Original Article

Evaluation of concentration and storage effects of mitomycin C in the diagnosis of Fanconi anemia among idiopatic aplastic anemia patients

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BACKGROUND: Fanconi anemia (FA) is a rare autosomal recessive genetic disorder that shows an increased sensitivity to the intercalating agents such as mytomycin C (MMC), measured as chromosomal aberrations. This study was conducted to differentiate between FA and "idiopathic" aplastic anemia on the basis of induced chromosomal breakage study with MMC.

MATERIALS AND METHODS: MMC stress tests in different final concentrations of 20 and 50 ng/ml of MMC were conducted on peripheral blood lymphocytes from 32 patients with aplastic anemia and 13 healthy controls. Fifty nanograms per milliliter of MMC from old, fresh and frozen stocks was used to check the sensitivity of diagnosis on FA-diagnosed patients. Statistical analysis was used for the assessment of aberrations, including chromatid and chromosome breaks and exchanges.

RESULTS: Eight patients (25%) with a very high percentage of chromosomal breakage were diagnosed as FA on the basis of the chromosomal breakage study. Six of these patients exhibited congenital anomalies at presentation, while another two lacked such anomalies or had minor physical problems. Freshly made MMC has shown more sensitivity to detect FA patients compared with frozen or 1-week-old MMC stock.

CONCLUSIONS: The study indicates that freshly made MMC stress test provides an unequivocal means of differentiation between FA and "idiopathic" aplastic anemia. Further, the study, the first of its kind from Iran, stresses on the need for conducting this test in all aplastic anemia cases, even those without congenital anomalies, for accurate and timely diagnosis of FA to implement appropriate therapy.

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Introduction

Aplastic anemia is an acquired disorder in more than 80% cases, while the remaining 20% are inherited forms consisting of Fanconi anemia (FA), dyskeratosis congenital and Schwachman syndrome.[1] FA is a rare autosomal-recessive genetic disorder showing progressive bone marrow failure, various phenotypic abnormalities and a high risk of developing malignant disease, particularly acute myelomonocytic leukemia. Cultured primary cells from FA patients show spontaneous or DNA damage-induced high frequency of chromosomal aberrations[2-4] and elongation in G2 transit.[5-9] However, a number of patients display only minor phenotypic variations or lack congenital abnormalities. Such patients present a diagnostic dilemma for clinicians.[3,10,11] Schroeder *et al.*[12] first demonstrated that FA is associated with abnormal susceptibility of the somatic chromosomes to spontaneous aberrations. Sasaki *et al.*,[13] who discovered that their chromosomes were highly sensitive to cross-linking agents such as mytomycin C (MMC) and nitrogen mustard, suggested that FA patients were defective in one of the repair metabolic pathways, especially the mechanism for removing the interstrand cross-links (ICLs) from the DNA. Cross-linking agents are DNA-damaging drugs able to form covalent bridges joining opposite strands of the DNA

