Cytogenetic Study of Aplastic Anaemia in Eastern Indian population

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Abstract

Background: *In India, ailment like Aplastic Anaemia (AA) is nothing but short of a curse. For people who belong to low socio-economic status, the treatment of this disease creates a huge financial encumbrance. Appropriate diagnosis in this circumstance can not only save treatment stretch but also significantly condense undesirable expenditures. Sporadic Cytogenetic Abnormalities (CAs) in Acquired Aplastic Anaemia (AAA) has long been a controversial issue from the treatment perspective. Fanconi anaemia (FA) is the most common Inherited Aplastic anaemia (InAA), generally aided by typical congenital malformation, absence of which makes clinicians confuse to make a diagnosis.*

Observations: *These gloomy areas have encouraged us to evaluate the Cytogenetic Abnormality(CA) in AAA patients and the frequency of FA patients in phenotypically normal AA patients in this first comprehensive study on AA patients from Eastern India.*

Methods: *170 AA patients participated in this ethically cleared study (2015-2018). Bone marrow aspiration (BMA) and Bone marrow biopsy (BMB) were done to confirm AA. On written consent; 51 peripheral venous blood (PVB), 19 BM-PVB paired samples and 100 BM samples were collected followed by leukocyte and/or BM culture. GTG banding-Karyotyping were performed. To discriminate InAA and AAA, Stress Cytogenetics (SC) using Mitomycin C was done (n=99, <50 years).*

Results: *4.11% (n=7) CA was observed. 9.09% (n=9) cases were found positive for SC suggestive for FA.*

Conclusion: *Negligible percentage of CA indicates the cytogenetic analysis of AA patient is not mandatory until the BMA-BMB is showing blasts, dysplasia, pre-leukemic changes, fibrosis etc. A significant percentage of SC positive patients in phenotypically normal AA indicate SC is indispensable in AA patients.*

Keywords: *Acquired aplastic anaemia; Cytogenetic abnormality; Bone marrow culture; Karyotyping, Stress cytogenetics*

1. INTRODUCTION

Research on the relation of Aplastic Anaemia (AA) and Cytogenetic Abnormalities (CAs) is still at a nascent stage in India. The morphological examination may not be enough for diagnosis and there comes the importance cytogenetic analysis. AAis a rare syndrome of bone marrow (BM) failure characterized by peripheral pancytopenia and marrow hypo-cellularity in the absence of an abnormal infiltrate and no increase in reticulin.[1]The disease can be categorized into two groups i.e. congenital/inherited and acquired aplastic anaemia. The major types of inherited AA (IAA) are Fanconi anaemia, Dyskeratosiscongenita, Shwachman diamond syndrome, Blackfan diamond syndrome, etc. Acquired aplastic anaemia (AAA)has relation

with some definite etiological agents like drugs, chemicals and virusesetc. However, in many AAA cases the definite etiological factor is unknown.In those cases AAA is called idiopathic AA.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 AAA.[2,3]Literatures have already documented that 4-15% patients with AAA showed cytogenetic abnormalities during diagnosis.[4-6] Due to differences in diagnostic criteria, patient population, treatment protocol, and the frequency of follow-up bone marrow examinations, acquisition of an abnormal karyotype in AA is frequent, but estimates have been variable between published studies.[4,7-16]Cytogenetic abnormalities in relation to AAA have been reported rarely in some Western studies.[3,17]The BM is hypo-cellular in AA, especially at the onset of the disease. So, it is very difficult to obtaining sufficient cells and metaphases for analysis. Historically cytogenetic abnormalities have a significant role in diagnosis of hypo-cellular myelodysplastic syndrome (MDS) and AA, as the treatment procedure in this two situation is different. [17,18]

Fanconi anaemia (FA) is a rare inherited bone marrow failure and autosomal recessive blood disorder.[19]Thirteen complementation groups, defined by somatic cell hybridization are associated with the development of FA.[20]The complementation groups have been designated as FANCA, B, C, D1, D2, E, F, G, I, J, L, M and N. The most frequent mutations are found in FANCA, FANCC or FANCG.[21]FA patients have a higher risk of getting cancer including acute myeloid leukaemia and squamous cell carcinoma. Maximum, but not all, affected individuals have one or more somatic abnormalities, including skin, skeletal, genitourinary, gastro-intestinal, cardiac and neurological anomalies etc. Positive Stress Cytogenetics has immense implications for the treatment and management of FA.

There are very few studies on CAs from India with AAA patients have been reported till date. The present study was carried out to evaluate the CAs in AAA patients and incidence of FA in the population of phenotypically normal AA patients in eastern India.

