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Cytogenetic findings in Polish patients with suspected Fanconi anemia

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Advances in Clinical and Experimental Medicine, ISSN 1899–5276 (print), ISSN 2451–2680 (online) *Adv Clin Exp Med*. 2024

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Funding sources
None declared

Conflict of interest
None declared

Acknowledgements
We would like to thank the families for the participation in the study and for their consent to publish the data.

Received on February 12, 2023
Reviewed on April 6, 2023
Accepted on June 26, 2023

Published online on August 4, 2023

Cite as
Repczyńska A, Jułga K, Lorenc A, et al. Cytogenetic findings in Polish patients with suspected Fanconi anemia [published online as ahead of print on August 4, 2023]. *Adv Clin Exp Med*. 2024. doi:10.17219/acem/168825

DOI
10.17219/acem/168825

Abstract

Background. The high sensitivity of cells of Fanconi anemia (FA) patients to DNA cross-linking agents (clastogens), such as mitomycin C (MMC), was used as a screening tool in Polish children with clinical suspicion of FA.

Objectives. The aim of the study was to compare chromosome fragility between 3 groups, namely non-FA, possible mosaic FA and FA patients.

Materials and methods. The study included 100 children with hematological manifestations and/or congenital defects characteristic of FA, and 100 healthy controls. Blood samples obtained from participants were analyzed using an MMC-induced chromosomal breakage test.

Results. Patients with clinical suspicion of FA were divided into 3 subgroups based on the MMC test results, namely FA, possible mosaic FA and non-FA. Thirteen out of 100 patients had a true FA cellular phenotype. The mean value of MMC-induced chromosome breaks/cell for FA patients was higher than for non-FA patients (6.67 ±3.92 compared to 0.23 ±0.18). In addition, the percentage of cells with spontaneous aberrations was more than 9 times higher in FA patients than in non-FA patients.

Conclusions. Our results confirmed that the MMC sensitivity test distinguishes between individuals affected by FA, those with possible somatic mosaicism, and patients with bone marrow failure for other reasons, who were classified as non-FA in the first diagnostic step. However, a definitive differential diagnosis requires follow-up mutation testing and chromosome breakage analysis of skin fibroblasts.

Key words: Fanconi anemia, mitomycin C, chromosome breakage test

Background

Fanconi anemia (FA) is the most prevalent cancer-prone inherited bone marrow failure syndrome (IBMFS). The phenotype of FA patients is characterized by microcephaly, radial ray defects, skin pigmentation abnormalities, short stature, and genitourinary defects,[1–4] although 25% of patients do not present abnormalities or pancytopenia at birth. In this patient population, the diagnosis is often not established until they develop cancers typical of adults with FA or family studies are conducted.[5–7]

Fanconi anemia is a rare genetic disease caused by pathogenic variants in any of the 23 FA complementation group A (*FANC*) genes, which are involved in the FA/breast cancer gene (*BRCA*) pathway. An autosomal recessive inheritance was demonstrated for 21 *FANC* genes, an autosomal dominant inheritance was demonstrated for 1 gene (*FANCR/RAD51*), and an X-linked recessive inheritance for another (*FANCB*). The incidence of FA is 1 in 300,000 live births, affecting from 1 to 9 people in 1,000,000, with a carrier frequency in the general population of around 1 in 181.[8]

The consequences of FA pathway failure manifest at the chromosomal, cellular and clinical levels, with characteristic structural aberrations formed at the chromosomal level. Cellular outcomes include increased apoptosis, cell cycle changes and heightened sensitivity to DNA cross-linking agents such as exogenous mitomycin C (MMC) and diepoxybutane (DEB), and endogenous aldehydes. At the clinical level, FA patients present a triad of symptoms that involve bone marrow failure, a high risk of cancer and congenital defects.[9]

Chromosomal aberrations found in metaphase spreads from FA patients include gaps, chromatid or isochromatid breaks, chromosome breaks, acentric fragments, dicentric chromosomes, chromatid interchange, and characteristic triradial and tetraradial figures.[10]

