ORIGINAL ARTICLE

Differentiation of Fanconi Anaemia (FA) from Idiopathic Aplastic Anaemia by Mitomycin - C (MMC) Stress Test

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Abstract:

Introduction and Background:
Fanconi Anaemia (FA), an autosomal recessive disorder is one of the most common amongst hereditary causes of idiopathic aplastic anaemia with a worldwide incidence of 1 in 1, 60,000 individuals. Apart from the haematological changes like pancytopenia and bone marrow failure, which are the most important clinical features, FA also presents with a variety of congenital anomalies and spontaneous chromosome instability. However, FA is difficult to diagnose on the basis of clinical features alone due to its heterogenous nature. Hypersensitivity to DNA cross linking agents like Mitomycin C (MMC), Diepoxybutane (DEB) is used as a differential diagnostic test while working up for aplastic anaemia.

Aims and Objectives:
Objective of our study was to throw light on the significance of Mitomycin stress test as a first line test in diagnosing FA while differentiating it from other causes of idiopathic aplastic anaemia. Since FA is a hereditary disease, an early identification of cases in at-risk families can help in haematological monitoring of the patients in these families.

Materials and Methods:
Patients of idiopathic aplastic anaemia with strong clinical suspicion of FA were taken up for chromosomal breakage evaluation by Mitomycin stress test (MMC Stress test) and results were compared using samples from age and gender matched controls.

Results and Discussion:
1. Out of 17, 4 patients showed chromosomal instability suggestive of Fanconi anemia.
2. On MMC stress test, we observed increased number of chromosomal breaks as compared to the controls. Also it was observed that patients showed triradial, quadriradial and complex chromosomal breakage figures in more than 90 percent of the cells analysed.

Conclusion:
Mitomycin induced stress test can be considered as a gold standard test in differentiating Fanconi anaemia patients from the other causes of aplastic anaemia.

Keywords: Idiopathic Aplastic anaemia, chromosomal breakage syndrome, Mitomycin, Fanconi anaemia

Introduction:
Fanconi anaemia belongs to group of disorders that cause hereditary aplastic anaemia. Apart from progressive pancytopenia, Fanconi anaemia is characterized by variable congenital anomalies, susceptibility to malignancies and induced chromosomal instability[1]. Chromosome instability is a characteristic cytogenetic feature of a number of genetically determined human disorders collectively known as chromosome breakage syndromes.
These disorders include Fanconi anemia (FA), Bloom's syndrome (BS) and Ataxia telangiectasia (AT)[2]. In each of these, chromosome instability exists in the form of

increased frequencies of breaks and inter-changes occurring either spontaneously or following treatment with various DNA-damaging agents [3,4]. In 1927, Guido Fanconi described a family in which three brothers, all between 5and 7 years of age, displayed pancytopenia and various birth defects[5]. In 1931, Naegeli proposed the name Fanconi Anemia (FA) to describe patients with pancytopenia, congenital physical anomalies and familial occurrence.

Sixteen genes associated with the FA phenotype have been identified since 1992 (Selenti N, et al., 2015**).** .At least 15 genes, which are responsible for the known FA complementation groups (A, B, C, D1 [BRCA2], D2, E, F, G, I, J [BRIP1], L, M, N [PALB2], O [RAD51C], and P [SLX4]) have been identified [6]. All FA genes are localized to autosomes, except FANC, which is X-linked and subject to X inactivation in female carriers[7]. It was recognized that the FA phenotype is extremely variable, making diagnosis difficult on the basis of clinical manifestations alone[8].

FA is heavily under diagnosed due to its rarity and variable presentation. The disease is usually seen between the ages of 5 and 10 years and presents with growth retardation and congenital defects in combination with thrombocytopenia followed by pancytopenia (life threatening bone marrow failure). The classical clinical presentation is progressive bone-marrow failure, which first manifests as low platelet count and eventually leads to transfusion dependent anaemia in first two decades of life[9]. Majority of patients with underlying undiagnosed FA do not respond to immunosuppression therapy, which is usually given to treat idiopathic aplastic anaemia. Moreover, patients with FA often succumb due to a hypersensitivity to chemotherapy agents and their toxicity, if given conventional conditioning for hematopoietic stem cell transplant (HSCT).

Hence, less myeloablative regimens have been used in this population[10].

