CHROMOSOMAL BREAKAGE TEST IN THE DIAGNOSIS OF FANCONI ANEMIA IN PATIENTS WITH APLASTIC ANEMIA

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Abstract
Fanconi anemia (FA) is a rare recessive genetic disorder characterized by bone marrow failure, developmental delay, congenital malformations, genomic instability and a high cancer risk. To date 15 different FA genes have been identified. Chromosomal breakage test (CBT) using mitomycin C in peripheral blood lymphocyte culture to diagnose FA is performed as a routine diagnostic test by our Cytogenetic Laboratory. We report here the findings of CBT on the blood samples of 82 patients with aplastic anemia (AA). Three out of 82 AA patients (3.7%) were positive for CBT. The diagnosis of FA was made only when CBT was positive, and the results consistent with clinical and hematological data. About 96.3% of the AA patients were negative for CBT. Genetic reversion in FA may result in ‘spontaneous’ hematologic improvement and lymphocyte mosaicism, thus giving a false negative result for CBT. In patients who were negative for chromosomal breakage blood test and if FA is highly suspected, then the next tier of tests should be considered. FA testing could be performed on skin fibroblasts to overcome misleading results in blood lymphocytes.

INTRODUCTION
Fanconi anemia (FA) is a rare recessive genetic disorder and its incidence is about 1 to 5 per million births in the overall population. However, the incidence is higher (less than 1 in 20,000 births) in some consanguineous ethnic groups (e.g. Ashkenazi Jews and Afrikaners in South Africa) [Castella et al, 2011]. To date, 15 different FA genes have been identified: FANCA, FANCB, FANCC, FANCD1/BRCA2, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCJ/BRIP1, FANCL, FANCN/PALB2, FANCO/RAD51C, and FANCP/SX4. All FA genes are autosomal recessive except FANCB which is X-linked recessive. At the molecular level, the FA proteins are involved in a common DNA repair signaling pathway (FA/BRCA pathway), which cooperates with other DNA repair proteins for resolving DNA interstrand cross-links during replication (Kee & D’Andrea, 2010).

FA is characterized by bone marrow failure, developmental delay, congenital malformations, endocrine dysfunctions, genomic instability, and an increased risk of developing leukemia and solid tumors. Clinically, the disease is very heterogeneous in nature. About 25% of FA patients have no major birth defects (Lipton, 2011). Many patients develop bone marrow failure between 5 and 15 years of age, and the diagnosis of FA is often made at this stage. During their teens or young adulthood, there is a high risk of acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS). In adult patients there is an increased risk of solid tumors, especially squamous cell carcinoma of head and neck. Throughout life, the hematopoietic condition can change spontaneously by genetic reversion or undergo clonal evolution including progression to MDS or AML (Soulier, 2011). Hematopoietic stem cell transplantation (HSCT) is currently the best treatment to cure FA, but however a high proportion of these patients develop solid cancers in later life.

At the cellular level, the manifestations of genomic instability are chromosomal breakage, cell cycle disturbance, and increased somatic mutation rates. Even though genomic instability due to mutational inactivation of the FA genes is detrimental to a majority of FA patients, about 10-30% of FA patients appear to benefit from genomic instability. Genomic instability also increases the chance of spontaneous genetic reversion of their constitutional mutations in the hematopoietic progenitor cells, and the reverted cells may correct the bone marrow failure partially or completely. Genetic reversion such as intragenic crossovers, gene conversion, compensatory deletions/insertions, and back mutations in cis resulting in the selective advantage of gene-corrected lymphocytes have been observed in FA patients (Gregory et al., 2001; Gross et al., 2002; Kalb et al., 2006). This phenomenon of ‘natural gene therapy’ or self corrections of disease causing
In the majority of cases, the diagnosis of FA can be made based on physical examination, careful history and a positive chromosomal breakage blood test. FA cells show hypersensitivity to DNA clastogenic (cross-linking) agents such as mitomycin C (MMC), diepoxybutane (DEB), and cisplatinum. In peripheral blood cells, after culture with a T-cell mitogen and a DNA clastogenic agent, FA cells show elevated rates of chromosome breakages or aberrations (breaks, gaps, rearrangements, radials, exchanges, etc). Although the chromosomal breakage test (CBT) induced by MMC or DEB is the ‘gold standard’ test for the diagnosis of FA, the test is not 100% specific. Some chromosome breakage syndrome such as Nijmegen breakage syndrome, cohesinopathies Roberts Syndrome, and Warsaw breakage syndrome may score positive in the test (Oostra et al., 2012). Lymphocyte mosaicism (presence of both FA and ‘normal’ cells) due to genetic reversion may give a false negative result. Since genetic reversion occurs only in the lymphocytes, and not skin fibroblasts, CBT could be performed on these cells to overcome misleading results in blood lymphocytes.