The hallmark of FA cells is high genomic instability, first described in 1966 by Schroeder as a high level of spontaneous chromosome breaks.[11] Some years later, studies showed that hypersensitivity to DNA cross-linking agents caused this genomic instability, leading to the development of the MMC and DEB tests, which are the "gold standard" diagnostic tests for FA. Although the high sensitivity to DNA cross-linking agents is the hallmark of peripheral blood T-lymphocytes of FA patients, it is not observed in all FA cases.[12,13]

Around 10–20% of FA patients present with a special type of hematopoietic somatic mosaicism that reduces or eliminates lymphocyte sensitivity to clastogens. Identifying individuals affected with mosaicism using a chromosome breakage test can be difficult. Various mechanisms, such as back mutation, gene conversion, intragenic crossover, and second-site mutation, may result in the restoration of the affected gene to wild type, and these mechanisms have been identified in cells of mosaic FA patients. Gene reversion may affect all hematopoietic cell lineages, causing a "natural gene therapy" with a stable phenotype or only individual cell lines and leading to a limited effect. The success of reversion depends on the stage of differentiation of the cell at which the gene correction occurs.[14,15]

Determining the level of chromosomal abnormalities is crucial for identifying patients with FA. The most challenging problem when interpreting the results of chromosome breakage tests is distinguishing between non-FA and mosaic FA patients. Some non-FA patients may have a proportion of T-lymphocytes with chromosome breaks after chemotherapy treatment, which can be interpreted by inexperienced cytogeneticists as mosaicism, leading to false-positive results. In contrast, a high proportion of reverted T-lymphocytes in FA mosaic patients can lead to false negatives.[16,17]

A spontaneous chromosome breakage analysis of cells not treated with MMC is also required since various levels occur among patients with different FA subtypes. Individuals with variants in *FANCD1/BRCA2* or *FANCN/*partner and localizer of BRCA2 (*PALB2*) genes have very high levels of spontaneous breakage and atypical aberrations compared with other groups of FA patients. Other DNA repair deficiency syndromes also show increased levels of spontaneous fragility, and it is possible to reveal specific types of chromosomal abnormalities for these syndromes. For example, telomeric rearrangements are present in cells of patients with dyskeratosis congenita, premature centromere separation occurs in Roberts syndrome, and Warsaw breakage syndrome and aberrations of chromosomes 7 and 14 are frequent in Nijmegen breakage syndrome and ataxia–telangiectasia. Additionally, analyzing breakages on G-banded chromosome preparations makes it possible to diagnose constitutional chromosome aberrations that may be responsible for the patient's clinical features in around 1–2% of patients with suspected FA.[10,18]

Objectives

The aim of the study was to assess chromosomal fragility using chromosome aberration analysis, including the number of chromosomal breaks and/or radial figures/cell, percentage of cells with aberrations, and breaks or radials/aberrant cell in Polish patients with suspected FA.

Materials and methods

Patients

One hundred patients (52 females and 48 males) with suspected FA were referred to the Department of Clinical Genetics of Collegium Medicum in Bydgoszcz (Nicolaus Copernicus University in Toruń, Poland). The age at diagnosis ranged from 1 month to 32 years (median: 15.36 years).

