patients, but also for female heterozygous carriers, at least for complementation groups FANCA and FANCD2.

Key words: diepoxybutane, Fanconi anemia lymphocytes, ionizing radiation, sister chromatid exchange

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INTRODUCTION

The Fanconi anemia (FA) molecular network consists of at least 15 FANC proteins identified thus far (HUCL and GALLMEIER, 2011). Studies on their functions revealed their main role in protecting against genotoxic stress by forming complexes with each other. However, all that proteins also participate in survival signaling pathways in hematopoietic and germ cells, and that second function does not necessarily require the nuclear complex formation. At least four FANC proteins (FANCA, FANCC, FANCG and FANCD2) have dual roles in distinct cellular pathways that respond to replication fork damage and oxidative stress (KONTOU *et al.*, 2003; MUKHOPADHYAY *et al.*, 2006; WILLERS *et al.*, 2008).

FA is characterized by variability of clinical phenotypes (JOENJE and PATEL, 2001; YAMASHITA and NAKAHATA, 2001), but the common, historically known feature associated with FA mutation is marked chromosomal instability (GRANT *et al.*, 2005) manifested as chromatid breaks and chromatid exchanges (SCHROEDER *et al.*, 1964). About 10 years after the discovery of spontaneous chromosomal instability as cytogenetic hallmark of FA, Sasaki and Tonomura (SASAKI and TONOMURA, 1973) showed its association with a high rate of induced chromosomal aberrations after treatment with DNA cross-linking agents, such as diepoxybutane (DEB) or mitomycin C (MMC). As the management of patients with FA differs from that of idiopathic bone marrow failure (BMF) syndromes, it is essential to differentiate these disorders at the earliest. The determination of cellular sensitivity towards DEB still serves as a gold standard for the diagnosis and exclusion of Fanconi anemia.

During the last decade, several *in vitro* assays have been developed to facilitate diagnosis of FA (LESKOVAC *et al.*, 2010; JOKSIC *et al.*, 2012). It is known that FA heterozygotes cannot be detected by the DEB test because their results are within the normal range (AUERBACH, 2003). Similar to that, Mohseni-Meybodi *et al.* (MOHSENI-MEYBODI *et al.*, 2007) underlined that differentiation between obligate carriers and the control group, based on chromosomal aberration analysis after MMC treatment and gamma irradiation is impossible. On the contrary, Barquinero *et al.* (BARQUINERO *et al.*, 2001) reported significantly higher incidence of bleomycin-induced chromatid breaks in G_2 lymphocytes of FA heterozygotes. Besides metaphase analysis (AUERBACH, 2003), sensitivity of FA cells to cross-linking agents or ionizing radiation usually was determined using cell cycle examination (SEYSCHAB *et al.*, 1995), comet assay (DJUZENOVA and FLENTJE, 2002), etc.

The sister chromatid exchange (SCE) analysis is used extensively to evaluate the presence and extent of chromosomal damage in human populations (TUCKER and PRESTON, 1996). Moreover, SCE is much more sensitive as a mutagenic biomarker than chromosomal aberrations and micronuclei (CINKILIC *et al.*, 2009). Spontaneous SCE frequencies are believed to be normal in FA patients, but studies on induced SCE have yielded conflicting results. Some studies using DEB found no considerable changes in SCE levels in FA cells (PORFIRIO *et al.*, 1983; GEBHART *et al.*, 1985), but example of increased level induced by MMC is also noted (MIURA *et al.*, 1983). However, it is worth noting that DEB and MMC differ in the mechanisms underlying their toxicity (PAGANO and KORKINA, 2000). In addition, most of the studies involving sensitivity of FA cells to cross-linking agents were performed without knowing patients' complementary groups. The results of our recent work have shown an *in vivo* prooxidant state of cells in FANCA and FANCD2 patients and clearly indicate that FA patients can be distinguished from idiopathic BMF patients based on the oxidant state of cells. Similar prooxidant state of cells was noted in female heterozygous carriers, but in none of the male

carriers (PETROVIC *et al.*, 2011). Knowing that the DEB-associated toxicities involve redox mechanisms, we analyzed DEB- induced SCE frequencies in FA patients and their mothers in order to ascertain whether or not that parameter can be used as an adjunct diagnostic tool for at least FANCA and FANCD2 complementation groups. In addition, frequencies of SCE induced by ionizing radiation are also examined.