2. MATERIALS AND METHODS

2.1. Sample Collection

In this study, a stratified sampling method was used to select AA patients. 1,39,518 patients with different type of haematological disorders were screened in the Department of Haematology, Nil Ratan Sircar Medical College & Hospital from 2013-2018. Diagnosis of AA was established according to the guidelines of International Agranulocytosis and Aplastic Anaemia Study Group, 1987. At least two of the three criteria with hypo-cellular bone marrow must be present to define AA which are, (1) haemoglobin<100 g/L, (2) platelet count <50 $\times 10^9$/L, (3) neutrophil count <1.5×10^9/L. Bone marrow aspiration and biopsy (BMA&BMB) examination was done of 4032 pancytopenia patients to confirm AA. BMA & BMBis an invasive procedure that cannot be done repeatedly. Considering all pancytopenia cases as potential case of AA we took BM and PVB samples. Later, only those cases were selected for the study, who was found to have hypo-plastic anemia or hypo-cellular BM in BMA & BMB reports.A total of 170AA patients were participated in this study.

Stress Cytogenetics using MitomycinC (MMC) was done of 99 AA patients (<50 years) and age (relaxed up to ± 10 years)-sex matched control to exclude inherited BM failure syndrome i.e. FA.[22,23]

Patients who are < 50 years of age, but negative for Stress Cytogenetics are categorized as AAA and those who ≥ 50 years old are considered as AAA case directly without SC analysis. For the standardization of SC protocol used in this study, the procedure was performed with the three known FA patients. Cytogenetic analysis and Stress Cytogenetics were performed in the Department of Genetics, Vivekananda Institute of Medical Sciences, Kolkata.

2.2. Ethics

Ethical clearances were obtained from Nil Ratan Sircar Medical College & Hospital, Kolkata and Vivekananda Institute of Medical Sciences, Ramakrishna Mission Seva Pratishthan, Kolkata.

2.3. Questionnaire & Consent

Patients who participated in this study were thoroughly informed about the research work and written consent was taken before sample collection. A detailed questionnaire was administered to record their demographic data (age, sex, residential information) as well as their lifestyle factors.

2.4. Analysis of Samples

PVB and BM samples were collected in the heparinized vacutainer (BD).500μl of PVB sample was added to the media [RPMI 1640 Gibco, USA (4ml) + Foetal bovine

serum(Gibco) (FBS) (1ml) + Phytohemagglutin in (0.2ml)]. The media composition for bone marrow culture was [RPMI 1640 Gibco, USA3.75ml +Foetal bovine serum (Gibco) 1.25ml].To get a good amount of pellet a bone marrow culture should contain 1.5 million WBC/ml of media. Two types of culture tubes were established for BM, Overnight (ON) culture and Overnight with Colcemid (ONC) culture. The ONC and ON culture would achieve a good number and good morphology of metaphases respectively; thereby reducing the chance of skipping chromosomal abnormality. Cultures were incubated at 37^0C, O_2 incubator. 100µl of Colcemid(working solution=1µg/ml) solution was added in the ONC culture at the time of inoculation whereas in ON and PVB culture 250µl and 300µlColcemid solution was added prior one hour of harvesting. Incubation period was16 hours for ONC, 17 hours for ON and 69 hours for PVB culture. These two kinds of cultures are helpful in obtaining metaphases from BM, because BM cultures are very much susceptible to contamination. After centrifugation, the supernatant was discarded, and the pellet was treated with hypotonic solution (0.075M KCl) by gentle flushing. The culture tubes were incubated again at 37^0C for 20 min. The tubes were again centrifuged carefully at 1200 rpm for 10 min. The supernatant was removed and 10ml of freshly prepared Conroy's fixative (3:1 methanol: glacial acetic acid) was added to the pellet and mixed thoroughly (3-4 times). The tubes were allowed to stand overnight in 4^0C. Next morning after the centrifugation at 1200 rpm for 10 min, 20µl of cell suspension was gently poured on the slide. Slides were aged at 90^0C for 20 minutes. Giemsa Trypsin Giemsa (GTG) banding was performed. At least 20 metaphases were analyzed by the microscope with DSS Cyto-Vision Karyotyperon well spread G-banded metaphase plates. Karyotyping was performed according to the International System for Human Cytogenetic Nomenclature 2016.