Sampling and culture conditions

Peripheral blood samples were collected with heparin (≥5 mL) to prepare cultures for routine cytogenetic analysis upon receiving signed consent forms from the parents of examined children and healthy controls. Four cultures were set up for each participant by adding 0.5 mL of blood to 4.5 mL of Roswell Park Memorial Institute (RPMI) 1640 culture medium containing 15% fetal bovine serum (FBS), phytohemagglutinin (PHA) and gentamicin (all from Thermo Fisher Scientific, Waltham, USA), according to the procedure described by Oostra et al.[10] Cultures were incubated for 72 h with 0, 50, 150, or 300 nM of MMC (Sigma-Aldrich, St. Louis, USA). Cells were harvested after the treatment with colcemid (0.1 µg/mL) for 50 min. The next step involved incubation with 0.075 M KCl for 20 min at 37°C, followed by fixation with methanol and acetic acid (3:1). Lastly, microscope slide were prepared by staining cytogenetic preparations in 5% Giemsa solution for 2 min (Sigma-Aldrich) using the no banding technique. Chromosome breakage was analyzed using the Nikon E600 microscope (Nikon Corp., Tokyo, Japan) with a computer-assisted metaphase system (ASI Technologies, Netzer Sereni, Israel).

Cytogenetic analysis

The analysis included at least 50 metaphases from MMC-treated and untreated cultures of every patient and healthy control (Table 1,2). According to the recommendations for chromosomal breakage testing with MMC, it is necessary to set up the cell cultures from healthy controls concurrent with the samples from patients with suspected FA.[10] The chromosome breakage analysis was performed using the aberrations outlined in Table 3, though chromosome and chromatid gaps were not scored.

The data were analyzed and scored for each patient and healthy control as a percentage of aberrant cells, mean chromosome breaks/cell, mean chromosome breaks/aberrant cell, and triradial, tetraradial and chromatid interchange figure frequency (Table 1,2).

Statistical analysis

The Shapiro–Wilk (S–W) test, with a statistical significance level of 0.05, was used to determine the distribution of MMC test data across all investigated groups – control, non-FA, possible mosaic FA, and FA. The highest p-value for all S–W tests was 0.04, indicating that the data were non-normally distributed. As such, further analysis required non-parametric statistical methods, with the Mann–Whitney U test with Bonferroni correction applied to compare FA, possible mosaic FA and non-FA groups to the control group. A statistical significance level of 0.05 was established for this test. All analyses employed PQStat v. 1.8.2 (PQStat Software, Poznań, Poland).

Results

The results of the chromosome breakage examination and cytogenetic analysis of a population of Polish patients presented herein were systematically obtained in the same laboratory under controlled conditions over a 6-year period (2015–2020). There are no studies on FA conducted in Poland, except for a small number of case reports.

The MMC test results included the percentage of aberrant cells, the number of breaks/analyzed cell, the number of breaks/aberrant cell, and the presence of radial figures characteristic of FA, which allowed for the stratification of patients into 3 subgroups: FA, displaying a typical MMC-sensitive cellular response, possible mosaic FA, and non-FA. The results of the spontaneous and MMC-induced breakage for all subgroups and controls are presented in Table 1 and Table 2.

Thirteen out of 100 examined patients (13%) had a cellular FA phenotype, with increased MMC-induced chromosomal breaks at 50-nM, 150-nM and 300-nM MMC concentrations (Fig. 1). Twelve of the 13 FA patients showed an increased rate of spontaneous breaks, while 1 patient had no spontaneous breaks. Meanwhile, 27 patients had possible mosaic FA, as they presented with only a slightly higher number of aberrant cells and breaks/aberrant cell and characteristic radial figures. The remaining patients presented with single chromosome breaks only, similar to the control group, and they were classified as non-FA.

