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A CLASTOGENICITY STUDY WITH MITOMYCIN-C IN APLASTIC ANEMIA: DISTINGUISHING FANCONI ANEMIA

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ABSTRACT

Objective: *Aplastic anemia is a rare condition characterized by bone marrow failure due to various etiologies including Fanconi anemia. Mitomycin C is an alkylating agent inducing chromosome breaks in aplastic anemia. The clastogenic effect of mitomycin C is most marked in Fanconi anemia. This study aims to demonstrate mitomycin C induced chromosomal breaks in patients with aplastic anemia and distinguish Fanconi anemia patients among them.*

Material and Method: *Clastogenicity test was applied to 147 patients with aplastic anemia, four siblings and 30 healthy controls. Both simultaneous and mitomycin C (MMC) treated lymphocyte cultures were prepared. Chromosome breaks per metaphase were calculated and cytogenetic abnormalities were analyzed.*

Results: *The mean number of chromosome breaks per metaphase increased from 0.02 to 0.37 after MMC treatment in patients with aplastic anemia. This number changed only from 0.01 to 0.16 in the healthy controls. In 25 patients (16.6%), the number of chromosome breaks per metaphase was ≥ 1 in MMC treated cultures, and MMC treatment induced a significant increase compared to spontaneous culture (2.88 vs 0.35), which assured the diagnosis of Fanconi anemia. We observed various multiple chromosomal aberrations in all of them and accompanying phenotypic features in 20.*

Conclusion: *Mitomycin C induced chromosomal breaks and cytogenetic abnormalities enable the diagnostic differentiation between aplastic anemia and Fanconi anemia.*

Keywords: *Aplastic anemia, Fanconi anemia, bone marrow failure, mitomycin C.*

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APLASTİK ANEMİDE MİTOMİSİN-C İLE BİR KLASTOJENİTE ÇALIŞMASI: FANKONİ ANEMİSİNİ AYIRT ETME

ÖZET

Amaç: Aplastik anemi, Fanconi anemisi de dahil olmak üzere çeşitli etiyolojilere bağlı kemik iliği yetmezliği ile karakterize nadir görülen bir hastalıktır. Mitomisin C, aplastik anemide kromozomal kırıklara neden olan alkilleyici bir ajandır. Mitomisin C'nin klastojenik etkisi en çok Fanconi anemisinde belirgindir. Bu çalışma, aplastik anemili hastalarda mitomisin C'ye bağlı kromozomal kırıkları göstermeyi ve böylece Fanconi anemili hastaları aplastik anemili hastalardan ayırt etmeyi amaçlamaktadır.

Materyal ve Metot: Klastojenite testi aplastik anemili 147 hasta, dört kardeş ve 30 sağlıklı kontrole uygulandı. Eşzamanlı mitomisin C (MMC) ile muamele edilmiş ve edilmemiş lenfosit kültürleri hazırlandı. Metafaz başına kromozom kırıkları hesaplandı ve sitogenetik anomaliler analiz edildi.

Bulgular: Aplastik anemili hastalarda MMC muamelesinden sonra metafaz başına ortalama kromozom kırık sayısı 0,02'den 0,37'ye yükseldi. Bu sayı sağlıklı kontrollerde sadece 0,01'den 0,16'ya değişti. Yirmibeş hastada (%16,6), MMC ile muamele edilen kültürlerde metafaz başına kromozom kırık sayısı ≥ 1 idi ve spontan kültüre kıyasla Fanconi anemisi tanısını kesinleştiren önemli bir artışa neden oldu (0,35'e karşı 2,88). Hepsinde çeşitli multipl kromozomal aberasyonlar ve 20'sinde eşlik eden fenotipik özellikler gözlemlendi.

Sonuç: Mitomisin C'nin neden olduğu kromozomal kırıklar ve sitogenetik anomaliler, aplastik anemi ile Fanconi anemisi arasında tanısal ayrım yapılmasını sağlar.