The culture harvesting and slide procedure for SC procedure was same as stated above for the PVB culture except, MMC was introduced to the culture after 24 hours of inoculation. Three cultures, each for case and control (0ng, 50ng, 100ng MMC/ml of culture) were set.Giemsa stain was used to stain the chromosomes. GTG banding is not needed to visualize the break and radial structures. A set of 60 metaphases each from case and control (positive & negative) (20 metaphases from each culture) was observed under microscope (Zeiss Axioskop Microscope) 100× magnification for detecting the presence of gap, break and radial structure (bi-radial, tri-radial, higher order radial structures) suggestive for FA. The standard formula was used to analyse the sensitivity of MMC [% of cells with tri-radials + (1.6 × Total number of radials in 50 & 100 ng MMC/ml of Culture)]; the cut-off for the sensitivity of MMC is >40. The bi-radial, tri-radial and higher order radial structures all are counted as tri-radial, whereas the gap and break structures are not considered in the formula.

2.5. Statistical Analysis

Quantitative datawas presented bypercentage, median and average and standard deviation. Chi-square (X^2) statistic was done with the data of 170 AA patientsto evaluate the role of cytogenetics and stress cytogenetics.

3. RESULTS

3.1. Results of Cytogenetics Study

A total seven AAA patients showed various type of CAs. The median age was 56 years (n=7). The age range was 16-72 years. Among them, 6(85.71%) were male and 1(14.29%) was female. Out of these 7 patients, 2(28.57%) came out with very severe aplastic anaemia (VSAA), 4(57.14%) came out severe aplastic anaemia (SAA) and 1(14.29%) came out non-severe aplastic anaemia (NSAA). Mean±standard deviation of neutrophil count was 0.39±0.16 ($\times10^9$)/L, haemoglobin count was 57±16.17 (g/L), absolute reticulocyte count was 19.74± 11.2($\times10^9$)/L and platelet count was 16.33± 13.18($\times10^9$)/L.

Table1. *Showing the haematological and abnormal cytogenetic profile of the AAA patients observed in the present study*

Sl. No.	Age /Sex	Severity	NC($\times10^9$)/ L	Hb (g/L)	ARC ($\times10^9$)/L	Plt ($\times10^9$)/L	Cytogenetic Profile
Case1	16Y/M	VSAA	0.15	83	0.2	4	45,XY,rob(14;21)(q10;q10)[20]
Case2	28Y /M	SAA	0.46	59	21	15	45,X,-Y[3]/46,XY[47]
Case3	66Y/M	SAA	0.48	45	25	10	53~92,XY[5]/46,XY[15]
Case4	72Y/F	SAA	0.46	43	20	2.35	92,XXXX[2]/46,XX[18]
Case5	52Y/M	VSAA	0.17	58	12	20	46,XY,-7(q11)[3]/46,XY[17]

| Case6 | 56Y/M | SAA | 0.42 | 39 | 24 | 22 | 46,XY,-2(q33.3),-4(p15.3),-6(q13),-13(p11.2),X(q22.3)[12]/46,XY[8] |
| Case7 | 65Y/M | NSAA | 0.6 | 72 | 36 | 41 | 47~57,XY[2]/45,XY,-7[2]/45,XY,19[2]/48,XY,+21,+mar[1]/45,X,-Y[3]/46,XY[10] |

The Mean (±) standard deviation of neutrophil count, haemoglobin count, absolute reticulocyte count and platelet count which are responsible for the pancytopenia are graphically represented in AA patients with cytogenetic abnormalities (n=7), AA patients without cytogenetic abnormalities (n=163) and control cases (n=170) (Figure 1) with no obvious differences observed between these parameters of AA patients with cytogenetic abnormalities (n=7), AA patients without cytogenetic abnormalities (n=163).
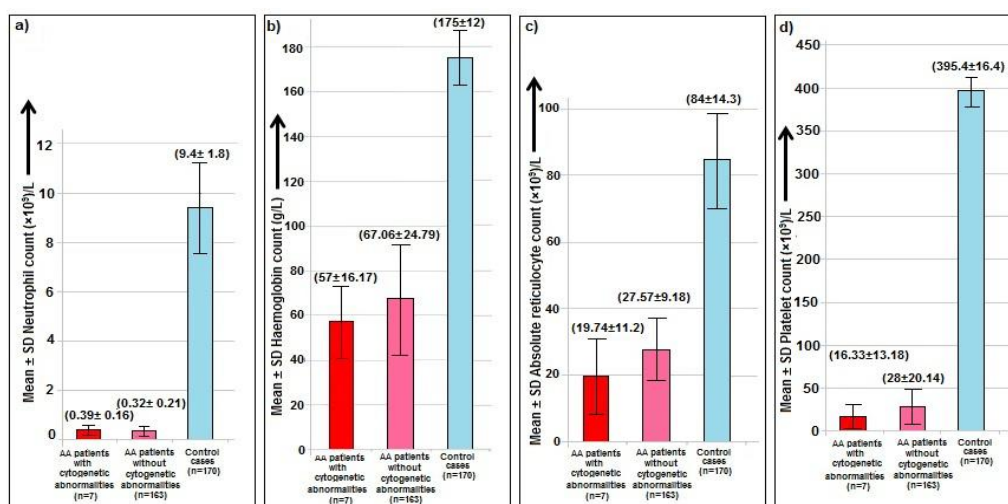
Figure1. *Showing the mean ± standard deviation of (a) neutrophil count, (b) haemoglobin count, (c) absolute reticulocyte count, (d) platelet count of the AA patients with cytogenetic abnormalities (n=7), AA patients without cytogenetic abnormalities (n=163) and control cases (n=170), with no obvious differences observed between these parameters of AA patients with cytogenetic abnormalities (n=7) and AA patients without cytogenetic abnormalities (n=163)*

