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Original Article

Cytogenetic study is not essential in patients with aplastic anemia

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Abstract: Depending on contemporary treatment approach of aggressive immunosuppression, Aplastic Anemia (AA) is caused by immunological destruction of otherwise normal hematopoietic stem cells. The aim was to summarize the cytogenetic abnormalities in AA patients and the frequency of Fanconi Anemia (FA) in morphologically normal AA patients in eastern India. Ethical clearances were obtained from both institutions involved in this study. Out of 72800 patients attending the outpatient department, 520 pancytopenia patients were screened for AA after Bone marrow (BM) aspiration and biopsy. Samples were collected from 117 cases in 3 phases. 51 peripheral venous blood (PVB) samples in the first phase, 19 BM & PVB paired samples in the second phase and 47 BM samples in third phase were collected followed by leukocyte and/or BM stem cell culture. Next GTG banding and karyotyping were performed. PVB was collected from 63 (< 50 years) AA patients and stress cytogenetics was done to diagnose FA. In the first phase of the study, out of 51 PVB samples, 1 (1.96%) showed a unique chromosomal abnormality, i.e. 45,XY,rob(14:21)(p10:q10)[20]. In the second phase of study, among 19 BM & PVB paired samples, 1 (5.26%) showed abnormal karyotype i.e. 45,X,-Y[3]/46,XY[47]. In the third phase of the study, 47 BM samples showed normal karyotype. Only 6 (9.52%) cases were found positive for stress cytogenetics. A negligible percentage showing cytogenetic abnormality in such a considerable number of AA cases indicates that routine cytogenetic analysis of AA patient is not essential. A significant percentage was positive for stress cytogenetics; suggestive for FA, even the patients were morphologically normal.

Keywords: Aplastic anemia, bone marrow failure, cytogenetic abnormalities, GTG banding, stress cytogenetics, radial formation

Introduction

Aplastic Anemia (AA) is a potentially life-threatening hematopoietic stem cell (HSC) disorder characterized by peripheral blood pancytopenia and bone marrow hypo-cellularity with no increase in reticulin [1]. In approximately 15-20% of cases, the disease is inherited. But the majority (70-80%) of the disease is categorized as idiopathic (Acquired Aplastic Anemia or AAA) [1, 2]. The incidence of AA in the western countries is about 2 per million per year, but in the Far East it is 2 to 3-fold higher [3, 4]. It shows a biphasic distribution with highest frequency occurring between the age of 15-25 years and a second peak after 60 years [5]. Historically, AAA has been strongly associated with exposure to toxic chemicals, viruses, auto-immune disorders, pharmaceuticals, heavy metals, radiations, addictions, etc. [5, 6]. The rarity of the disease and lower incidence of observing chromosomal abnormalities have made it more difficult to understand the clinical relevance of cytogenetic abnormality in AA [7, 8]. The literatures have already documented that 4-15% patients with AAA showed cytogenetic abnormalities during diagnosis [9-11]. Due to differences in diagnostic criteria, patient populations, treatment protocols, and the frequency of follow-up BM examinations, acquisition of an abnormal karyotype in AA is frequent, but estimates have been variable between published studies [12-22]. Previous studies reported that Fanconi Anemia (FA) patients face some typical abnormalities like developmental delay in 56% cases, hypogonadism in 22% cases, strabismus in 26% cases, abnormalities of the gastrointestinal system in 7% cases, abnormal skin

