Chromosomal instability in patients with Fanconi anemia from Serbia

Hromozomska nestabilnost kod bolesnika sa Fankonijevom anemijom u Srbiji

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Abstract

Background/Aim. Fanconi anemia (FA) is a rare hereditary disease in a heterogeneous group of syndromes, so-called chromosome breakage disorders. Specific hypersensitivity of its cells to chemical agents, such as diepoxybutane (DEB), was used as a part of screening among patients with clinical suspicion of FA. The aim of this study was to determine chromosomal instability in patients with FA symptoms in Serbia. **Methods.** A total of 70 patients with phenotypic symptoms of FA, diagnosed at the Mother and Child Health Care Institute of Serbia "Dr Vukan Čupić", Belgrade and University Children's Hospital, Belgrade from February 2004 to September 2011, were included in this study. Cytogenetic instability analysis was performed on untreated and DEB-treated 72 h-cultures of peripheral blood. **Results.** Ten patients in the group of 70 suspected of FA, showed increased DEB induced chromosome breakage and were classified into the FA group. The range of DEB induced aberrant cells percentages in the FA group was from 32% to 82%. DEB sensitivity of 58 tested patients were bellow FA values (range: 0–6%) (non-FA group), with no overlapping. The remaining two patients showed borderline sensitivity (borderline FA group – FA*), comparing to the healthy controls. **Conclusion.** This study revealed 10 patients with FA on the basis of cytogenetic analysis of DEB induced chromosome aberrations. Our results are in consistency with those from the literature. Early and precise diagnosis of FA is very important in further treatment of these patients, considering its cancer prone and lethal effects.

Key words:
fanconi anemia; diagnosis, differential; cytogenetics; chromosome aberrations; chromosome disorders.

Apstrakt

Uvod/Cilj. Fankonijeva anemija (FA) je retka nasledna bolest hromozomske nestabilnosti sa specifičnom hipersenzitivnošću ćelija na dejstvo DNK-unakrsno-vezujućih agenasa kao što je diepoksibutan (DEB) i mitomicin C. Cilj rada bio je da se utvrdi hromozomska nestabilnost kod bolesnika sa simptomima FA u Srbiji. **Metode.** Hromozomska senzitivnost na DEB ispitivana je kod 70 bolesnika klinički suspektnih na FA, koji su dijagnostikovani u Institutu za zdravstvenu zaštitu majke i deteta Srbije „Dr Vukan Čupić", Beograd i Univerzitetskoj dečijoj klinici, Beograd u periodu 2004–2011. godine. Analiza je sprovedena na netretiranim i DEB-om tretiranim 72-časovnim kulturama limfocita periferne krvi bolesnika i zdravih osoba. **Rezultati.** Kod 10 bolesnika je uočen povećan broj DEB-om indukovanih hromozomskih prekida (FA grupa), kako u odnosu na ostale pacijente (ne-FA grupa), tako i u odnosu na zdrave kontrole. Procenat aberantnih ćelija u FA grupi kretao se u rasponu 32–82%, dok je raspon u grupi od 58 ne-FA bolesnika iznosio 0–6%, a u kontrolnoj grupi 0–8%. Preostala dva bolesnika pokazala su granični odgovor na DEB (FA* grupa) u poređenju sa zdravim kontrolama. **Zaključak.** Na osnovu citogenetske analize DEB-om indukovane hromozomske nestabilnosti u limfocitima periferne krvi bolesnika suspektnih na FA, dijagnoza bolesti je postavljena kod njih 10. Postavljanje rane i precizne dijagnoze FA je od velikog značaja za dalje lečenje ovih bolesnika, s obzirom na to da se radi o veoma teškom oboljenju sa letalnim ishodom.

Ključne reči:
anemija, fankoni; dijagnoza, diferencijalna; citogenetika; hromosomi, aberacije; hromosomi, anomalije.