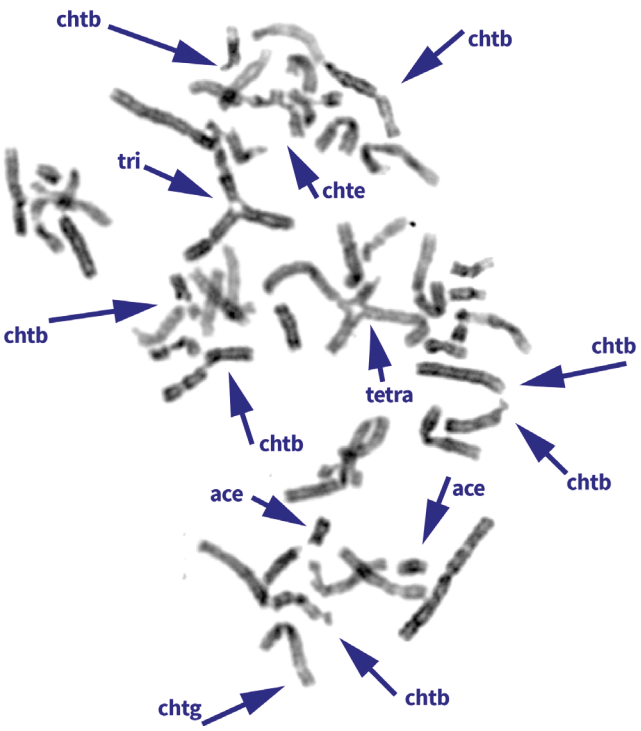

Fig. 1. Metaphase spread from a Fanconi anemia (FA) patient

ace – acentric fragment; chtb – chromatid break; chtg – chromatid gap; tri – triradial figure; tetra – tetraradial figure; chte – chromatid interchange figure.

Table 1. Evaluation of spontaneous and MMC-induced chromosome breakage in Fanconi anemia (FA), possible mosaic FA and non-FA patients

Culture type	Group	Analyzed cells, n	Ace	Ace (%)	Dic	Dic (%)	Chtb	Chtb (%)	Gaps	Triradial	Triradial (%)	Tetraradial	Tetraradial (%)	Multiradial	Multiradial (%)
Spontaneous chromosome breakage test	controls	3940	0	0.00	0	0.00	24	100.00	3	0	0.00	0	0.00	0	0.00
	non-FA	1914	0	0.00	1	3.85	25	96.15	0	0	0.00	0	0.00	0	0.00
	possible mosaic FA	854	2	5.56	0	0.00	31	86.11	9	1	2.78	1	2.78	1	2.78
	FA	551	2	3.57	0	0.00	50	89.29	1	2	3.57	1	1.79	1	1.79
Mitomycin C – 50 nM	controls	4496	0	0.00	0	0.00	386	99.48	34	1	0.26	1	0.26	0	0.00
	non-FA	2246	2	1.02	0	0.00	187	95.41	39	3	1.53	4	2.04	0	0.00
	possible mosaic FA	1088	0	0.00	0	0.00	170	92.90	8	4	2.19	8	4.37	1	0.55
	FA	578	4	0.58	2	0.29	626	90.59	115	23	3.33	26	3.76	10	1.45
Mitomycin C – 150 nM	controls	4605	0	0.00	0	0.00	972	98.38	57	1	0.10	13	1.32	2	0.20
	non-FA	2238	1	0.26	4	1.03	368	94.36	32	7	1.79	8	2.05	2	0.51
	possible mosaic FA	1076	0	0.00	0	0.00	245	93.87	37	4	1.53	11	4.21	1	0.38
	FA	543	32	1.48	2	0.09	1852	85.62	174	90	4.16	115	5.32	72	3.33
Mitomycin C – 300 nM	controls	5003	1	0.05	0	0.00	1822	96.91	119	25	1.33	31	1.65	1	0.05
	non-FA	2973	2	0.29	0	0.00	656	94.80	135	12	1.73	20	2.89	2	0.29
	possible mosaic FA	1333	6	0.59	1	0.10	882	86.81	111	31	3.05	85	8.37	11	1.08
	FA	672	74	1.65	0	0.00	3748	83.55	376	195	4.35	223	4.97	246	5.48

chtb – chromatid break (the interruption which is more than 2 times the width of the chromatid, or displacement of the fragment of chromatid); ace – acentric fragment (fragment of a chromosome with 2 chromatids without a centromere); dic – dicentric (a chromosome with 2 centromeres). Triradial figure is a chromosome arrangement formed by fusion of broken translocated chromatids in a three-armed way. Tetraradial figure is a chromosome arrangement formed by the incorrect joining of 2 chromosomal breaks involving 2 homologous or non-homologous chromosomes.