Anahtar kelimeler: Aplastik anemi, fankoni anemisi, kemik iliği yetersizliği, mitomisin C.

INTRODUCTION

Aplastic anemia is a rare disease characterized by deficiency of hematopoietic cells (< 25%) in the bone marrow. Many factors may be responsible for its etiology. Among these, radiation and benzene exposure are the best known. However, an underlying genetic disease such as Fanconi anemia, dyskeratosis congenita, Diamond-Blackfan anemia, TAR syndrome and Dubowitch syndrome may also cause it.[1,2]

Fanconi anemia is a genetic disorder with progressive bone marrow failure, congenital anomalies, growth retardation and predisposition to leukemia and other cancers.[3] It occurs with an incidence of 1 case per 100,000 live births and a prevalence estimated at 1 to 5 per million with a carrier frequency as high as 1 in 300.[4] The average life expectancy is approximately 20 years. Most patients are diagnosed at childhood but diagnosis may be delayed if they don't have the typical phycical features. It has been reported that up to 25-40% of patients didn't have a congenital anomaly.[3,5-7]

Cells from patients with Fanconi anemia exhibit faulty DNA repair of interstrand crosslinks. Those covalent links between two strands of DNA inhibit DNA replication and induce chromosomal breakage. This process particularly affects stem cells resulting in bone marrow failure. Treatment of the cultured lymphocytes with DNA interstrand crosslinking agents such as diepoxybutane (DEB) or mitomysin C (MMC) stimulates the breakage of DNA resulting in increased numbers of chromosomal abnormalities including translocations and radial chromosomes. This profound hypersensitivity has provided the gold standard test, chromosomal breakage analysis to diagnose Fanconi anemia.[5,7-10]

In this study we performed chromosomal breakage test in patients with aplastic anemia to identify the Fanconi anemia patients among them.

MATERIAL AND METHOD

Chromosomal breakage analysis was performed in a total of 147 patients referred with a preliminary diagnosis of aplastic anemia to rule out Fanconi anemia. Four siblings of Fanconi anemia patients were also consulted and included in the study group. A control group consisted of 30 healthy individuals. Among this total of 181 individuals, 79 were female and 102 male with a mean age of 13.3 ± 1.4 years (3-36 years) (Table 1).

Each case was evaluated with a detailed medical history and physical examination. Any radiological intervention or blood transfusion was queried as they could affect the results.

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of University Istanbul, Istanbul Medical Faculty (Date 11.19.2021/No 21).

Cell Cultures, Treatments, and Chromosome Preparations

Peripheral blood samples were obtained to prepare lymphocyte cultures. Two cell cultures were prepared for each participant. The first one was a spontaneous culture

(control) and no clastogenic agent was added. The second culture was treated with 0.1 μ/ml MMC (Sigma) at the 24th hour. Both cultures were processed under the same conditions for routine karyotype analysis. After routine harvesting five metaphase spreads were prepared and a hundred metaphases were examined in each culture for breaks, gaps, acentric fragments and exchange figures. G banding was also performed to detect derivative chromosomes and translocations.

Chromosomal breaks per metaphase were counted. Dicentric chromosomes, translocations, and ring chromosomes were counted as having two breaks while acentric chromosome parts as well as chromosome and chromatid breaks were each counted as one. When the number of chromosome breaks per metaphase was ≥ 1 or a minimum 3-fold increase in the percentage of chromosomal breaks in the MMC treated culture compared to the spontaneous culture, this assured the diagnosis of Fanconi anemia.[9-15]

Statistical Analysis

The data were analyzed using the Statistical Package for the Social Sciences (SPSS) ver21 statistical software (SPSS Inc., Chicago, IL, USA). P value less than 0.05 was considered statistically significant.

RESULTS

A total of 147 patients were referred for MMC induced chromosome breakage test. We demonstrated an increased rate of chromosomal breakage in 21 patients (20.7%), which leaded to the diagnosis of Fanconi anemia. Four siblings of the patients with positive test results participated subsequently. The patient characteristics and the test results are summarized at Table 1.