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Introduction

Chromosomal instability diseases are a heterogeneous group of inherited diseases which is determined with the growth and development disorders, defects of the immune system and bone marrow function, as well as increased predisposition to cancer [1, 2]. These conditions occur as a result of mutations in genes involved in the process of repairing and maintaining genome stability. Beside Fanconi anemia (FA) and Nijmegen Brekage syndrome (NBS), this group includes Ataxia-telangiectasia, Bloom syndrome, Xeroderma pigmentosum, Cockayne syndrome, Werner syndrome, Trichothiodystrophia, Rothmund-Thompson syndrome, and immunodeficiency syndrome with centromeric heterochromatin instability (ICF) [1]. The common characteristic of these diseases is the increased spontaneous chromosome breakage in the cells of affected patients, as a result of mutations in genes responsible for DNA repair [1]. However, the percentage of spontaneous chromosomal aberrations is very variable and nonspecific. For the purpose of accurate differential diagnosis, the induction of chromosomal aberrations with the number of specific mutagenic agents such as mitomycin C, mephalan or diepoxybutane (DEB) for FA or bleomycin for NBS, etc., is used to increase the baseline sensitivity [2, 3].

There are no available data in the literature about the frequency of affected with chromosome instability syndromes in Serbia. However, the most of the childhood patients referred for the genetic testing to the Laboratory for Medical Genetics Mother and Child Health Care Institute of Serbia "Dr Vukan Čupić" in Belgrade had clinical signs of two such disease: NBS and FA. FA is a rare autosomal recessive and X-linked polygenic disease characterized by pancytopenia, aplastic anemia (AA) with progressive bone marrow failure, short stature, developmental abnormalities, increased susceptibility to cancer development and cellular hypersensitivity to DNA cross-linking agents such as DEB [4, 5].

The aim of this study was to determine chromosomal instability in FA patients using DEB sensitivity test in a large group of patients from Serbia, with clinical suspicion of FA.

Methods

From February 2004 to September 2011, 70 children with FA symptoms, such as pancytopenia, AA, congenital anomalies and other, were treated at the Mother and Child Health Care Institute of Serbia "Dr Vukan Čupić" and University Children's Hospital in Belgrade. They all underwent DEB test in the laboratory for medical genetics, in order to establish the differential diagnosis of FA.

Chromosome fragility tests on blood samples from the patients with clinical suspicion of FA and controls (healthy family members) were performed as described by Auerbach [3], with minor modification [2]. Two cultures from each patient and a healthy individual (control) were treated with DEB in the final concentration of 0.1 μg/mL for the last 24 h, and the remaining two cultures were left for the evaluation of spontaneous chromosome fragility [2]. After 70 hours of cultivation, colcemid (2.5 μg/mL) was added and cytogenetic analysis was performed according to standard protocol [2]. A total of 100 metaphases from each culture were analyzed, using G banding, for the presence of chromosome/chromatid breaks and other aberrations [6]. Chromatid and chromosome breaks, and acentric fragments were counted as one break, while dicentrics, ring chromosomes and radial structures are counted as two breaks [2]. The parameters of chromosomal instability evaluation were: the percentage of aberrant cells, the number of breaks per cell and the number of breaks per aberrant cell [2, 3].

Statistical analysis

Chi-square test was used to determine a level of differences between two groups of aberrant cells of each patient and its control counterpart [2].

The obtained values of cytogenetic sensitivity to DEB divided patients into two main subgroups: FA and non-FA patients. Statistical analysis of differences between these groups included Mann-Whitney test, which revealed that there were no overlapping ranges of values among them, and with the third one – the subgroup of patients with borderline sensitivity.

Results

Clinical features of patients with suspecion of FA treated at the Mother and Child Health Care Institure of Serbia "Dr Vukan Čupić and University Childrens Hospital, Belgrade, from February 2004 to Septembre 2011, are shown in Table 1.