Table 2. Results of statistical analysis of spontaneous and mitomycin C (MMC)-induced chromosome breakage in Fanconi anemia (FA), possible mosaic FA and non-FA patients

Culture type	Group	Analyzed cells, n	Sample size, n	Analyzed cell numbers per patient (range; Me; Q1; Q3)	General number of breaks in the analyzed group	Abnormal cells, n	Aberrant cells (%)	Number of breaks/ cell (Me; Q1; Q3)	Mann–Whitney U test with Bonferroni correction (Z; p) for number of breaks/cell	Number of breaks/ aberrant cell (Me; Q1; Q3)	Mann–Whitney U test with Bonferroni correction (Z; p) for number of breaks/ aberrant cell
Spontaneous chromosome breakage test	controls	3940	100	12–100; 20; 20; 50	24	26	0.55	0; 0; 0	4.87; <0.01	0; 0; 0	4.87; <0.01
	non-FA	1914	60	0–100; 21; 20; 25.75	26	20	0.84	0; 0; 0	5.59; <0.01	0; 0; 0	5.32; <0.01
	possible mosaic FA	854	27	19–100; 21; 20; 30	36	24	1.55	0; 0; 0.02	3.76; <0.01	0; 0; 0.50	2.83; <0.01
	FA	551	13	16–100; 22; 21; 50	56	44	8.31	0.09; 0.07; 0.17	–	1.00; 1.00; 1.40	–
Mitomycin C – 50 nM	controls	4496	100	0–117; 50; 50; 50	388	389	8.66	0.06; 0.00; 0.12	10.25; <0.01	1.00; 0.00; 1.00	10.32; <0.01
	non-FA	2246	60	0–50; 50; 34.5; 50	196	172	7.66	0.04; 0.00; 0.08	4.10; <0.01	1.00; 0.00; 1.00	3.83; <0.01
	possible mosaic FA	1088	27	0–50; 50; 50; 50	183	121	11.55	0.10; 0.00; 0.19	3.45; <0.01	1.00; 0.00; 1.48	2.91; <0.01
	FA	578	13	0–80; 50; 50; 50	691	259	44.58	1.09; 0.5; 1.44	–	2.18; 1.92; 3.00	–
Mitomycin C – 150 nM	controls	4605	100	0–100; 50; 50; 50	988	872	18.87	0.14; 0.06; 0.28	12.08; <0.01	1.00; 1.00; 1.17	12.13; <0.01
	non-FA	2238	60	0–51; 50; 27.75; 50	390	321	14.29	0.08; 0.00; 0.19	4.12; <0.01	1.00; 0.00; 1.17	4.06; <0.01
	possible mosaic FA	1076	27	0–50; 50; 50; 50	261	200	19.78	0.14; 0.03; 0.29	3.57; <0.01	1.00; 0.42; 1.42	3.49; <0.01
	FA	543	13	0–57; 50; 50; 50	2163	386	71.36	2.54; 1.10; 5.02	–	3.55; 2.89; 6.26	–
Mitomycin C – 300 nM	controls	5003	100	11–94; 50; 50; 50	1880	1421	28.24	0.30; 0.16; 0.54	13.29; <0.01	1.22; 1.00; 1.28	13.30; <0.01
	non-FA	2973	60	14–60; 50; 50; 50	692	605	20.12	0.18; 0.10; 0.30	5.63; <0.01	1.00; 0.79; 1.31	5.63; <0.01
	possible mosaic FA	1333	27	0–50; 50; 50; 50	1016	442	33.81	0.64; 0.55; 1.02	5.06; <0.01	2.18; 1.92; 2.74	5.05; <0.01
	FA	672	13	36–86; 50; 50; 50	4486	575	85.62	4.86; 4.24; 8.34	–	6.18; 5.14; 8.34	–

Me – median; Q1 – 1st quartile; Q3 – 3rd quartile.