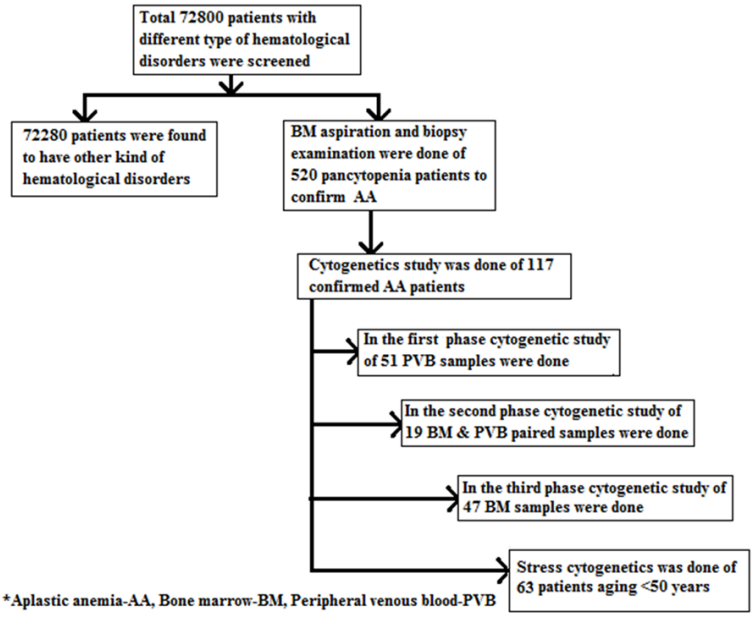

Figure 1. Showing the schematic representation of study design. A total 72800 patients with various hematological diseases were screened. Among them BM aspiration and biopsy were done on 520 pancytopenia patients to confirm AA. Finally 117 cases were participated in this study. In the first phase, cytogenetic study of 51 PVB samples, in the second phase, a cytogenetic study of 19 BM & PVB paired samples and in the third phase, a cytogenetic study of 47 BM samples was done. In 117 cases, 63 cases were less than 50 years old. PVB samples were collected from these 63 cases and stress cytogenetics was performed to find out Fanconi anemia.

pigmentation in 75% cases, café-au-lait spots, microcephaly in 43% cases, malformations of the skeletal system in 60-80% cases, the absence or malformation of thumb, abnormality in radius & ulnar bone in 60% cases, abnormality in kidney and urinary tract in 28% cases, with other complications in the heart, eyes, central nervous system, ears, genitalia system etc. [23-25]. In our study all patients were morphologically normal. It is known that the carrier frequency of FA is 1:181 in USA and 1:93 in Israel [26]. This study is the first documentation in its category and was aimed to summarize the possible cytogenetic abnormalities in AA patients and the frequency of FA patients in morphologically normal AA patients in populations of eastern India.

Methods

Study population

In this study, a stratified sampling method was used to select AA patients. 72800 patients with different types of hematological disorders were screened in the Department of Hematology, Nil Ratan Sircar Medical College & Hospital, Kolkata from January 2015 to December 2016. Among them, 520 pancytopenia patients were screened for AA by performing bone marrow aspiration and biopsy examination. A Cytogenetic study of 117 AA patients was done. Stress cytogenetics was performed of 63 patients aging less than 50 years. 63 Controls samples for stress cytogenetics were collected from healthy age and sex matched persons without pancytopenia. The schematic representation of the study design is shown in **Figure 1**.

Diagnosis of AA was established according to the guidelines of International Agranulocytosis and Aplastic Anemia Study Group, 1987. At least two of the three criteria with hypocellular bone marrow must be present to define AA which is, (1) hemoglobin < 100 g/L, (2) platelet count < 50 × 10^9/L, (3) neutrophil count < 1.5 × 10^9/L. Cytogenetic analysis and Stress cytogenetics were done at Vivekananda Institute of Medical Sciences, Kolkata.

Ethics

Ethical clearances were obtained duly both from the Ethics Committee of Vivekananda Institute of Medical Sciences and Nil Ratan Sircar Medical College & Hospital.

Questionnaire and consent form administration

Patients and control cases were thoroughly informed about the research work. Written consent was taken before the collection of samples. A detailed questionnaire was administered to record their data.

Collection and analysis of sample

In the first phase of the study 51 PVB, in second phase 19 BM & PVB paired samples and in

third phase 47 BM samples were collected in heparinized vacutainer (green vial). 500 µl of PVB sample was added to the media [RPMI 1640 Gibco, USA (4 ml) + Foetal bovine serum (FBS) (1 ml) + Phytohemagglutinin (0.2 ml)]. In case of BM, two types of culture tubes were established, Overnight (ON) culture and Overnight with colcimid (ONC) culture [RPMI 1640 Gibco, USA (3.75 ml) + FBS (1.25 ml)]. Colcimid solution (10 µg/ml) was added in ONC at the time of inoculation and in ON culture prior one hour of harvesting. Cultures were incubated at 37°C, O_2 incubator for 72 hours (PVB) and 24 hours (BM) respectively. After centrifugation harvesting was done using freshly prepared hypotonic (KCL-0.075M) and fixative solution (3:1 methanol:glacial acetic acid). Slides were dipped into ethanol and chilled water consecutively for 3-5 seconds. 20 µl of cell suspension was gently poured on the slide. Slides were aged at 90°C for 20 minutes. Giemsa Trypsin Giemsa (GTG) banding was performed. At least 20 metaphases were analyzed by DSS image tech karyotyper for each case. Karyotyping was performed according to the International System for Human Cytogenetic Nomenclature 2013 (ISCN 2013).