MATERIALS AND METHODS

Study subjects. Subjects included in this study were twelve patients (six boys and six girls) with BMF of unknown etiology (non-FA BMF), five Fanconi anemia patients (one boy and four girls), and their parents. The patients were aged 8±5 years. Hematological parameters were within the range typical of bone marrow failure patients. Clinical diagnosis of FA was supported by cellular hypersensitivity to diepoxybutane (AUERBACH, 2003). The FA complementation group analysis (Universität Würzburg, Institut für Humangenetik, Germany and Department of Genetics Microbiology, Universitat Autonoma de Barcelona, Spain) showed that four patients (girls) belong to the complementation group FANCD2, whereas one patient (boy) belongs to the complementation group FANCA. The study was approved by the Ethical Committee of the Mother and Child Health Care Institute of Serbia, and parents of patients signed an informed consent regarding this investigation. A control group was consisted of twelve healthy volunteers with normal blood profiles. Peripheral blood samples were obtained in accordance with current Health and Ethical Regulations in Serbia.

Blood cultures. Aliquots of heparinized whole blood (0.5 mL) of patients, their parents and controls were added to culture tubes containing 4.5 mL of a PBmax karyotyping medium (Invitrogen-Gibco, Paisley, UK). Three lymphocyte cultures from each subject were set up for assessment of sister-chromatid exchanges under different conditions of treatment: one culture served as a base-line control, the second one was treated with DEB, while the third was set up using irradiated peripheral blood samples.

Sister chromatid exchange analysis. The SCE analysis was performed following the addition of 5-bromo-2-deoxyuridine (BrdUrd) (Sigma Chemicals Co., Germany) at a final concentration of 15 μg/mL to achieve sister chromatid differentiation. BrdUrd was added one hour after culture initiation, and cells were incubated for 72 hours; the last three hours in a presence of Colchicine (Sigma Chemicals Co., Germany) at a final concentration of 2.5 μg/mL. The cells were harvested and processed through treatments with a hypotonic solution (0.56% KCl) and fixative (3:1 methanol:glacial acetic acid). Differential staining of sister chromatids was performed by the standard fluorescence-plus-Giemsa technique (PERRY and WOLFF, 1974). A total of 50 well-spread and complete second-division metaphases per culture were scored for SCE using a Ziess-Axioplan2 microscope (Carl Zeiss, Jena, Germany). The frequency of SCE per cell was recorded.

DEB treatment. Lymphocyte cultures were treated with diepoxybutane (Sigma Chemicals Co., Germany) (final concentration 0.1μg/mL) one hour after culture initiation and incubated for 72 hours.

Irradiation. Heparinized whole blood was irradiated using a ^{60}Co γ-ray source. The radiation dose employed was 2 Gy, the dose rate was 0.45 Gy/min, the dimensions of the radiation field were 20 × 20 cm, and the distance from the radiation source was 74 cm. Aliquots of irradiated whole blood (0.5mL) were set up into cultures and incubated for 72 hours.

Statistical analysis. A statistical analysis was carried out using statistical software package Statistics, version 6 for Microsoft Windows. Student's *t* test was used, and the *p* value < 0.05 was considered to be significant.

RESULTS

In this study, we analyzed frequencies of SCE in lymphocytes obtained from FA patients, non-FA BMF patients, their mothers, and healthy controls. Individual data of baseline, DEB-induced and radiation-induced frequencies of SCE are presented on Tables 1-3. The results of the current study showed that no significant difference in spontaneous frequencies of SCE between FA patients, non-FA BMF patients and healthy controls was observed (Table 4). The similar results were seen when mothers of FA and non-FA BMF patients, and control were analyzed.

Table 1. The frequencies of SCE in lymphocytes of FA patients and their mothers.

FA group	Patients	Baseline	DEB -induced	Radiation-induced	Mothers of patients	Baseline	DEB - induced	Radiation-induced
FANCD2	1	3.33±1.78	4.79±2.24	5.20±2.12	1a	3.83±1.62	3.96±1.65	5.30±2.19
	2	5.29±0.80	6.05±3.22	6.72±3.13	2a	3.71±1.78	5.65±2.06	6.78±1.73
	3	5.14±1.46	6.38±2.93	6.32±2.30	3a	4.00±2.16	6.00±1.97	6.70±1.75
	4	3.71±1.68	6.82± 2.77	6.20±2.80	4a	3.97±2.04	6.57±2.03	6.73±2.06
FANCA	5	3.52±2.53	7.06±3.26	6.97±3.00	5a	3.78±2.03	5.45±2.34	6.40±2.76