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double helix. These bridges, known as ICLs, are a class of particularly toxic DNA lesions. Indeed, for their capability to prevent strand separation, ICLs strongly affect both transcription and replication.[14,15] Several hereditary human diseases associate genetic or chromosomal instability and hypersensitivity to DNA-damaging agents and cancer predisposition. The key features that define these pathologies have been related to abnormalities in DNA repair, cell cycle checkpoint activation or control of apoptosis. Among these pathologies, FA has been unambiguously associated to ICL response due to its chromosomal and cellular hypersensitivity to ICL inducers.[16,17] This forms the basis of the cytogenetic technique for the diagnosis of FA. Auerbach and Wolman[3] showed that the bifunctional alkalyting agent diepoxybutane (DEB), in a nontoxic concentration for the lymphocytes of normal individuals, increased the chromosome breakage in those of FA homozygotes. Auerbach *et al.*[18] were the first to claim that it was possible to distinguish between controls, heterozygotes and homozygotes using the DEB system. Cohen *et al.*[19] demonstrated the possibility of misdiagnosing non-FA patients when the DEB concentration was too high, and claimed that it was impossible to distinguish between controls and heterozygotes. They found that the amount of chromosomal aberrations per cell obtained from heterozygotes overlap with those of controls. They also found the use of DEB or MMC equally reliable in the cytogenetic diagnosis of FA. Kwee *et al.*[20] described an FA patient who showed hypersensitivity to bifunctional alkalyting agents in only a minority of the cultured lymphocytes, the majority being as sensitive as those from healthy controls. They speculated that the clastogen-resistant cells had arisen *de novo*. The same phenomenon was reported by Auerbach *et al.,*[21] raising the question whether the occurrence of clastogen nonresponsive cells, as documented, may be more common among FA patients. As the management of patients with FA differs from that of "idiopathic" aplastic anemia, it is essential to differentiate these disorders at the earliest. Generally, the number of suspected FA patients referring to cytogenetic laboratories is not very much and, therefore, the MMC solution should be frozen and thawed. This made us to investigate whether this frozen MMC can have the same effect of fresh or old MMC or not? In the present study, MMC with two different concentrations was used to analyze induced chromosomal breakage in 32 patients with aplastic anemia with the aim to differentiate these cases and to determine by means of a statistical evaluation of the yield of chromosomal aberrations obtained in lymphocyte cultures to which MMC was added, the accuracy of differentiating between FA homozygotes and controls.

Materials and Methods

Individuals studied

The study group included 32 patients (20 males and 12 females) from Iranian families with aplastic anemia with or without congenital malformations visiting the Blood transfer organization of Tehran. The median age of the patients at presentation in the present study was 8.37 ± 2.44 years (range: 5–12 years). Family history was recorded in predesigned proformas and a detailed pedigree was taken in each case. Informed consent was taken from the patients or their parents, in case of minors. Thirteen phenotypically normal nonsmoker healthy individuals, who had not received any antibiotic or drug for at least 2 months, were randomly selected regardless of sex, race or age, to serve as the control group. The study was approved by the Ethical Committee of the Faculty of Medical Sciences of the Tarbiat Modares University (Tehran, Iran). Parents of patients gave their informed written consent. All healthy donors completed a written questionnaire to obtain information related to their life style, such as dietary habits, medical history and exposure to chemical and physical agents.

Sampling and culture conditions

Blood samples were collected by venipuncture with heparinized tubes. For each individual, three lymphocyte cultures were prepared. One of them was used as a control and the others were used to determine the effect of different concentrations of MMC in order to verify the patients. The bifunctional alkylating agent MMC was used to induce chromosomal aberrations. MMC was diluted with commercially available sterile saline solution to final concentrations of 20 and 50 ng/ml and added to the medium at the time of culture initiation. In all cases,

0.5 ml peripheral blood was cultured for 48 h in 5 ml RPMI-1640 medium supplemented with 15% fetal calf serum, antibiotics and phytohemagglutinin (1%) at 37°C. Colcemid at a final concentration of 0.4 µg/ml was added 2 h before harvesting and mitotic cells were arrested at their first division. MMC prepared as fresh, old (1 week after making) and frozen at a concentration of 50 ng/ml was used to treat only FA-diagnosed samples as described. Slides were prepared from all cultures, stained in 5% Giemsa solution and chromosomal aberrations were analyzed using a ×1000 Nikon light microscope.

Cytogenetic analysis

Chromosome analyses were carried out on first-division metaphases containing 46 centromeres. Lesions were classified according to the International System of Cytogenetic Nomenclature for Acquired Chromosome Aberrations (ISCN, 1985).[22] For each sample, at least 50 metaphases were observed. All metaphases with chromosome abnormalities were independently analyzed. The chromosome-type abnormalities taken into account were dicentric chromosomes and ring chromosomes, only considered when the acentric fragment was present, and acentrics plus chromosome breaks, which were recorded together as extra acentrics. The number of breakages in structural rearrangements, such as chromatid exchange configurations, dicentrics and rings, were scored by the number of centromeres.