The utmost reliable diagnostic test for FA is stress cytogenetics. The most consistent and accepted cellular phenotype of FA cases, is hypersensitivity to DNA crosslinking agents like mitomycin c (MMC) or diepoxybutane (DEB) which produce Interstrand crosslinks (ICLs) [27]. ICLs include chromosome breakage and radial formation, which is found in metaphase chromosome spreads. FA patients have mutant/dysfunctional DNA repair protein. Thus, normal cell cycle functions are hampered, leading to the damage of hematopoietic stem cells and bone marrow failure. This property is used as a diagnostic test of FA [28, 29]. PVB was collected from 63 AA patients (< 50 years) for stress cytogenetics. All 63 AA patients did not have any morphological abnormalities. Echocardiogram and ultrasonography were done to rule out cardiac and renal abnormalities, although the results showed normal. Stress Cytogenetics was done using MMC. Three cultures, each for cases and controls (0 ng MMC/ml, 50 ng MMC/ml, and 100 ng MMC/ml) were set. The media composition and harvesting procedure were same as PVB, stated above. Only exception, MMC was introduced to the PVB culture after 24 hours from inoculation. After slide preparation, Giemsa staining was done. A set of 60 metaphases each from cases and control (20 metaphases from each culture) was observed under the Zeiss Axioskop Microscope (100 × magnification) for the presence of break, radial, bi-radial and tri-radial structures, which are suggestive for FA. Standard formula [percentage of cells with tri-radial + (1.6 × Total Number of radials in 50 & 100 ng MMC/ml Culture)] was used to analyze the sensitivity of MMC; the cutoff value of sensitivity to MMC was > 40.

Statistical analysis

Qualitative data presented with number and percentage. Quantitative data presented by mean ± standard deviation. The chi-square (X^2) test was done to evaluate the statistical significances of the data. The X^2 test is the most commonly used non-parametric test in statistical estimation. The quantity X^2 describes the magnitude of the discrepancy between the theory and actual observation. The chi-square (X^2) test is also termed as 'test of goodness of fit' because it enables us to ascertain how appropriately the theoretical distribution fit empirical distributions i.e. those obtained from sample data.

Results

In 117 patients, the mean and standard deviation of four cell lineages responsible for pancytopenia were as follows. Mean hemoglobin was 56.70 ± 19.31 g/L, mean neutrophil count was 0.29 ± 0.07 (× 10^9/L), mean reticulocyte count was 13 ± 1.33 (× 10^9/L), mean platelet count was 20 ± 1.1 (× 10^9/L). The results are described below according to the phase of the study.

Results of the first phase of study

The median age of the patients was 35 years (n=51). The age range of the patient was (3-68 years). Among them, 36 (70.58%) were male and 15 (29.41%) were female. Out of these 51 patients, 21 (41.17%) had Very Severe Aplastic Anemia (VSAA), 27 (52.94%) had Severe Aplastic Anemia (SAA) and 3 (5.88%) had Non Severe Aplastic Anemia (NSAA). Out of 51 peripheral blood samples only one (1.96%) patient (15 Y/M) had a unique chromosomal translocation i.e. 45,XY,rob(14:21)(p10:q10)[20] never previously reported with AA (**Figure 2**).