Table 3. Scoring aberrations in chromosomal breakage test with mitomycin C (MMC)

Type of aberration	Break events, n
Chromatid/chromosome break (chtb)	1
Acentric fragment (ace)	1
Dicentric chromosome (dic)	2
Triradial figure	2
Tetraradial figure	2
Complex reciprocal chromatid exchange (chte)	sum of centromeres and open breaks

The most reliable results were obtained using the highest MMC concentration (300 nM), with an increased aberration rate in FA patients. Moreover, statistically significant differences in aberration rates were observed between patient and control blood samples (Fig. 2,3).

In the present study, the maximal percentage of MMC-induced breaks/aberrant cell at 300-nM MMC was 100%. The median number of MMC-induced chromosome breaks/cell for the FA patients was 4.86, with a Q1 value of 4.24 and a Q3 value of 8.34. The MMC-induced breaks/

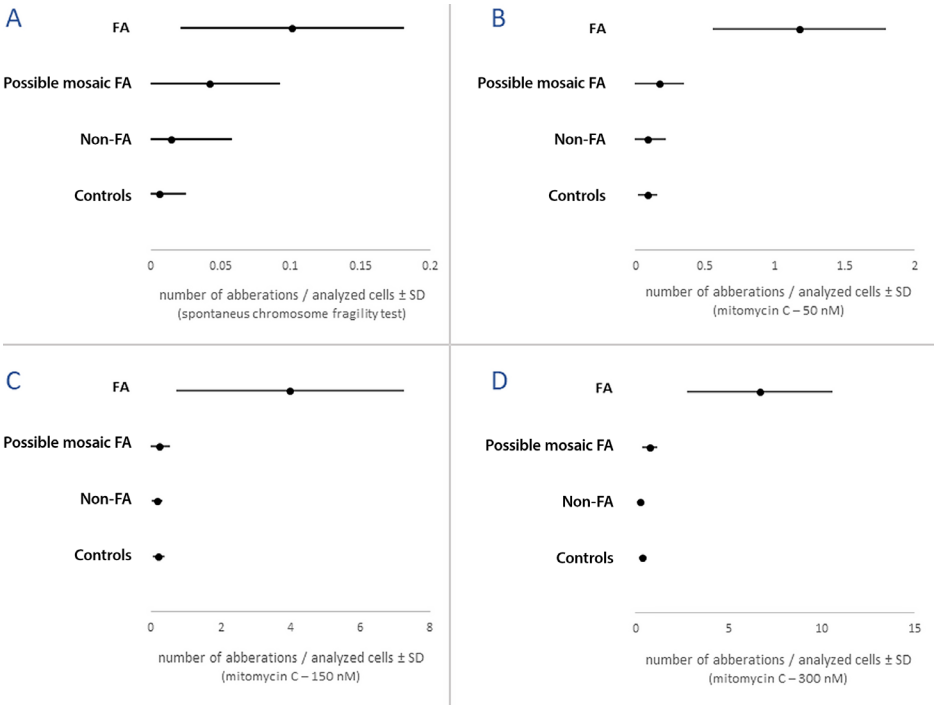

Fig. 2. Differentiation between Fanconi anemia (FA) and other groups by comparing mean breaks/cell observed in the different study groups after treatment with various concentrations of mitomycin C (MMC). A. Culture without MMC; B. Culture with 50 nM of MMC; C. Culture with 150 nM of MMC; D. Culture with 300 nM of MMC

SD – standard deviation.