We observed the classical phenotypic features besides bone marrow failure in 20 of the patients with positive test results. Only one patient had no clinical signs of Fanconi anemia but aplastic anemia. The other 4 cases (3 women and a man) with positive chromosomal breakage test having normal phenotype without hematological manifestations were the siblings of three patients. They were detected when family mapping.

The chromosomal breakage test revealed negative results in 126 of the 147 patients with bone marrow failure. The mean number of chromosome breaks per metaphase in the spontaneous and MMC induced cultures were 0.02 ± 0.02 and 0.37 ± 0.12 respectively in patients with aplastic anemia, 0.35 ± 0.26, 2.88 ± 1.46 in Fanconi anemia patients and 0.01 ± 0.007 and 0.16 ± 0.04 in the control group consisting healthy volunteers (Table 2).

Table 1. Patient characteristics

	Mean Age Years (Age interval)	Male / Female	MMC Testi (+)	MMC Testi (-)	Total
Aplastic Anemia	11.1 (3-32)	77/49	0	126	126
Fanconi Anemia	13.3 (3-36)	10/15	25*	0	25
Healthy Controls	12.6 (5-35)	15/15	0	30	30
Total	13.3 ± 1.4 (3-36)	102/79	25	156	181

*20 patient with phenotypic features, 1 patient without phenotypic features, 4 siblings without phenotypic features or hematological manifestations of 3 patients
MMC: Mitomisin C

Table 2. Results of chromosomal breakage analysis

	Chromosomal Breaks Per Metaphase	
	Spontaneous culture Mean ± SD (interval)	MMC treated culture Mean ± SD (interval)
Aplastic Anemia	0.02 ± 0.02 (0.01 - 0.05)	0.37 ± 0.12 (0.02 – 0.37)
Fanconi Anemia	0.35 ± 0.26 (0.00 - 1.00)	2.88 ± 1.46 (1.00 - 6.54)
Healthy Controls	0.01 ± 0.007 (0.00 - 0.01)	0.16 ± 0.04 (0.01 – 0.16)

MMC: Mitomisin C, **SD:** standard deviation.

The breakdown of the cytogenetic analysis of 25 patients with Fanconi anemia is seen at Table 3. We observed chromosome and chromatid breaks, gaps, acentric fragments, and ring chromosomes in the metaphase spreads of those patients (Figure 1). Triradial and tetraradial exchanges were demonstrated in Figure 2.

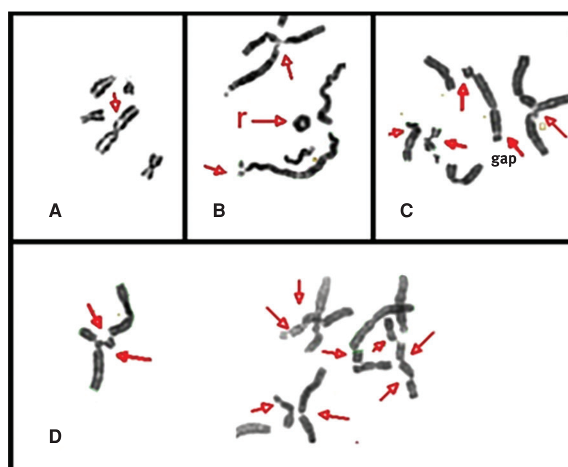

Figure 1. Chromosome breaks in Fanconi anemia patients
A: Chromosome break; **B:** Ring chromosome (r) and chromosome breaks; **C:** Gap and chromosome breaks; **D:** Acentric fragment and chromosome breaks