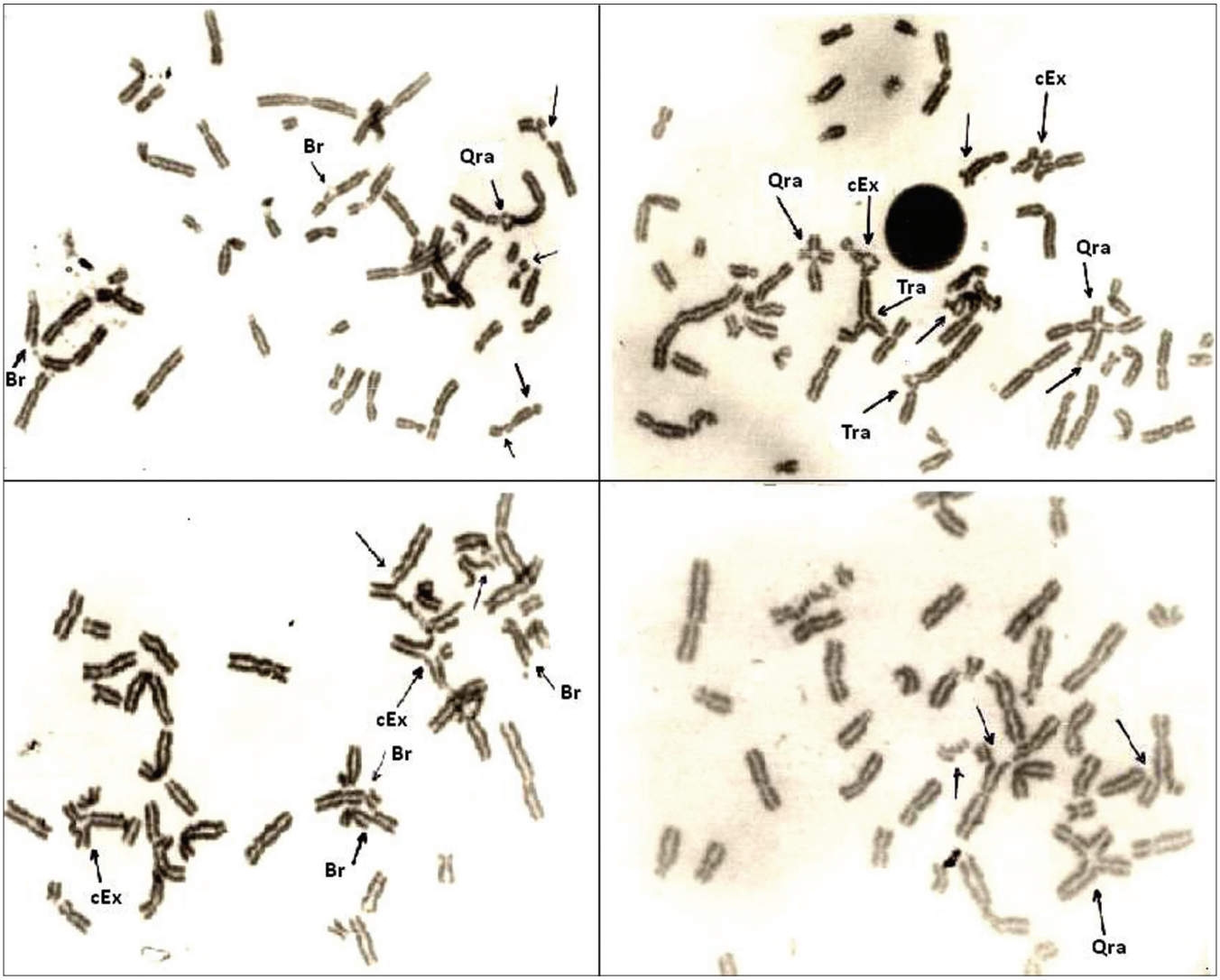

Figure 1: Examples of chromosomal aberrations observed in the lymphocytes of Fanconi anemia patients after mitomycin C treatment. Br = break; Tra = triradial; Qra = quadriradial; cEx = complex exchange