The management of FA is essentially supportive and differs from that of other causes of aplastic anaemia and hence a proper diagnosis is essential.

In 1964, Schroeder and colleagues discovered that cells derived from FA patients displayed higher levels of spontaneous chromosomal aberrations when compared to cells derived from unaffected individuals. This cellular phenotype of FA cells is even more pronounced following exposure to DNA cross linking agents such as diepoxybutane (DEB) [11,12] or mitomycin C (MMC)[13]. The hypersensitivity of FA cells to D NA cross-linking agents is now recognized as a unique cellular marker for the disorder, and is also an essential diagnostic tool for the disease[7].

Mitomycin C is an antibiotic which was first isolated in 1956 from the broth of Streptomyces caespitosus. It belongs to the group of agents which are also known as clastogens (Figure1) or DNA interstrand cross linkers. The other chemicals included in this category are diepoxybutane (DEB), nitrogen mustard, and cyclophosphamide etc. MMC is biologically inactive in its natural state, but upon chemical or enzymatic reduction, it is reduced in the cell to hydroquinone derivatives by a quinine reductase (diaphorase) and these components are then able to alkylate and extensively cross link DNA[14].

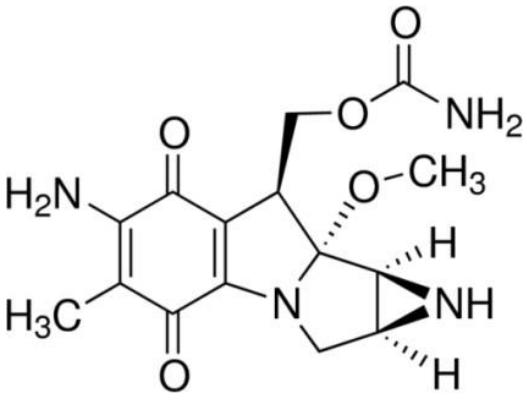


Figure 1: Chemical structure of Mitomycin C [15]

The test using these cross-linking agents to the peripheral blood cultures is regarded as the gold standard for diagnosing FA[11]. Chromosomal breakage tests can identify the suspected patients in at risk families by assessing their chromosomal damage. This can pave the way to genetic counseling and preventing the cases in future generations.

Aims and Objectives:

With this background, the objective of this study was to:

1. Standardize and validate laboratory protocol of MMC - stress test.
2. To apply MMC - stress test to diagnose FA from the pool of suspected idiopathic aplastic anaemia from peripheral blood cultures.
3. To describe chromosomal breakage pattern with respect to the number of chromatid breaks, gaps and radial structures in the patient samples.

Materials and Methods:

This study was done as a part of an internal project at the Centre for DNA Fingerprinting and Diagnostics (CDFD), Hyderabad. This was a prospective observational study on patients of idiopathic aplastic anaemia. A total of 17 patients with aplastic anaemia or with a clinical suspicion of FA, referred to the Diagnostic division (CDFD), from September 2015 to February 2016, were taken up for chromosome breakage evaluation by MMC test.

The patients were having history of recent blood transfusion and having history of any malignancy and / or on treatment with alkylating agents were excluded from the study. Informed consent was taken from the patients. In case patient was minor, consent was taken from the parents and ascent from the minor. For standardization of the protocol, 17 subjects who were phenotypically normal individuals of both sexes were taken as controls.

Peripheral blood samples (3- 4ml) collected in sodium heparin vaccutainer was obtained from each patient and 72hr cultures were set up, using 0.5ml of heparinised blood sample and 5 ml of RPMI culture medium (Flow-chart). Replicate cultures were set up with different concentrations set in both control and patient samples. Harvesting was started at 69th hour by adding colchicine. Slides were prepared and analysed. Fifty Giemsa-stained metaphases were analysed and was scored for chromosome number and for the any changes in the chromosome structures.

Flow chart for MMC-stress test

Patient/ control Blood Sample
↓
72 hours PHA stimulated culture
↓
After 24hours: MMC added
↓
Hypotonic treatment and Fixing
↓
(Colchicine at 69th hour followed by harvesting at 72 hour)
↓
Slide Preparation
↓
Slide staining (Giemsa Stain)
↓
Microscopic Analysis

Standardization of the MMC Concentration for Breakage Analysis:

Two to three ml peripheral blood was collected from 27 controls. Cultures were set up with four different concentrations (50µl, 100µl, 200µl, 300µl) of MMC of working concentration of 0.01mg/ml.Cultures from the healthy controls were not affected at all concentrations except 300 µl, where around 30% of the cells show 1 to ≤ 5 break events/cell. Hence the target concentration was set at 200µl for further experiments in both controls and patients.