In the sample of 70 children, 10 patients with a positive response to DEB and a significant difference comparing individually to their control counterparts (Chi-square test: $p < 0.05$), were classified into the FA group. No significant level of chromosomal response was found in 58 patients and they were classified as non-FA group. The remaining of two patients revealed chromosomal breakage values between FA

Table 1

Clinical features of 70 patients with suspicion of Fanconi anemia

Clinical features	Patients n (%)
Haematologic abnormalities (aplastic anemia, pancytopenia, trombocytopenia, myelodisplasy, etc)	6.7 (95.71%)
Physical abnormalities (congenital anomalies, short stature, etc)	4 (5.71%)
Malignacy (tumor, ALL, lymphoma, etc)	6 (8.57%)

ALL – acute lymphoblastic leukemia.

and non-FA ranges (FA* group). The results of spontaneous and DEB-induced chromosomal instability analysis for all the groups of patients, including the control group, are presented in Table 2.

The percentage of DEB-induced aberrant cells in the FA group, ranged from 32% to 82%, and the mean value was 52.52%, which is about 40 times more than in the control group (range 0–8% ; mean: 1.30%), and about 32 times

Table 2
Spontaneous and diepoxybutane (DEB) chromosome breakage findings in Fanconi anemia (FA), borderline-FA (FA*), non-FA and control groups

| Disease (n) | Chromosome instability ($\bar{x} \pm SD$, x_{min}-x_{max}) | | | | | |
| | Break/cell | | Aberrant cells (%) | | Breaks/aberrant cell | |
	S	DEB	S	DEB	S	DEB
FA (10)	0.14 ± 0.13	1.47 ± 1.16	10.50 ± 9.17	$52.52 \pm 18.50^{+}$	1.11 ± 0.43	2.58 ± 0.17
	0.00–0.39	0.48–4.39	0.00–29.00	32.00–82.00	0.00–1.50	1.20–5.35
FA* (2)	0.06 ± 0.01	0.23 ± 0.04	6.00 ± 1.41	17.00 ± 7.07	1.07 ± 0.14	1.43 ± 0.35
	0.06–0.07	0.20–0.26	5.00–7.00	12.00–22.00	1.00–1.20	1.18–1.67
Non-FA (58)	0.01 ± 0.01	0.02 ± 0.02	0.81 ± 1.22	1.60 ± 1.50	0.55 ± 0.61	0.86 ± 0.51
	0.00–0.06	0.00–0.08	0.00–5.00	0.00–6.00	0.00–2.00	0.00–2.67
Control (healthy) (97)	0.00 ± 0.01	0.02 ± 0.02	0.34 ± 0.64	1.30 ± 1.54	0.24 ± 0.44	0.69 ± 0.76
	0.00–0.03	0.00–0.17	0.00–3.00	0.00–8.00	0.00–1.50	0.00–3.40

n – number of respondents; S – spontaneous chromosome instability; DEB – DEB-induced chromosome instability; $\bar{x}$ – mean value; SD – standard deviation.
Notes: (+) Mann-Whitney test revealed a statistically significant difference between the groups FA and non-FA ($p < 0.001$).

The main criteria for the determination of chromosome fragility were as follows: the percentage of aberrant cells, the number of breaks per cell and the number of breaks per aberrant cell. Ten patients (14.3%) revealed an increased number of induced chromosome and chromatid breaks, and other chromosome aberrations (Figures 1 and 2).
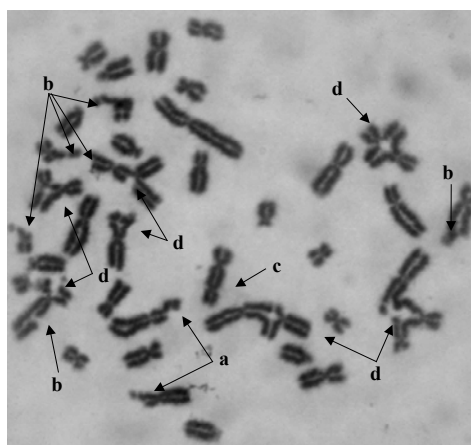
Fig. 1 – Diepoxybutane induced chromosome aberrations in patient with Fanconi anemia: a – chromosome break, b – chromatid break, c – dicentric, d – chromatid exchanges.
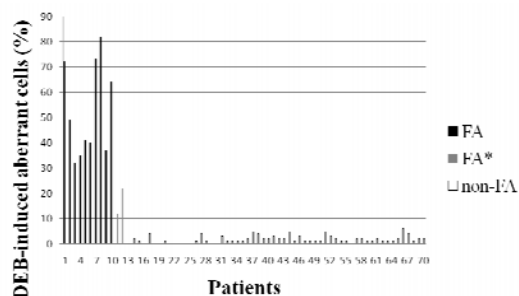
Fig. 2 – Diepoxybutanol – induced chromosomal breakage in Fanconi anemia (FA), borderline FA* and non-FA groups of patients.