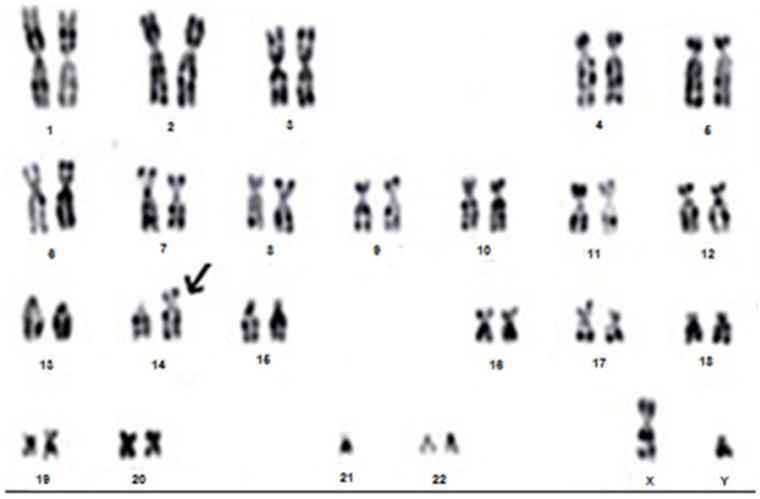

Figure 2. A unique Robertsonian translocation between chromosome 14 and 21 resulting 45,XY,rob(14:21)(p10:q10)[20] was observed in the PVB sample of an aplastic anemia patient. Robertsonian translocation (also known as whole-arm translocations or centric-fusion translocations) is a rare chromosomal rearrangement. Generally it occurs in acrocentric chromosomes like 13, 14, 15, 21, and 22. During the Robertsonian translocation, chromosome breaks at their centromere and the long arms fuse to form a single, large chromosome with a single centromere. The short arms join to form a small reciprocal product, which typically contains only non-essential genes also present elsewhere in the genome, and is usually lost within a few cell divisions. This type of translocation is cytologically visible and can reduce chromosome number.

Results of the second phase of study

The median age of the patients was 42 years (n=19). The age range of the patient was (3-69 years). Among them, 89.47% (n=17) were male and 10.53% (n=2) were female. Out of these 19 patients, 31.57% (n=6) had VSAA, 63.15% (n=12) had SAA, and 5.26% (n=1) had NSAA. One patient (28 Y/M) showed 45,X,-Y[3]/46, XY[47] (**Figure 3**). A total of 50 cells in this case was analyzed, as it showed two types of cell line. Other cases showed normal karyotype.

Results of the third phase of study

The median age of the patients was 38 years (n=47). The age range of the patient was (7-85 years). Among them, 91.41% (n=43) were male and 8.59% (n=4) were female. Out of these 47 patients, 29.78% (n=14) had VSAA, 63.82% (n=30) had SAA, and 10.6% (n=3) had NSAA. All patients showed normal Karyotype. Neither structural nor numerical abnormality was found.

Results of stress cytogenetics study

The median age of the patients was 22 years (n=63), whereas the median age of the cases positive for stress cytogenetics was 6 years. The age range of the patient was (3-50 years). Out of these 63 patients, 46 (73.01%) were male, and 17 (26.99%) were female. Among them, 44.44% (n=28) had VSAA, 49.22% (n=31) had SAA and 6.34% (n=4) had NSAA. 6 (9.52%) cases were found positive for stress cytogenetics which is suggestive for FA. In every positive case break, gap, radial, and Tri radial structures were observed under the microscope (100 × magnification) (**Figure 4**). Breaks and radial structure were not found in other AA cases or in control samples.

Results of statistical analysis

The first observation of this study was cytogenetic study is not essential in AA patients. The calculated value of X^2 is 22.1714, which was much greater than the chi-square table value, i.e. 5.99 at 5% confidence level (degrees of freedom 2). The two tailed p value was 0.000015. The result is significant at $p < 0.05$. By conventional criteria, this difference is considered to be statistically significant.

The second observation was stress cytogenetics in morphologically normal AA patients (< 50 years) should be done to exclude FA. In our analysis the calculated value of X^2 was 12, which is greater than the chi-square table value, i.e. 9.21 at 1% confidence level (degrees of freedom 1). Two tailed p value is 0.000532. The result is significant at $p < 0.01$. This statistical analysis is supporting that the observation is extremely statistically significant.

Discussion

Aplastic anemia (AA) is a very rare hematological disorder which is thought to be immune mediated. The main pathologic procedure is a contraction of the stem cell compartment leading to reduced numbers of available hematopoietic stem cells. Cytogenetics has long been used as a very important diagnostic tool in AA.