Table 2. The frequencies of SCE in lymphocytes of non-FA BMF patients and their mothers.

non-FA BMF patients	Baseline	DEB - induced	Radiation-induced	Mothers of patients	Baseline	DEB - induced	Radiation-induced
6	3.80±2.05	6.71±2.37	5.38±3.58	6a	4.83±2.07	5.67±2.29	5.51±1.62
7	3.87±2.62	7.50±2.72	5.32±2.93	7a	3.36±2.09	4.20±2.35	5.53±2.46
8	4.06±2.29	3.57±3.10	5.97±2.73	8a	3.80±1.80	4.35±2.36	6.12±1.63
9	5.06±2.84	3.93±3.67	6.94±3.26	9a	3.93±1.64	4.21±2.68	4.85±1.93
10	3.70±2.05	3.57±1.78	5.69±2.45	10a	3.57±2.31	4.79±3.77	5.80±2.38
11	3.91±2.47	3.81±2.45	5.35±2.88	11a	4.67±2.51	5.03±2.42	7.02±2.01
12	4.97±2.59	3.64±2.18	5.84±2.05	12a	3.97±1.81	5.04±2.41	6.90±1.90
13	5.54±2.93	4.65±2.46	5.87±2.86	13a	4.57±3.17	4.79±2.44	7.14±2.05
14	3.13±1.3	4.16±2.27	6.19±2.15	14a	4.39±2.16	5.17±3.33	7.25±2.68
15	3.41±2.71	1.81±0.88	6.63±2.29	15a	3.54±1.49	2.23±1.16	6.56±2.35
16	2.79±2.45	2.07±1.79	6.09±2.04	16a	3.77±2.32	1.83±0.94	7.24±1.94
17	3.30±1.62	2.86±1.90	5.50±2.28	17a	4.27±1.51	2.17±1.37	5.06±1.52

Table 3. The frequencies of SCE in lymphocytes of healthy controls.

Control subjects	Baseline	DEB -induced	Radiation- induced
18	4.90±3.07	4.43±1.39	5.29±2.63
19	3.90±1.56	3.97±1.26	4.67±1.45
20	3.07±1.37	2.27±1.29	3.97±1.61
21	4.08±2.72	5.52±3.46	5.45±2.93
22	4.87±2.06	4.26±2.78	4.85±1.82
23	5.67±2.28	4.60±1.31	5.95±2.21
24	6.27±2.54	5.67±3.64	6.67±2.44
25	4.81±2.27	3.37±1.64	5.55±2.32
26	3.19±1.31	2.13±1.32	5.95±1.58
27	5.53±2.08	4.43±1.62	5.90±2.09
28	4.25±2.12	3.19±1.62	5.53±2.24
29	5.84±2.33	5.12±2.75	6.80±2.39

Table 4. The baseline, DEB-induced and radiation-induced frequencies of SCE (mean ± S.D) in FA and non-FA BMF patients, their mothers, and healthy controls.

	Baseline frequency of SCE	*t* test	Frequency of DEB – induced SCE	*t* test	Frequency of radiation-induced SCE	*t* test
Control	4.70±1.03	-	4.08±1.15	-	5.55±0.80	-
FA patients	4.20±0.94	*ns **ns	6.22±0.89	$^{*}p < 0.05$ $^{**}p < 0.05$	6.28±0.68	*ns **ns
Mothers of FA patients	3.86±0.12	*ns **ns	5.53±0.97	$^{*}p < 0.05$ $^{**}p < 0.05$	6.38±0.62	*ns **ns
non-FA BMF patients	3.96±0.83	*ns	4.02±1.66	*ns	5.90±0.51	*ns
Mothers of non-FA BMF patients	4.06±0.48	*ns	4.12±1.30	*ns	6.25±0.88	*ns

*comparison with control, **comparison with non-FA BMF, ns- no significant

However, in response to DEB FA patients displayed the highest frequency of SCE, over 50% higher compared to both non-FA BMF patients and control, and 48% higher than the untreated self-control ($p < 0.05$) (Figure 1).

The data obtained from FA female carriers showed the similar pattern as they had significantly higher DEB-induced frequency of SCE compared to both mothers of non-FA BMF patients and control. In the same way, mothers of FA patients displayed 43% higher frequency of SCE compared to self-state before DEB treatment ($p < 0.05$).

On the other hand, statistical analysis failed to show any significant differences in DEB-induced SCE frequencies in non-FA BMF families. The frequency of DEB-induced SCE in healthy controls was significantly reduced ($p < 0.05$) compared to untreated self-control (Figure 1). Figure 2 shows metaphases displaying 13 SCE and asymmetrical chromatid exchange in FA cells treated with DEB.