higher than in non-FA group (range 0–6%; mean: 1.60%). The patient No. 8 reached a maximum percentage (82%) of cells with aberrations induced by DEB, while patient No. 3 had the minimum value of 32% for the same parameter (Table 3).

The mean value of DEB-induced breaks per cell in the FA group (mean: 1.47 breaks/cell, range of values: 0.48 to 4.39 break/cell) was 73 times higher than the mean value in the non-FA group (mean: 0.02 break/cell, range of value 0.00–0.08 break/cell). patient no. 8 reached the maximum number of break/cell (4.39) and the minimum value was found in the patient no. 6 (0.48 breaks/cell) (Table 3). Statistical analysis showed a significant difference between the FA and non-FA groups (Mann-Whitney test: $p < 0.001$) with ranges of values that were not overlapped (Table 2).

However, two patients, according to DEB test, were classified in the FA* group showing borderline sensitivity for DEB-induced percentage of aberrant cells (range: 12–22%; mean 17%), about 10 times higher than in the non-FA group and three times lower than in FA group, as well as for break/cell findings (range: 0.20–0.26 breaks/cell; mean: 0.23 breaks/cell) (Table 2).

Spontaneous chromosomal instability values (percentage of aberrant cells and the number of breaks/cell) for 10 FA patients are partially overlapped with the values in the groups non-FA and FA-borderline (Table 2). Nevertheless, the mean percentage of spontaneously aberrant cells in the FA group was 12 times higher (mean 10%; range 0–29%) comparing to those from the non-FA group (mean: 0.81%; range 0–6%) (Table 2). Baseline chromosomal instability of the borderline FA* patients was also in the ranges of values for other groups of examinees (non-FA group ranges: 0.06–0.07 breaks/cell and 5–7% of aberrant cells vs. control group ranges: 0.00–0.03 breaks/cell and 0–3% of aberrant cells) (Table 2).

Spontaneous and diepoxybutane induced chromosome instability in 10 patients with Fanconi anemia (FA)

N° of FA patient	Chromosome instability					
	Break/cell (n)		Aberrant cells (%)		Breaks/aberrant cell (n)	
	S	DEB	S	DEB	S	DEB
1.	0.01	2.15	1.00	72.22	1.00	2.97
2.	0.07	1.50	5.00	49.00	1.40	3.06
3.	0.00	0.95	0.00	32.00	0.00	2.97
4.	0.08	0.68	8.00	35.00	1.00	1.94
5.	0.27	0.58	18.00	41.00	1.50	1.41
6.	0.12	0.48	9.00	40.00	1.33	1.20
7.	0.18	1.75	15.00	73.00	1.20	2.40
8.	0.39	4.39	29.00	82.00	1.34	5.35
9.	0.03	0.91	3.00	37.00	1.00	2.46
10.	0.22	1.31	17.00	64.00	1.29	2.05
Total ($\bar{x} \pm$ SD)	0.14 ± 0.13	1.47 ± 1.16	10.50 ± 9.17	52.52 ± 18.50	1.11 ± 1.16	2.58 ± 1.17

n – number; S – spontaneous chromosome instability; DEB – DEB-induced chromosome instability; $\bar{x}$ – mean value; SD – standard deviation.

Discussion

FA is a rare autosomal recessive disease that occurs with a frequency of about 2.5 : 100,000, depending on the population [7]. This disease is both clinically and genetically heterogeneous [8–11]. FA diagnosis based on clinical indications is difficult because of variations in phenotypic expression, which significantly reduces the possibility of distinguishing FA from other clinically similar disorders (patients with AA and other signs of bone marrow failure) [5, 12]. However, FA patients are characterized clinically from other patients in specific hypersensitive response to DNA cross-linking agents such as DEB or MMC, which results in the presence of a large number of chromosome and chromatid breaks in the cells of these patients. This characteristic of FA cells is now widely used as a differential diagnostic test tool in the screening of FA patients.