Case Number	Gender*	Age (years)	Chromosomal Breaks Per Metaphase		Number of exchange chromosomes
			Spontaneous culture	MMC treated culture	
1	M	15	0.4	2.49	0
2	M	17	0.25	3.0	0
3	M	7	0.4	3.08	0
4	M	5	0.24	3.3	5
5	M	22	0.02	2.28	3
6	M	36	0.06	1.56	6
7	M	8	0.18	1.68	2
8	M	14	0.15	2.38	2
9	M	3	0	5.34	7
10	M	14	0.06	1.0	6
11	F	12	1.0	3.5	5
12	F	21	0.64	1.9	0
13	F	16	0.2	2.2	0
14	F	18	0.7	1.6	0
15	F	16	0.55	2.8	0
16	F	15	0.56	4.9	2
17	F	9	0.36	1.2	0
18	F	13	0.4	1.38	2
19	F	9	0.4	2.0	0
20	F	10	0.5	6.54	4
21	F	17	0.02	3.0	0
22	F	6	0.6	5.0	0
23	F	8	0.02	1.6	3
24	F	11	0.92	3.5	2
25	F	11	0.2	4.76	5
TOTAL	10 M/15 F	13.3	0.35 ± 0.26	2.88 ± 1.46	

Table 3. Chromosomal breakage analysis in patients with Fanconi anemia

* **M:** male, **F:** female

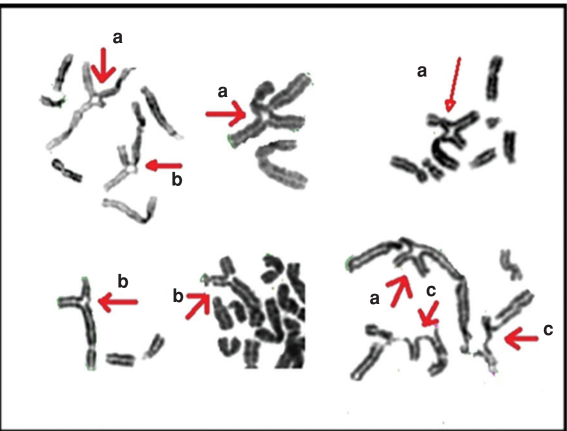

Figure 2. Exchange between chromosomes in Fanconi anemia patients

a) Quadriradial (tetraradial) chromosome structures
b) Triradial chromosome structures
c) Chromosome breaks

DISCUSSION

Fanconi anemia is a heterogonous disease in terms of the age of onset, phenotypic expression, clinical course and laboratory signs. Alan and D'andrea reported that the age at diagnosis ranged from 0 to 48 years with a median age of 6.5 years for boys and 8 years for girls.[6] Our patients with Fanconi anemia had an age range of 3 to 36 years, three patients were over twenties. The median age was 12 years, indicating a delayed diagnosis. Early diagnosis and scheduled cancer screening can prolong life expectancy. We contributed to the diagnosis of Fanconi anemia in 21 patients among the 147 patients (14.2%) referred with bone marrow failure with an additional 4 cases detected during risk assessment for family members, reaching a total of 25 cases (16.6%). Tootian *et al.*, reported that they diagnosed 61 patients cytogenetically among the 318 suspected referrals (19.2%).[16]

The classical phenotype with obvious physical abnormalities (short stature, skeletal malformations, abnormal skin pigmentation, microcephaly, ophthalmic and genital tract anomalies) may be easily recognized by an experienced physician, however a lot of cases will not conform this conversant picture. Therefore, it is difficult to diagnose on the basis of clinical manifestations solely.[5,17-20] It was reported that approximately one-third of the patients did not manifest major clinical malformations.[20,21] An analysis of 754 patients enrolled in the International Fanconi Anemia Registry revealed that 20% of patients didn't have bone marrow failure.[22] Auerbach *et al* classified their patients with Fanconi anemia into four groups with respect to the presence or absence of congenital malformations or aplastic anemia. Both hematologic abnormalities and congenital malformations were present in only 44% of them. 51% had either malformations or hematologic abnormalities and 5% had neither.[7] One of our patients with aplastic anemia didn't have the typical clinical features despite positive clastogenicity test. Dutta *et al*, reported that 6 out of 63 (9.3%) phenotypically normal aplastic anemia patients showed sensitivity towards MMC.[23] A study conducted on 318 patients referred for Fanconi anemia diagnosis revealed that there was no clinically significant difference with respect to several hematologic and clinical parameters between Fanconi positive and non-Fanconi patients.[16] Because of the variable clinical spectrum, the diagnosis of Fanconi anemia should not depend only on the phenotypic expression, but supported by a reliable laboratory test.