The objective of this retrospective study was to analyze the findings of CBT using MMC for the diagnosis of FA in patients with aplastic anemia (AA).

MATERIALS AND METHODS

Patients and Controls

CBT for the diagnosis of FA is performed by our Cytogenetic Laboratory, Hematology Unit, Institute for Medical Research (IMR), Kuala Lumpur as a routine diagnostic test. Some of the reasons clinicians request for FA testing are aplastic anemia, bone marrow failure, sibling with FA, clinical features of FA, MDS or AML at a young age, or to rule out FA (e.g. patients suspected of having FA or potential donors for HSCT). About 5-10 ml of peripheral blood was collected in sterile tubes containing sodium heparin from each patient and ‘matched’ healthy control. The control was matched for age and sex, and must not be a sibling of patient. The blood samples collected were then sent to our Cytogenetic Laboratory for CBT.

A total of 82 patients with AA were included in this retrospective study. The patients were aged from 6 months to 49 years, and the male: female ratio was 1:0.84. Out of 82 patients, 64 (78%) were children (age 14 years and below) and 18 (22%) were adults (age above 14 years).

Lymphocyte Culture for Chromosome Breakages

Standard cytogenetic culture for peripheral blood lymphocytes was performed according to the procedure of Moorhead et al. (1960) with modifications. Blood was cultured for 72 hours in complete culture media containing RPMI 1640 (Flowlab), fetal bovine serum (Hyclone), penicillin-streptomycin (Hyclone), L-glutamine (Hyclone) and phytohaemagglutinin (Sigma, M Form). For each culture, one ml of blood was added to 20 ml of complete culture media in a culture flask. For each individual, three cultures were performed, one culture without MMC, one culture with 10 ng/ml MMC, and one culture with 50 ng/ml MMC. After 71 hours of incubation at 37°C, 0.2 ml of 5 ug/ml of colcemid was added to each culture for one hour. Cell harvesting was performed at 72 hours. Hypotonic solution (0.075M potassium chloride) was added for 4 minutes, followed by fixation in methanol-acetic acid (3:1). The cell suspension was then dropped onto slides and air dried at room temperature. The slides were stained with Leishman stain. Metaphase spreads were captured using an automated metaphase capture system (GSL 120).

For each individual, a minimum of 50 unband metaphase spreads were examined for chromosome breakages (chromatid gaps and breaks) and radials, in each of the three cultures: without MMC treatment, with 10 ng/ml MMC, and with 50 ng/ml MMC. The percentage of cells with chromosome breakages were calculated, and radials (if present) were recorded. The matched healthy control was used to establish a baseline for chromosome breakage. For each individual, chromosome banding (G-banding by trypsin) according to the procedure of Seabright (1971) with modifications, was performed on prepared slides without MMC treatment to exclude constitutional cytogenetic abnormalities.

RESULTS

The CBT results of the 82 AA patients (Table 1) were as follows: 56 patients (68.3%) had 0-2% of cells with chromosome breakages, 21 patients (25.6%) had 3-10% aberrant cells, 2 patients (2.4%) had 11-20% aberrant cells, and 3 patients (3.7%) had more than 30% aberrant cells. The result is positive for CBT when more than 30% of cells examined show chromosome aberrations (breakages and/or radial formation). Three out of 82 AA patients were positive for CBT. Fig.1 shows the metaphase spread of a AA patient with chromosome aberrations (multiple chromosome breakages and the presence of a radial) induced by MMC and positive for CBT. About 0-2% aberrant cells were detected in the 82 normal controls, and 97.5% of them do not have any chromosome breakage. One break is observed only in the aberrant cells.