Results:

1. A total of 50 metaphases were scored and analyzed for chromosome breakage as well as the formation of radials. Results were compared with those from normal control cells. While counting chromosomal breakage, following things were followed:

- Cultures without the MMC were used to measure the spontaneous breakage rate.
- Results are reported as the average number of breaks/cells.
- The number of cells with radial forms is recorded.
- Types of aberration taken into consideration for scoring were chromatid gaps, chromatid breaks, triradial chromosomes, quadriradial chromosomes, other chromatid interchanges. Chromatid gaps or breaks are counted as single break events, tri- and quadriradials as two break events each.

2. According to the results of the MMC test, the patients were divided into two subgroups: FA, displaying typical MMC sensitive cellular response, and non-FA (Table: 1).

Table No. : 1 Results and Scoring for aberrations:[13]

Sr.No	Case No.	Breaks present/absent	No. of breaks /cell	Type of aberrations visualized	Inference
1.	Case-1	Present	>10 breaks/cell*	Breaks,triradials, complex figures	**FA**
2.	Case-2	Present	>10 breaks/cell*	Complex figures	**FA**
3.	Case-3	Absent	-	-	NON-FA
4.	Case-4	Absent	-	-	NON-FA
5.	Case-5	Absent	-	-	NON-FA
6.	Case-6	Present	>10 breaks/cell*	Breaks, Triradials present	**FA**
7.	Case-7	Absent	-	-	NON-FA
8.	Case-8	Absent	-	-	NON-FA
9.	Case-9	Absent	-	-	NON-FA
10	Case-10	Absent	-	-	NON-FA
11	Case-11	Absent	-	-	NON-FA
12	Case-12	Present	>10 breaks/cell	Triradials breaks present.	**FA**
13	Case-13	Absent	-	-	NON-FA
14	Case-14	Absent	-	-	NON-FA
15	Case-15	Absent	-	-	NON-FA
16	Case-16	Absent	-	-	NON-FA
17	Case-17	Absent	-	-	NON-FA

- Hence out of the 17 patients 4 (Case 1, 2, 6 and 12) were found to have FA cellular phenotype with increased number of MMC-induced chromatid and chromosome breaks and a variety of chromatid and chromosome interchanges. The increased sensitivity to MMC was present regardless of phenotype, congenital anomalies, or severity of the disease.