Spontaneous chromosomal breakage was once suggested as a cellular marker of Fanconi anemia, but this finding turned out to be inconsistent by longitudinal studies.[20,24] Therefore, spontaneous chromosomal fragility should not be used as a diagnostic tool.[25,26]

The classic diagnostic test for Fanconi anemia is the assessment of cellular hypersensitivity and abnormal response to DNA interstrand crosslinking agents, DEB and MMC being the most commonly used.[5,20] Exposure of lymphocytes derived from the patients to these clastogenic agents results in high levels of chromosomal aberrations, particularly chromosomal breaks and radial formations.[27] The markedly increased chromosomal breakage after treatment with MMC or DEB compared with the breakage in spontaneous culture reveals a positive test for Fanconi anemia.[5] As shown in Table 2, chromosome breaks per metaphase in spontaneous and MMC treated cultures raised from 0.35 to 2.88 in Fanconi anemia compared to 0.02 to 0.37 in aplastic anemia and 0.01 to 0.16 in healthy controls. These results favor using chromosomal breakage analysis to distinguish Fanconi anemia from aplastic anemia. It enables identification of patients with aplastic anemia or leukemia with or without the classical physical stigmata.[20]

Some laboratories prefer DEB, while others choose MMC or use both.[3] Various concentrations and exposure times of the clastogenic agents have been tested.[12,28,29] Both DEB and MMC tests were demonstrated to be sensitive, specific and reproducible.[13,20,30,31] However, when using DEB, it is difficult to provide appropriate laboratory conditions since it is a volatile substance. For this reason, Mitomycin C, a non-volatile and equally effective agent, was studied.[10]

Four new, completely asymptomatic cases were detected after the diagnosis of their siblings, by family mapping (Table 1). They may develop hematological manifestations later in their life. This study perhaps provided to identify them in the pre-anemic phase. The siblings of the affected proband may exhibit weak or absolutely no phenotypic signs, but all of them should be tested for chromosome breakage.[5] This is especially important in countries with high rates of consanguinity. Talmoudi *et al.* evaluated 26 siblings of a total of 38 Fanconi anemia patients in a cohort of 171 aplastic anemia patients with a 93% consanguinity rate. Five of them were diagnosed on the basis of MMC test.[26] In particular siblings considered as potential bone marrow transplant donors should be tested regardless of whether symptomatic or not, because manifestations of Fanconi anemia in the donor sibling may emerge after transplantation.[10] We demonstrated significantly increased number of chromosomal breaks (2.45) per metaphase in the induced cultures of the four first degree relatives of Fanconi anemia patients. However, this ratio was normal in the spontaneous cultures (0.5).

Molecular genetic tests (single gene testing, a multigene panel, and genomic testing) are available but they are complicated and expensive. In the diagnostic algorithm, if the chromosomal breakage testing reveals negative result, no further testing is recommended unless strong clinical suspicion. In case of a positive result, targeted gene panel or whole exome/genome sequencing should be performed.[5] When the resources are limited, molecular genetic testing should be reserved for the following settings: inconclusive chromosomal breakage analysis, prenatal or preimplantation genetic diagnosis.

CONCLUSION

We conclude that chromosomal breakage analysis distinguishes Fanconi anemia from aplastic anemia even when the clinical manifestations are subtle or before the presentation of bone marrow failure. Thus, all patients with bone marrow failure regardless of phenotypic characteristics should be referred for the clastogenicity test. The siblings of Fanconi anemia patients should also be tested.

*The authors declare that there are no conflicts of interest.

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