Table 1: Cytogenetic results obtained for controls, non-FA patients (aplastic anemia patients) and FA patients after treatment with different concentrations of MMC

Study groups	MMC treatment (ng/ml)	No. of analyzed cells	Gaps*	Breaks*	Exchanges*	Mean % Ab cells ± SD	Mean break/ cell ± SD
Controls	0	770	12	12	0	1.8 ± 3.3	0.02 ± 0.02
	20	800	70	66	10	10.5 ± 2.3	0.1 ± 0.02
	50	800	96	116	26	19 ± 6.7	0.2 ± 0.07
Aplastic anemia patients	0	1176	19	16	0	1.3 ± 2.5	0.32 ± 0.02
	20	1200	118	99	28	13.5 ± 2.4	3.06 ± 0.03
	50	1200	188	151	65	21.5 ± 5.6	5.44 ± 0.22
FA patients	0	395	67	56	19	23.1 ± 9.5	0.23 ± 0.1
	20	440	269	385	241	68.4 ± 10.1	1.93 ± 0.74
	50	380	371	579	346	71.5 ± 29.9	3.28 ± 1.07

*Gaps, breaks and exchanges refer to chromatid-type aberrations. Chromosome-type aberrations were rarely seen

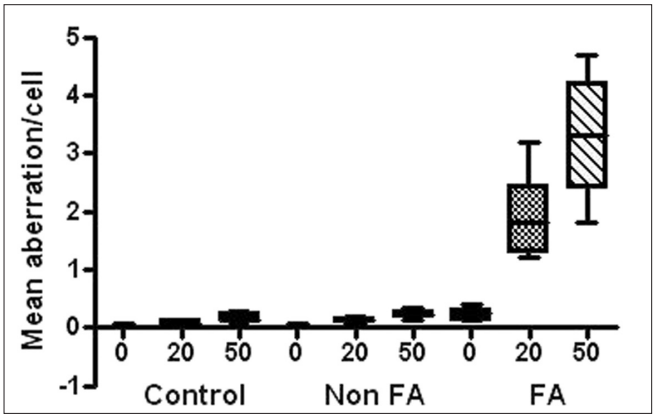

Figure 2: Bar chart showing mean aberration per cell observed in the different study groups after treatment with various concentrations of mitomycin C (MMC). MMC treatment at both concentrations used could effectively differentiate between Fanconi anemia (FA), non-FA patients and controls

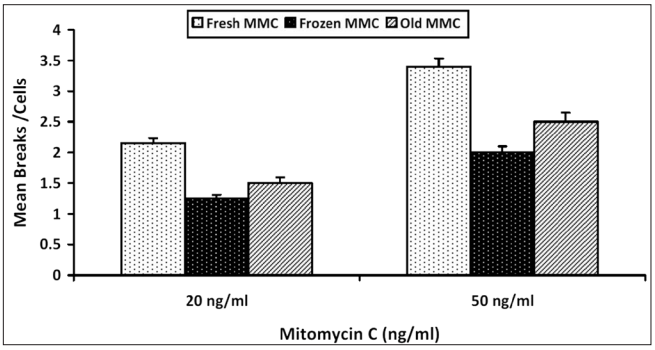

Figure 3: Comparison of the mean breaks per cells in Fanconi anemia patients after treatment with differently made mitomycin C

Chromatid and chromosome breaks were scored as one breakage event. The numbers of normal and aberrant metaphases were also scored for each individual. Figure 1 shows an example of the aberrations scored in this study.