DISCUSSION

Three out of 82 (3.7%) AA patients were positive for CBT, with more than 30% of cells having chromosome breakages. Chromosome breaks in the cells can vary from single to multiple breakages (gaps and breaks) with/without radials. The diagnosis of FA was done when only MMC-induced breakages are significantly increased in comparison to the controls (that is positive for CBT), and the results consistent with clinical and hematological data (Farjadian et al., 2008). Nijmegen breakage syndrome, Roberts Syndrome, and Warsaw breakage syndrome may also give positive results for CBT.

About 79 out of 82 AA patients who were negative for CBT had 0–20% aberrant cells. Two out of 79 AA patients had between 11% to 20% of cells with chromosome breakages. In these 2 patients, chromosome breakages in the cells also vary from single to multiple breakages with/without radials. Single break is usually observed in patients with 1% to 10% of aberrant cells. The existence of lymphocyte mosaicism may complicate diagnosis of FA by giving a false negative result. About 10-30% of FA patients have this result due to genetic reversion. The level of mosaicism varies, from low-level to
Table 1: Results of chromosomal breakage test induced by Mitomycin C to diagnose Fanconi anemia in aplastic anemia patients

<table>
<thead>
<tr>
<th>% of Aberrant Cells</th>
<th>0-2%</th>
<th>3-10%</th>
<th>11-20%</th>
<th>21-30%</th>
<th>Above 30%</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of Patients⁸ (%)</td>
<td>56 (68.3%)</td>
<td>21(25.6%)</td>
<td>2(2.4%)</td>
<td>0(0%)</td>
<td>3(3.7%)</td>
</tr>
<tr>
<td>No. of Breaks in cell (if present)</td>
<td>1</td>
<td>1</td>
<td>*varies</td>
<td>-</td>
<td>*varies</td>
</tr>
</tbody>
</table>

* : Varies from single to multiple breaks with/without radial forms.
# : Total no. of patients = 82

Fig. 1: Chromosomal aberrations induced by mitomycin C in a aplastic anemia patient positive for chromosomal breakage test
Arrows: Chromosome breakages (chromatid gaps or breaks)
Note the presence of a radial (solid arrow)

high-level mosaicism. Sometimes, the percentage of reverted cells has reached such a high level as to produce a false negative result. If CBT is negative, and FA is highly suspected, then the next tier of tests should be done. A skin biopsy should be done to provide fibroblast (which does not undergo genetic reversion) for FA testing. Other laboratory tests for the diagnosis of FA include cell cycle analysis via flow cytometry, FANCD2 Western Blotting, complementation studies, and molecular genetic testing (Alter & Kupfer, 2011). It is important that a correct and early diagnosis of FA be made so that these patients could be given the appropriate treatment/ management. FA patients with bone marrow failure (BMF) will not respond to immunosuppression drugs used to treat idiopathic aplastic anemia. They are hypersensitive to chemotherapy agents, and will die of toxicity if given conventional conditioning for HSCT. FA patients who do not develop BMF will have an increased risk of malignancies, and hence require continuous monitoring.

Variations in the chromosomal breakage test are used in different Cytogenetic Laboratories, and this include the type of clastogenic agents used (MMC or DEB, or both), different exposure time and clastogenic agent concentration. Ways in scoring chromosome aberrations are very diverse. Cytogenetic laboratories score chromosome aberrations in either one or more of the following ways: percentage of aberrant cells, number of breaks per cell, breaks per aberrant cell, or/and number of cells with radial forms (LaGrave & South, 2012; Oostra et al., 2012; Talmoudi et al., 2013). Hence, each Cytogenetic Laboratory must establish its own protocol for CBT, determine its own scoring criteria for chromosome breakages or aberrations, and also the baseline for chromosomal breakage rate. To obtain sufficient statistical power of the chromosome breakage data at least 50 metaphase spread must be examined per culture.

In conclusion, three out of 82 AA patients (3.7%) were positive for CBT using MMC. The result is positive when more than 30% of the cells have chromosome aberrations (e.g. gaps, breaks, with/without radials). The diagnosis of FA was made when chromosomal breakage blood test is positive, and the results consistent with the clinical and hematological data. About 96.3% of AA patients were negative for CBT. Genetic reversion occurs in the hematopoietic stem cells of about 10-30% FA patients, thus resulting in improved bone marrow function and lymphocyte mosaicism. This may give a false negative result in the CBT.
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REFERENCES


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