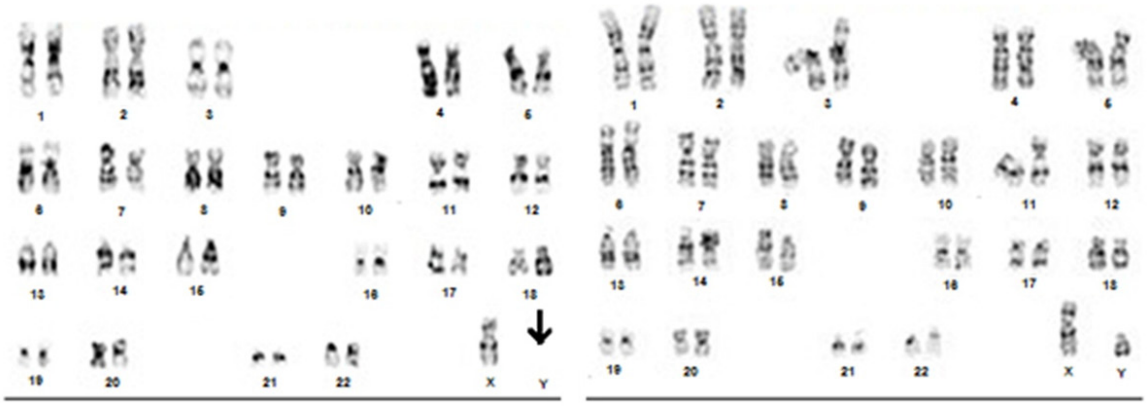

Figure 3. Showing the deletion of Y chromosome in three cells, resulting 45,X,-Y[3]/46,XY[47]. Loss of a single chromosome must be detected in at least three such cells according to ISCN 2013. The loss of the Y chromosome was obtained in the BM culture from one of the 19 BM & PVB paired sample. However, the PVB sample of the same case showed 46,XY cells. Loss of the Y chromosome is previously found to be associated with AA and may cause fatal diseases in men. But the exact cause and mechanism of this loss are still unknown.