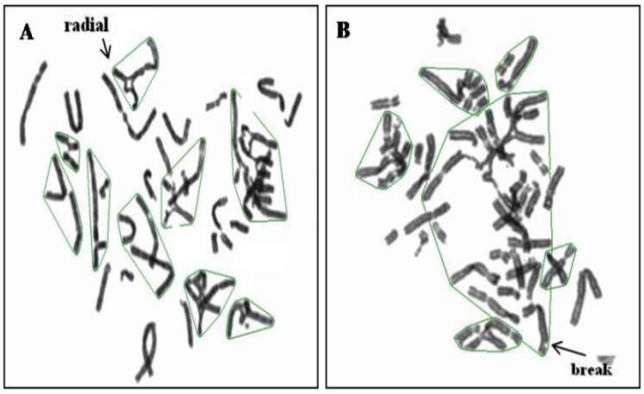


Figure - 2: Case 1 showing triradial formation and chromatid breaks

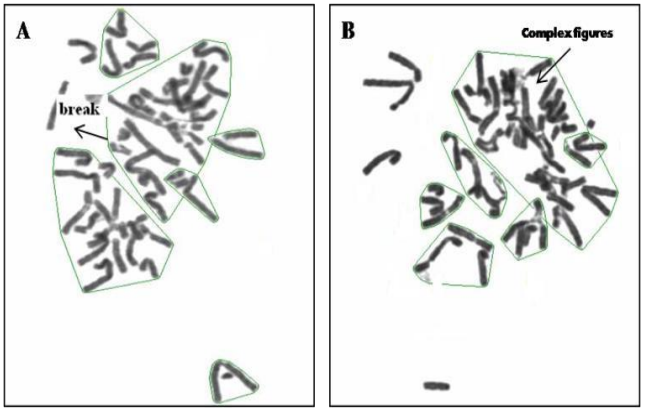
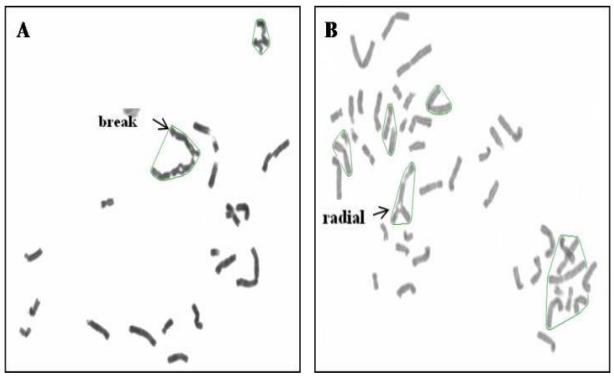


Figure - 3: Case 2 chromatid breaks and complex figures



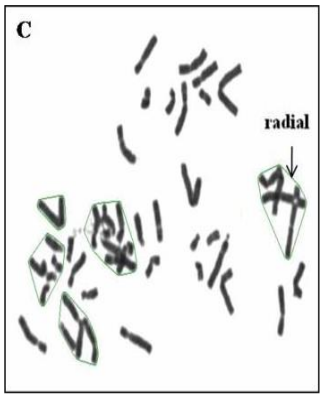


Figure - 4: Case 12 showing triradial formation and chromatid breaks

Discussion:

Chromosome instability is a characteristic cytogenetic feature of a number of genetically determined human disorders collectively known as chromosome breakage syndromes. These disorders include Fanconi anemia (FA), Bloom's syndrome (BS) and Ataxia telangiectasia (AT)[2]. In each of these, chromosome instability exists in the form of increased frequencies of breaks and inter-changes occurring either spontaneously or following treatment with various DNA-damaging agents[3],[4]. The test using these clastogens like DEB and MMC, to the peripheral blood cultures is regarded as the gold standard for diagnosing FA. In the present study, attempt was made to identify patients with the MMC induced stress test on peripheral blood cultures. It was found that 4 out of 17 examined patients had a FA cellular phenotype with increased

number of MMC-induced chromatid and chromosome breaks and a variety of chromatid and chromosome interchanges. This was in congruence with other researchers from India[16].

Although these patients were diagnosed after the onset of aplastic anemia, MMC-stress test can be used to detect such patients even in the pre-anemic phase. This would help in avoiding drugs that are usually administered in acquired or idiopathic aplastic anemia. Further, screening parents of FA patients can help detect 'silent' cases[17]. Such testing would also help patients who were diagnosed in pre anaemic phase, since it would help in avoiding drugs that are usually administered in acquired or idiopathic aplastic anemia. A delay in the diagnosis of FA can have serious consequences for patients and their families. An earlier diagnosis in FA patients (*i.e.,* before onset of haematological abnormalities) could provide more time to find a suitable HLA compatible donor for bone marrow transplantation. Further, at-risk families (with an affected child) should be identified early and offered genetic counselling and prenatal diagnosis, as FA is an autosomal recessive disorder with a recurrence risk of 25 %[18],[19]. Such a delay in identification of FA patients and at-risk families can be avoided by performing MMC-stress test in patients with macrocytosis and decreased platelet count as observed during screening of complete blood count with differentials[17],[20].

Hence, Mitomycin induced stress test can be considered as a gold standard test in differentiating FA patients from the other causes of aplastic anaemia. It also helps in identification of the at-risk families of FA so that genetic counseling and prenatal testing may be offered to such families.

Conflict of interest: Nil
Sources of Support: Nil

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Received date: 25/07/2020 Revised date: 28/08/2020 Accepted date: 02/09/2020

How to cite this article: Sowmya Gayatri C, Ashwin B. Dalal and Usha R. Dutta, Differentiation of Fanconi Anaemia (FA) from Idiopathic Aplastic Anaemia by Mitomycin-C (MMC) Stress Test. Walawalkar International Medical Journal 2020; 7(2):52-58. http://www.wimjournal.com