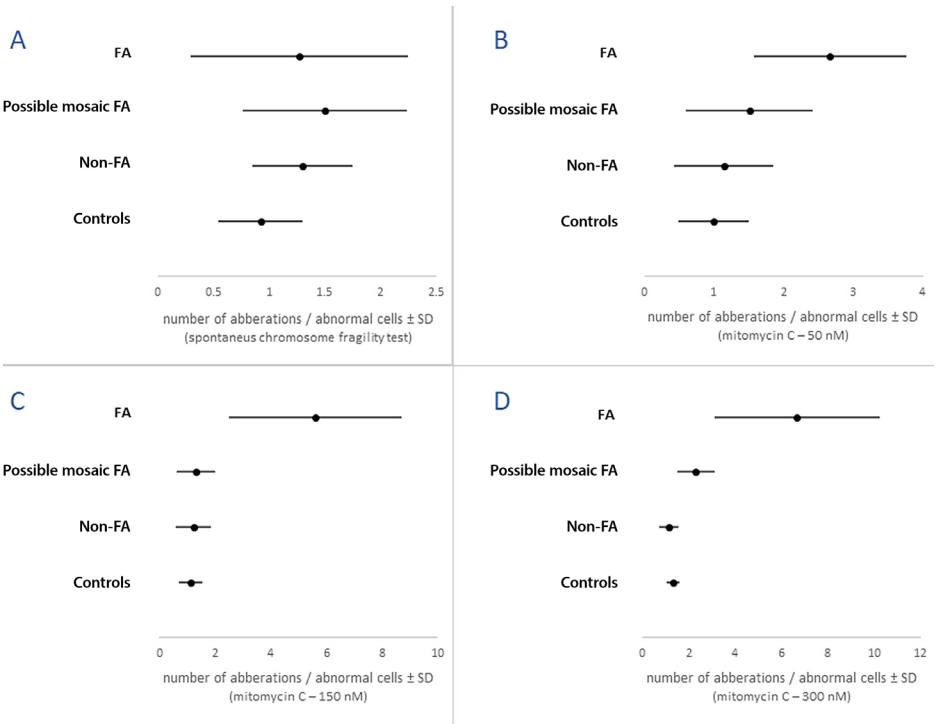

Fig. 3. Differentiation between Fanconi anemia (FA) and other groups by comparing mean breaks/abnormal cell values in the different study groups after treatment with various concentrations of mitomycin C (MMC). A. Culture without MMC; B. Culture with 50 nM of MMC; C. Culture with 150 nM of MMC; D. Culture with 300 nM of MMC

SD – standard deviation.

aberrant cell ranged from 3.44 to 16.18, while the median was 6.18 (Q1 = 5.14, Q3 = 8.34) (Table 2). Meanwhile, patients who presented with possible mosaic FA had a median breaks/cell value of 0.64 (Q1 = 0.55, Q3 = 1.02) and median MMC-induced breaks/aberrant cell value of 2.18 (Q1 = 1.92, Q3 = 2.74) (Table 2). Patients with no MMC hypersensitivity (non-FA group) had a median breaks/cell value of 0.18 (Q1 = 0.10, Q3 = 0.30) and a median MMC-induced breaks/aberrant cell value of 1.00 (Q1 = 0.79, Q3 = 1.31) (Table 2). In the control group, the median breaks/cell was 0.30 (Q1 = 0.16, Q3 = 0.54), and the median MMC-induced breaks/aberrant cell was 1.22 (Q1 = 1.00, Q3 = 1.28) (Table 2).

Data from other cultures (0-nM, 50-nM and 150-nM MMC) were analyzed similarly. Patients with FA were visibly different from non-FA individuals and those with possible mosaic FA (Table 2). The statistical analysis showed significant differences between the FA group and other groups ($p < 0.01$ for all comparisons using the Mann–Whitney U test with Bonferroni correction).