Statistical methods

The aberrations per cell were calculated after analyzing 50 consecutive well-spread metaphases in all samples. The mean of the aberrations per cell was calculated for each sample. The differences between the mean aberrations per cell were calculated by SPSS software, analyzed using the nonparametric Mann-Whitney U-test and one-way analysis of variance (ANOVA) and summarized by means, standard deviation and 95% limits of agreement. *P*-values of less than 0.05 were considered as significant.

Results

Induced chromosomal breakage study was successful in all patients and healthy controls. Of these successful cultures, MMC-stress test revealed a striking increase in both chromosomal breaks and chromatid exchange radial formations in eight patients (25%), slight increase in six patients (18.75%) and no induced chromosomal breakage in 19 patients (56.25%) when compared with controls. Eight patients with hematologic abnormalities diagnosed as FA showed a very high frequency of induced chromosomal breakage, with almost every cell exhibiting multiple chromatid breaks and exchanges after exposure to MMC. Of these, six patients also manifested congenital malformations like skeletal anomalies (absence of radius and/or thumb), ectopic kidney, hypo- and hyperpigmentation of skin, café-au-lait spots, clinodactyly, polydactyly, microcephaly and growth retardation with failure to thrive. The hematologic abnormalities included aplastic anemia and thrombocytosis. Six patients exhibited only slight

increase in cells with chromosomal breakage as compared with baseline cultures. Four of these revealed two populations of lymphocytes, majority of the cells (50–70%) treated with 20 ng/ml of MMC appeared to have no chromosomal breakage while the remainder of the cells in each case exhibited a high number of breaks and exchanges characteristic of FA patients. The mean breakage rate in these four was greater than controls, and these were diagnosed as mosaics for FA. The results are summarized in Table 1 and shown in Figure 1. Table 1 represents the sensitivity to different concentrations of MMC in the control group, patients and FA-detected patients measured by the chromosome aberrations present. It summarized data by means, standard deviation and 95% limits of agreement in each group with different concentrations of MMC treatments. The basal (background) frequencies of chromosomal and chromatid abnormalities were higher in FA patients than in controls ($P < 0.05$). Induced aberrations by MMC treatment were slightly higher for 24 aplastic patients than for controls. This difference was not statistically significant enough to allow accurate distinction between the two groups ($P = 0.856$). The number of every kind of aberration in nontreated patients was obviously higher than the nontreated controls. The frequency of breakages after MMC treatment was much more significantly higher in FA patients compared with the other groups ($P < 0.0001$). In all groups, MMC-induced aberrations were significantly higher than basal background aberrations. As shown in Figure 2, the frequency of aberrations induced by MMC in FA samples is concentration-dependent, and there was a statistically significant difference between the frequency of aberrations induced by 50 and 20 ng/ml MMC ($P < 0.05$). This figure also indicates that there was a small interindividual difference for sensitivity to MMC treatment in all study groups.

The results also indicate that fresh MMC induces more chromosomal aberrations in FA patients than old or frozen MMC. Moreover, the chromosomal aberrations made by old MMC are slightly higher than those made by frozen and thawed MMC, but in the concentration of 20 ng/ml is not statistical significant ($P > 0.05$). The same results were obtained in the concentration of 50 ng/ml, but it was significant ($P < 0.05$). The results are shown in Figure 3.