In this paper we presented the results of DEB test as a screening method for FA patients in the group of patients from Serbia with clinical suspicion of FA. The frequency of FA in the group of children with AA and other signs of bone marrow failure from Serbia was lower (14.3%) as compared to the published ones (25–30%), which could be explained with the fact that not all such patients were referred to DEB test [4].

In this study, parameter values of baseline chromosomal instability (% aberrant cells and breaks/cell) in the FA group overlapped with the corresponding values in the non-FA group (Table 2), meaning that these two groups could not be distinguished on the basis of spontaneous chromosomal breaks. Our results are consistent with the same results of a study published in the International Registry of Fanconi anemia (Fanconi's Anemia International Registry – IFAR) [13] as well as in similar works [12, 14].

Based on hypersensitive response to DEB, the mean values of chromosomal breakage parameters were much higher in the FA than in the non-FA group (32 times higher percentage of aberrant cells and 73 times higher breaks/cell), which confirmed the ability of DEB test to differ FA affected from other patients with similar symptoms, and corresponded to previously published studies [12, 14].

It should be noted that the number of DEB-induced aberrant cells in the group of 10 FA patients varied much ranging from 32% to 82%. Also, there were some difficulties in classifying the rest of two patients with borderline sensitivity to DEB, and possible somatic mosaicism pointing to FA phenotype. These variations and deviations are probably caused by the presence of patients with the mosaic form of FA in our group of patients in which the values of induced aberrant cells are generally lower (< 60%) compared to non-mosaic forms [12, 14–17]. Specifically, somatic mosaicism is a phenomenon of FA mutations reversion in certain hematopoietic cells, so that in the blood sample of the same patient can be found two clones of cells: FA clone and clone with a normal (insensitive to DEB) cells with reverted mutation [17–19]. According to previously published studies, FA patients with values of aberrant cells < 40% are classified as mosaic FA, while those with values of aberrant cells ranging from 40% to 60% are considered potential mosaics; FA patients with aberrant cells values ≥ 60% are considered complete, non-mosaic type of FA [20]. Our data indicated that FA patients with values: aberrant cells < 50%, may be considered as a form of mosaic FA, while those with non-mosaic form of FA had values greater than > 60%. For the remaining six patients with FA values of aberrant cells between 32% and 49%, further evaluation in order to confirm/exclude mosaicism is required. The presence of borderline FA patients and the variation of DEB-induced aberrations in non-FA patients (0–6%) can also be the consequence of a limiting sensitivity of patients with mosaic form of FA to DEB. Based on these results and the fact that there are some non-FA patients with increased sensitivity (up to 16%) to DEB and mitomycin C, which cannot be explained, identifying of mosaic FA forms is more complicated and could be done with less certainty [20].

The main characteristic of FA cellular phenotype is high sensitivity to DNA intercalating agents, so that in the case of mosaicism it is difficult to make accurate diagnosis of FA. The study of Castella et al. [20] proposed a new index of chromosome fragility that includes both % of aberrant cells and breaks/multiaberrant cell, and allows unambiguous diag-

nostic differentiation of mosaic and non-mosaic patients with FA, as well mosaic forms of FA, compared to non-FA patients. This approach could be applied to our group of patients in order to more accurately distinguish these groups of affected patients.

Early and precise diagnosis of this disease is very important for further treatment of the patients and, also, for providing accurate information, concerning genetic counseling of families with affected members [21].

Conclusion

The diepoxybutane test proved to be very effective and relatively simple diagnostic tool in the process of screening among Fanconi anemia patients with aplastic anemia and other symptoms of bone marrow failure.

Molecular testing and identification of complementation groups of Fanconi anemia for each Fanconi anemia patient sensitive to diepoxybutane and further clarification of borderline sensitivity, examining the cellular functionality of other tissue are perhaps the next steps in establishing the final diagnosis of Fanconi anemia.

Acknowledgements

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