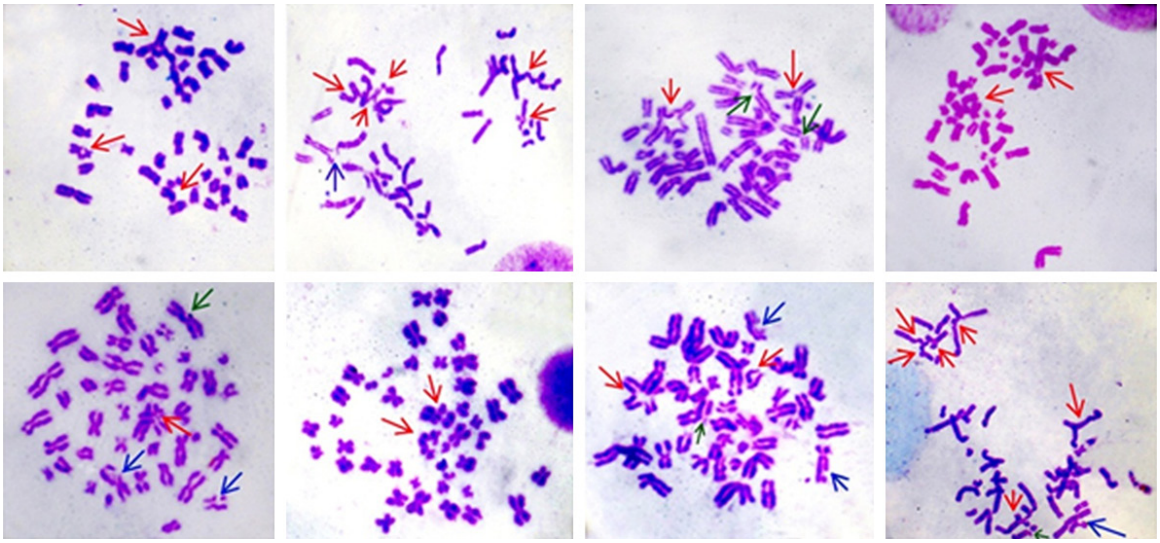

Figure 4. Showing break (green arrow), gap (blue arrow), radial and Tri radial (red arrow) found in MMC sensitive cases. Chromosome breakage and radial formation are nothing but Interstrand crosslinks (ICLs). It can occur before and after DNA replication in the homologous region of sister chromatid. If ICLs occur in post-replication state, two possibilities are there. Either homologous repair between sister chromatids produce an error free repair or mitosis proceeds normally and one of the daughter cells inherits an unrepaired ICL. In case ICLs occur in the pre replication state, a homologous sister chromatid is not available for re-combinational repair. There are three possible mechanisms for repair of pre-replication ICLs, 1) Non-homologous end joining, 2) Excision repair and/or lesion bypass and 3) Homologous repair between homologous chromosomes. Whereas first two mechanisms are error-prone, the third one is error-free. Unrepaired ICLs generate a mandatory chromatid-break during mitosis.

There is a gloomy part between the hypo-cellular myelo-dysplasia and AA. BM cytogenetics can help in establishing the proper diagnosis. It was previously assumed that the presence of an abnormal cytogenetic clone in otherwise apparent AA is indicative of an underlying hypo-accumulative clonal myeloid disease like, Myelodysplastic syndrome or hypocellular myelogenous leukemia [7]. Now it is evident that abnormal cytogenetic clones may be present in up to 12% cases with typical AA at diagnosis [1], as well as arise during the course of the disease [14, 22]. The development of the clonal cytogenetic abnormalities such as monosomy 7 or trisomy 8 in a patient with AA indicates the evolution of an acute leukemia or Myelodys-

plastic syndrome. In cases with disappearance of monosomy 7, hematologic improvement occurred [30]. Persistent monosomy 7 is a poor prognosis as compared to trisomy 8 [31, 32]. Some believe that typical AA is compatible with the normal karyotype. However, some random transient chromosomal abnormalities can appear and such abnormalities usually disappear after therapy.

A study carried out in the USA, established the fact that AA patients with the presence of cytogenetic abnormality have a higher risk of developing leukemia. They showed a 4% SAA patients with clonal cytogenetic abnormalities in unstimulated BM cells [7]. A study in Japan stated the presence of 46,XY,t(1;20)(p22;q13.3) in an AA patient (3 Y/M) suffering from ataxia [33]. Another study depicted the presence of a constitutional translocation i.e. t(13;14) in a woman with thrombocytopenia progressing to AA, and her brother, with persistent thrombocytopenia [34]. A British study also reported an SAA case with constitutional translocation 46,XY,t(6;10)(q13:q22) [35]. A Diamond-Blackfan Anemia (a type of inherited AA) case was even reported with balanced reciprocal translocation 46, XX,t(X;19)(p21;q13) [36]. A study was done on 1501 patients of North India, which concentrates only on epidemiology, clinico-hematological profile and management of AA [37]. Another study from the same region showed five (11.9%) AA patients with chromosomal abnormalities like trisomy 12, trisomy 8, monosomy 7, deletion 7q and translocation (5;12) [38]. The percentage of cytogenetic abnormality in this study is almost consistent with published Western data [1]. Certain South Indian hospital based studies are also there, depicting the present situation and clinico-hematological profile of AA, but not on the cytogenetic aspect [39-41]. In Western India, there are few studies that focused on epidemiology, pathogenesis, diagnosis and the treatment procedure of AA [42, 43]. A review article from the western India raised a question of the relation between the evolution of the disease and Cytogenetic abnormality in pediatric AA patients [44]. Finally, coming to the Eastern part of the country, a review literature said that a cytogenetic examination of the BM is useful to exclude hypoplastic myelodysplastic syndromes [45]. Another study on clinico-hematological analysis of AA among children was performed in the northern districts of West Bengal but did not focus on cytogenetic analysis [46]. Other than these studies, there is no instance of specific study in India that emphasized on the cytogenetic aspect of AA solely.

We have tried to focus on the cytogenetic abnormality in AA patients coming from different parts of Eastern India. In our study, we have reported a unique numerical abnormality, i.e. 45,XY,rob(14:21)(p10:q10)[20] (1.96%) in the PVB cytogenetic analysis of the first group of 51 AA patients. But the cytogenetic study from the BM of the same patient could not be performed due to much diluted marrow sample. For this reason, it cannot be assured that the translocation is solely evolved from the marrow failure, but not of constitutional origin. For further confirmation, fluorescence in situ hybridization (FISH) study will be performed later.