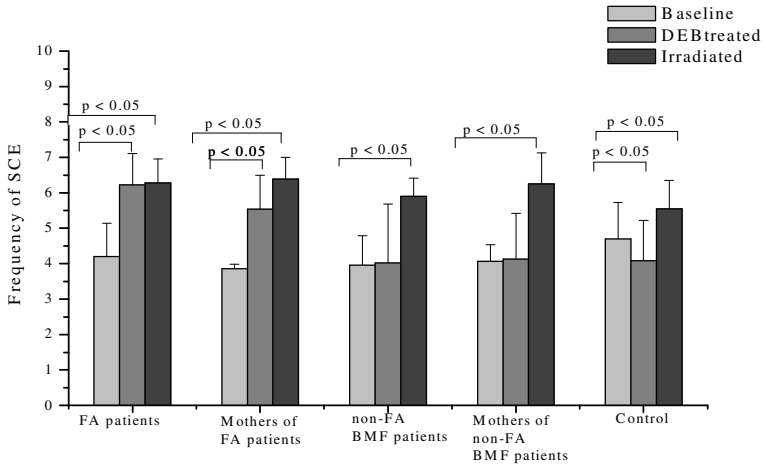

Figure 1. The frequency of SCE induced by DEB and ionizing radiation (mean ± S.D) in FA and non-FA BMF patients, their mothers and controls.

Figure 2. Representative metaphases analyzed for SCE: (A) SCE at chromosomal termini, (B) incomplete asymmetrical exchange with chromatid gap in FANCD2 cell

Considering the radiation-induced SCE frequencies, excess values, significantly higher than the unirradiated self-controls are noted in all studied groups (Figure 1). However, there were no differences in SCE frequencies between patients and control. Comparable results were seen when mothers of FA and non-FA BMF patients, and control were analyzed (Table 4).

The limited number of FANCA patients made it difficult to analyze complementation group-related differences in a base-line as well as in induced SCE frequencies. FANCA patient displayed slightly reduced baseline frequency of SCE compared to both FANCD2 patients and control. The frequencies of SCE induced by DEB and ionizing radiation in FANCA patient were modesty increased compared to frequencies observed in FANCD2 patients.

DISCUSSION

The role of the FANC protein network is both to promote translesion synthesis (TLS) at stalled/blocked replication forks and to promote homologous recombination (HR) repair at broken replication forks. A deficiency in the latter process could be expected to result in reduced spontaneous SCE in FA cells which is not observed (THOMPSON and HINZ, 2009). This finding suggests that there is more than one pathway of HR that participates in restarting broken replication forks. In agreement with that, in the current study, no differences in spontaneous frequencies of SCE between FA patients, non-FA BMF patients and healthy controls were observed.

As mentioned before, studies on induced SCE in FA cells mostly suggest decreased SCE frequencies in response to diepoxybutane. However, in our study DEB-induced SCE frequencies in FA patients and their mothers were significantly increased compared to both non-FA BMF families and healthy controls. In control, progression of SCE in response to DEB is significantly suppressed, which is probably due to activity of RAD52 that increases the level of resistance to the cross-linking agents (PARK, 1995; JOHNSON *et al.*, 1996).

Diepoxybutane is a highly reactive compound. Upon epoxide hydrolysis, DEB can covalently bind to DNA bases. Likewise, DEB generates reactive oxygen species that, in turn, can either damage DNA or produce H_2O_2 (EREXSON and TINDALL, 2000). The fact that DEB induces elevated frequency of SCE in FA patients and female carriers shedding new light on the redox-dependent mechanisms in DEB toxicity, and suggests a direct association of oxidative stress with the primary genetic defect in FA, at least for complementation groups FANCA and FANCD2. DEB toxicity includes modulation of catalase and MnSOD activities, and glutathione depletion (PAGANO and KORKINA, 2000). In addition, DEB-sensitivity has been suggested to be due to the lack of glutathione S-transferase (GST) T1 (LANDI *et al.*, 1996), which should be further examined in FA patients. It has been reported that hypoxia or antioxidants both correct spontaneous as well as DEB-induced chromosomal instability. Moreover, catalase is specifically involved in DEB and MMC detoxification hence its defective expression is consistent with FA cell sensitivity to these agents (KORKINA *et al.*, 2000). The inefficient removal of peroxide can result in high rates of sister chromatid exchange (NICOTERA *et al.*, 1989). A recent study from our laboratory has shown that FA patients (FANCA and FANCD2) and their mothers display decreased catalase activity (PETROVIC et al., 2011), which may account for at least a part of the observed outcomes in the current study. In other words, the extensive chromosome aberrations often seen in FA cells may be results of increased DNA damage by excessive generation of ROS along with compromised DNA damage response processes linked to HR.