Discussion

Fanconi anemia is a rare human genetic condition associated with hematological manifestations and a high risk of solid tumors. The clinical picture of FA may present with hypopigmentation or hyperpigmentation of the skin, short stature, skeletal defects of the limbs such as radial aplasia and hand and thumb abnormalities, congenital heart defects, and kidney malformations. Bone marrow failure usually develops between 7 and 10 years of age, while neoplasms can occur in up to 20% of patients. Acute myeloid leukemia is most frequent in adolescence, while head and neck tumors usually develop in adulthood.[2,13]

The chromosome breakage protocol used in the present study has been widely applied to detect FA for around 30 years. It requires a time-consuming analysis and specialized laboratory staff. The DNA cross-linking agents such as MMC, DEB and cis-diamminedichloroplatinum(II) (cisplatin) are used to demonstrate the hypersensitive phenotype of FA cells. According to the International Fanconi Anemia Registry (IFAR), DEB sensitivity is much more accurate for FA diagnosis than other cross-linking agents. However, most laboratories perform chromosome break induction in lymphocyte cultures of possible FA patients using MMC, since DEB is on the Special Health Hazard Substance List, as it is a volatile carcinogen that should be handled with great caution. In contrast, MMC and cisplatin are clinically approved chemotherapeutic agents that undergo rigorous quality control and are stable when stored in the vials provided by the manufacturer.[10,19]

Diagnosing patients with somatic hematopoietic mosaicism is challenging since a subset of their peripheral blood cells undergoes a molecular event in which 1 *FANC* allele reverts to normal while the second allele remains mutated. Furthermore, mosaicism presenting in the blood or bone marrow of those with FA does not protect from the development of clonal chromosome abnormalities, hematologic malignancies or solid tumors.[15]

In the present study, the results of the MMC breakage test revealed a higher percentage of MMC-induced aberrant cells in FA patients compared to non-FA patients and possible mosaic FA patients. The study revealed that 13 (13%) out of 100 examined patients had a cellular FA phenotype with increased MMC-induced chromosome fragility. The number of MMC-induced breaks/cell after 300-nM MMC treatment was more than 23 times higher in FA patients than in non-FA patients, and a clear discrimination was observed between FA and non-FA subgroups.

According to the IFAR study, significant differences were observed between the FA and non-FA groups based on DEB-induced chromosome breaks. Also, patients from the possible mosaic FA group had lower chromosome fragility than FA patients. However, there was an overlap between the possible mosaic FA group, non-FA group and controls. These findings may have resulted from a low number of cells with substantial breaks and/or radials in healthy individuals, a phenomenon also described by Castella et al.[20]

According to the current study, the incidence of FA is lower in the Polish population than in other European countries,[3,17] which may result from the routine testing of children, but not adults, with a clinical suspicion of FA in Poland. Moreover, it would be reasonable to perform simultaneous blood and skin tests in all patients with suspected FA at the beginning of the diagnostic process to detect those with mosaic FA.

Limitations

An important limitation of our work is that we only performed a cytogenetic study on peripheral blood T-lymphocytes to distinguish possible mosaic FA patients from definite FA patients. Chromosome breakage tests on skin fibroblasts and *FANC* gene sequencing of blood and skin in possible mosaic FA patients are necessary for the next step of our research. However, the strength of our study is that it is the first to directly compare cytogenetic results of patients classified as FA, possible mosaic FA and non-FA in a Polish population with suspected FA.

Conclusions

The results of the current study highlight the importance of performing standard diagnostic MMC tests in patients with suspected FA. The role of FA laboratory tests in this regard is extensive:

1. The heterogenic nature of FA is often the reason for late clinical diagnosis. Moreover, 3 well-known syndromes can yield positive results in the chromosomal breakage test,

including Warsaw breakage syndrome, Nijmegen breakage syndrome and Roberts syndrome, though all are characterized by specific chromosomal aberrations[10];

2. Patient control and care by hematologists, oncologists and other specialists are required for life-long monitoring and treatment of FA[21,22];

3. Positive MMC-induced breakage results are crucial for patients requiring bone marrow transplantation or chemotherapy. Patients with FA require a modified pre-transplantation conditioning protocol, with a lower than usual dose of chemotherapeutic agents[21];

4. The results of the present study indicate that testing MMC-induced chromosome breakage is useful for distinguishing FA patients from others manifesting some FA clinical features. An accurate diagnosis in these patients is critical for therapeutic decision-making.

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