We have also observed another abnormality, i.e. 45,X,-Y[3]/46,XY[47] (5.26%) in the BM & PVB paired cytogenetic analysis of the second group of 19 AA patients. This observation was consistent with two more studies, one reported from Japan addressing the loss of Y chromosome in a 33 year old male AA patient [47] and another study showed loss of Y chromosome in PVB cells is associated with increased risk of mortality, different forms of Cancer, Alzheimer's disease as well as other fatal conditions in men [48]. Thus, loss of Y chromosome may have relation with the BM failure disorder, but it is common in diseased men. However, we observed normal Karyotype in the third group of BM cytogenetic analysis of 47 AA patients. Depending on the percentage of cytogenetic abnormality and statistical analysis, it is too difficult to come to any definite inference. Hence, we conclude that, cytogenetic study as a routine examination in otherwise typical AA is not essential in this population until the bone marrow aspiration and biopsy is showing blasts, dysplasia, a pre-leukemic changes, fibrosis etc. Such cytogenetic abnormalities will not change the treatment precession in classic AA patients.

FA is a rare inherited bone marrow failure and autosomal recessive blood disorder. It is the most common inherited form of aplastic anemia. Our second aim was to find out the frequency of FA in morphologically normal AA patients. A study was done in North India with 94 AA cases and found 13.8% cases affected

by FA [49]. Another North Indian study found 11.3% FA cases [50]. However, In Delhi, India a larger study with 300 AA patients found 9.20% with FA [51]. A study from Kashmir among 50 AA patients revealed 14% FA cases [52]. Another study of 488 patients reported 13.1% cases with breaks in comparison to their respective control [53]. A case report was published from the Eastern India which showed FA with the incidental hemoglobin E trait in a 5 year old child who was presented with weakness, epistaxis, melena and intermittent fever [54]. Another study emphasized on the molecular screening in FA [55]. One study from Western India emphasized on the need of an accurate cytogenetic analysis in FA patients along with a clinico-hematological correlation [56].

The percentages of FA positive cases in the above mention studies are different because the study population and the number of patients participated are significantly different. In these studies, researchers considered all kinds of AA patients, irrespective of their morphological condition for the chromosomal breakage test. We tried to find out the frequency of FA in morphologically normal AA patients, thus making the study noble again. If a person is having pancytopenia with hypocellular bone marrow and the age is < 50 years; this situation criticality demands stress cytogenetics test for the exclusion of FA. Sometimes physicians get confused by the absence of typical physical features, which are very much frequent in FA. In this scenario, FA case might escape from being diagnosed with a high chance of erroneous treatment and disease management. Although in our study, patients were morphologically normal, still significant percentage (9.52%) showed sensitivity to MMC which indicates, the stress cytogenetics study in morphologically normal AA patients is very important to find out FA. Nonetheless the study of larger sample size is required to confirm the findings.

Limitation

In our study, very low percentage of cytogenetic abnormality was found. This was may be due to small sample size.

Especially, the number of bone marrow sample was smaller. AA is a bone marrow failure disorder; the chance of getting chromosomal abnormality from the BM culture was higher.

The PVB or BM samples were collected only from Nil Ratan Sircar Medical College & Hospital as it is the nodal center for hematological diseases in Eastern India. Many patients from rural parts of Eastern India are unable to obtain the government medical facility. Sample collection from more than one center could improve the sample size.

Adequate follow up for the patients after diagnosis of the disease were not made in our study. As many patients died due to incapability of receiving treatment for their financial constrain. It could give us additional information about the disease.

Male dominant societal condition of the country. The Male-female ratio in our sample is significantly disproportional. So, any gender biases associated with AA or FA were not determined by this study.

Most of the patients participated in this study belong to low socioeconomic status and their lack of affordability make it even more difficult to diagnose such patients.

However, Cytogenetics being a manual procedure, chances of skipping abnormality is there. Flow cytometry (cell marker assay), can be used in AA for discrimination from very similar other hematological diseases.

In West Bengal there is a shortage of FA testing center. In the rural areas of the state, clinical centers are not in proximity. Thus, in many cases disease remain undetected.

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Disclosure of conflict of interest

None.

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