The finding of increased SCE suggests inability of the cell to repair efficiently replication-blocking lesions, which results in the fork collapse and the initiation of HR. In addition, defects in single strand break repair (SSBR) also lead to enhanced SCE frequencies, trough formation of double strand breaks upon a replication fork collapse at the SSB site, when HR than usually uses sister chromatid to facilitate accurate repair. In normal DNA replication, SCE occurs upon a natural fork collapse with estimates being 3-4 exchanges per cell per cycle. However, Thyagarajan and Campbell (THYAGARAJAN and CAMPBELL, 1997) reported that in FA fibroblasts recombination levels are nearly 100-fold higher than that in controls, which contribute to the genomic instability and cancer predisposition that characterized Fanconi anemia.

The results of the present study showed that in response to ionizing radiation SCE frequencies were significantly increased in all studied groups, but no differences between FA patients and non-FA BMF patients were seen. These results suggest that radiation-induced SCE frequencies cannot be used as a diagnostic tool for discrimination of FA in the large group of BMF patients.

In conclusion, our data confirm and expand previous findings by showing that SCE induced by DEB can be used as an adjunct diagnostic test not only for FA patients, but also for female heterozygous carriers, at least for complementation groups FANCA and FANCD2.

ACKNOWLEDGEMENT

We wish to express our gratitude to Prof. Dr**.** H. Hoehn and Prof. Dr. D. Schindler (Universität Würzburg, Institut für Humangenetik, Germany) and Prof. Dr**.** J. Surralles (Department of Genetics and Microbiology, Universitat Autonoma de Barcelona, Spain) for FA complementation group analysis. This research was supported by Serbian Ministry of Education and Science, Grant No 173046.

Received August 03th, 2012
Accepted April 06th, 2013

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POVEĆANA UČESTALOST IZMENA SESTRINSKIH HROMATIDA DIEPOKSIBUTANOM JE SPECIFIČNA ODLIKA ĆELIJSKOG FENOTIPA FANKONIJEVE ANEMIJE

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Izvod

Fankonijeva anemija (FA) je genetički heterogena, retka bolest, koju karakterišu razvojne anomalije, progresivna aplazija kostne srži i sklonost ka oboljevanju od karcinoma. Kliničke manifestacije bolesti u odredjenom satrosnom dobu, mogu biti veoma slične aplaziji kostne srži drugog porekla. Diferencijalna dijagnostika ove dve kategorije uobičajeno se radi testom indukcije hromozomskih aberacija alkilirajućim agensima, tzv. Diepoksibutan (DEB) testom na limfocitima periferne krvi *in vitro*, ali su dragoceni svi drugi testovi koji mogu pomoći u postavljanju pravilne dijagnoze.U ovom radu prikazani su rezultati ispitivanja spontane i indukovane učestalosti izmena sestrinskih hromatida (SCE) u ćelijama poreklom od FA pacijenata, pacijenata obolelih od aplazije kostne srzi, njihovih roditelja, i odgovarajućih kontrola. Rezultati ispitivanja su pokazali da se bazalna učestalost izmena sestrinskih hromatida ne razlikuje izmedju tri navedene grupe. Tretman DEB-om, u trajanju od 24h finalne concentracije 0.1µg/ml indukuje dvostruko povećanje učestalosti SCE samo u grupi FA pacijenata i njihovih majki. Iznenadjujuće je da se heterozigotni nosioci mutacije u FA genima ženskog pola (majke), *in vitro*, po učestalosti SCE ne razlikuju od homozigotnih nosilaca mutacije. Ćelije poreklom od pacijenata sa drugim uzrokom aplazije kostne srži ponašaju se slično kontrolnim. Učestalost SCE u in vitro ozračenim uzorcima krvi navedenih grupa, ne pokazuje statistički značajnu razliku. Na osnovu dobijenih rezultata može se zaključiti da indukovana učestalost SCE alkilirajućim agensima može da bude od značaja kao dodatni test u diferencijalnoj dijagnostici FA ćelijkog fenotipa.

Primljeno 03. VIII 2012.
Odobreno 06. IV. 2013.