Discussion

A considerable interpatient variation was observed in the chromosome breakage results obtained spontaneously and with clastogenic stressing [Table 1]. The abnormalities of nontreated patients are significantly higher than in the other groups ($P < 0.001$). This difference can show the genomic instability of these patients. It also can be related to the deficiency of repair mechanisms and can describe the high amount of cancers in these people. In the IFAR database,[11] the mean baseline frequency of chromosomal breakage was 0.25 breaks per cell (range: 0.02 ± 0.8), which is comparable to the result of our study (0.23 breaks per cell). The wide range of the baseline chromosomal breakage score means that some FA patients overlap with normal individuals and, for this reason, it cannot be used for diagnostic purposes. The aberrations, however, both in number and in type, seen in the clastogen-stressed lymphocyte cultures of FA homozygotes are distinctive for these patients and differ remarkably from that found in the lymphocyte cultures of other aplastic anemia patients and controls. It is important to keep in mind that the amount of MMC in the culture medium is very small, and a small variation in technique may have a substantial effect on results obtained, because higher concentrations of clastogen in the medium are known to cause diagnosis of mosaics of FA patients. Moreover it seems likely that MMC is a very sensitive solution and potentially an unstable molecule. Changing of the pH and freezing may decrease its function. This alkylating agent needs to be reduced for activation and, therefore, it is sensitive to the changing of pH. It seems that freezing the MMC solution crystallizes this molecule, which may lead to a reduction in its function.[23] Chromosome instability is a characteristic cytogenetic feature of a number of genetically determined human disorders collectively known as chromosome breakage syndromes. These disorders include FA, Bloom's syndrome and Ataxia telangiectasia. In each of these, chromosome instability exists in the form of increased frequencies of breaks and interchanges, occurring either spontaneously or following treatment with various DNA-damaging agents.[1,10,24] Several findings support the idea that FA

proteins function during the S phase of the cell cycle. ICLs, the major genotoxic challenge for FA cells, are processed through generation of DNA double-strand breaks intermediates, which are generated specifically during the S phase[14,25-28] and repaired by the process of homologous recombination.[29] As we treated the cells before S phase of the cell cycle, the high frequency of aberrations can show the deficiency in S phase repair as well. It is important to keep in mind that the amount of MMC in the culture medium is critical and if inappropriate might induce adverse effects by itself. Although hypersensitivity to MMC and DEB in mitogen-stimulated peripheral blood lymphocyte cultures is accepted as a diagnostic criterion in FA, interpretation is difficult in mosaic patients.[11] In the present study, four patients showed mixed populations of lymphocytes with hypersensitivity and normal sensitivity to MMC. Such a mosaicism has been reported earlier in 25–30% of FA patients.[11,30,31] Aplastic anemia in the remaining 24 of the 32 patients with relatively no or slight-induced chromosomal breakage could be due to other bone marrow failure syndromes or nutritional deficiencies. Further evaluation of these patients is needed at the molecular level to screen for FA-like gene mutations.[32] MMC-stress test not only identified patients with congenital anomalies but also two patients who lacked any congenital malformations. Giampietro *et al.*[33] reported 25–30% of FA patients without any congenital malformations, all of which were diagnosed only after onset of hematologic abnormalities. A similar delay in diagnosis was observed in the present study. In the present study, induced chromosomal breakage studies could diagnose FA in eight patients with a history of affected siblings [Table 1, Figure 1]. Although these patients were diagnosed after the onset of aplastic anemia, MMC-stress test can be used to detect such patients even in the preanemic 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.[33-35] 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 the onset of hematological 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 counseling and prenatal diagnosis as FA is an autosomal-recessive disorder with a recurrence risk of 25%.[34,36] Such a delay in identification of FA patients and at-risk families can be avoided by performing the MMC-stress test in patients with macrocytosis and decreased platelet count, as observed during screening of complete blood count with differentials.[34,37] In a recent report, it is shown that MMC-induced DNA damage also can be used as an index for FA identification.[38] We suspect a correlation between sensitivity of cells to alkylating agents, measured as breakage index, and the amount of cells containing aberrations, measured as the percentage of aberrant cells. We conclude that induced chromosome breakage, as used for the diagnosis of FA homozygotes, still remains an important diagnostic tool, as it is the only general screening strategy for detecting FA homozygotes of known and as yet unknown mutations. The variety of mutations and possible founder effects will determine future screening strategies based on mutation screening. Also, appropriate concentration and freshly made MMC [Figure 3] has revealed to be more reliable for FA identification.

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