3.1.1. Results of Cytogenetic Analysis of 51PVB Samples

The median age was 35 years (n=51). The age range 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%),27 (52.94%) and 3 (5.88%) were VSAA, SAA and NSAA respectively. Only one (1.96%) patient (15 Y/M) had a unique chromosomal translocation i.e. 45,XY,rob(14;21) (q10;q10) [20] (Figure 2).
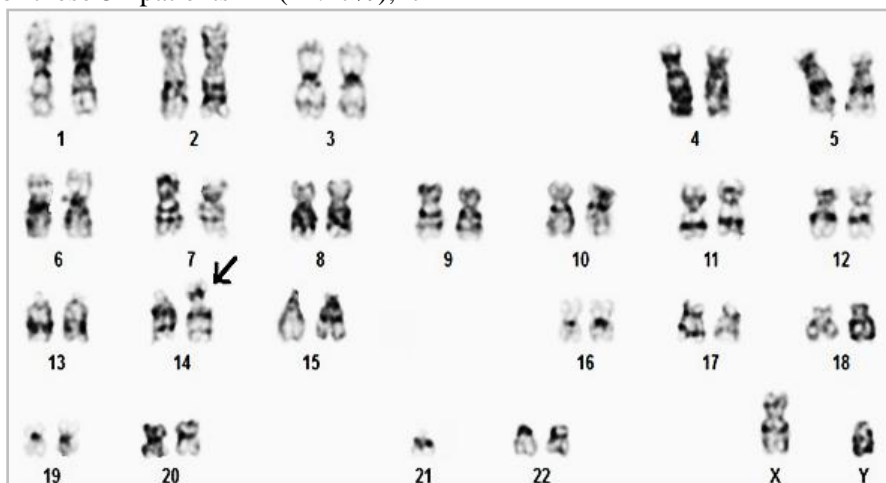

Figure 2: *A unique robertsonian translocation between chromosome 14 and 21 resulting 45,XY,rob(14;21) (q10;q10)[20] was observed in the PVB sample of an aplastic anaemia patient.*

3.1.2. Results of Cytogenetic Analysis of 19PVB&BM Paired Samples

The median age was 42 years (n=19). The age range 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. The abnormality was found in BM whereas the PVB showed normal karyotype.
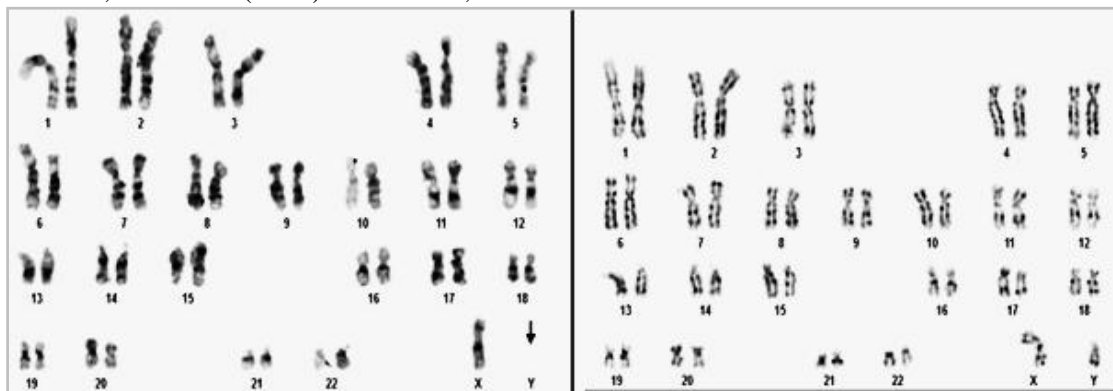

Figure3: *Showing the deletion of Y chromosome in three cells, resulting 45,X,-Y[3]/46,XY[47]. The loss of the Y chromosome was obtained in the BM culture from one of the 19 BM & PVB paired samples*.
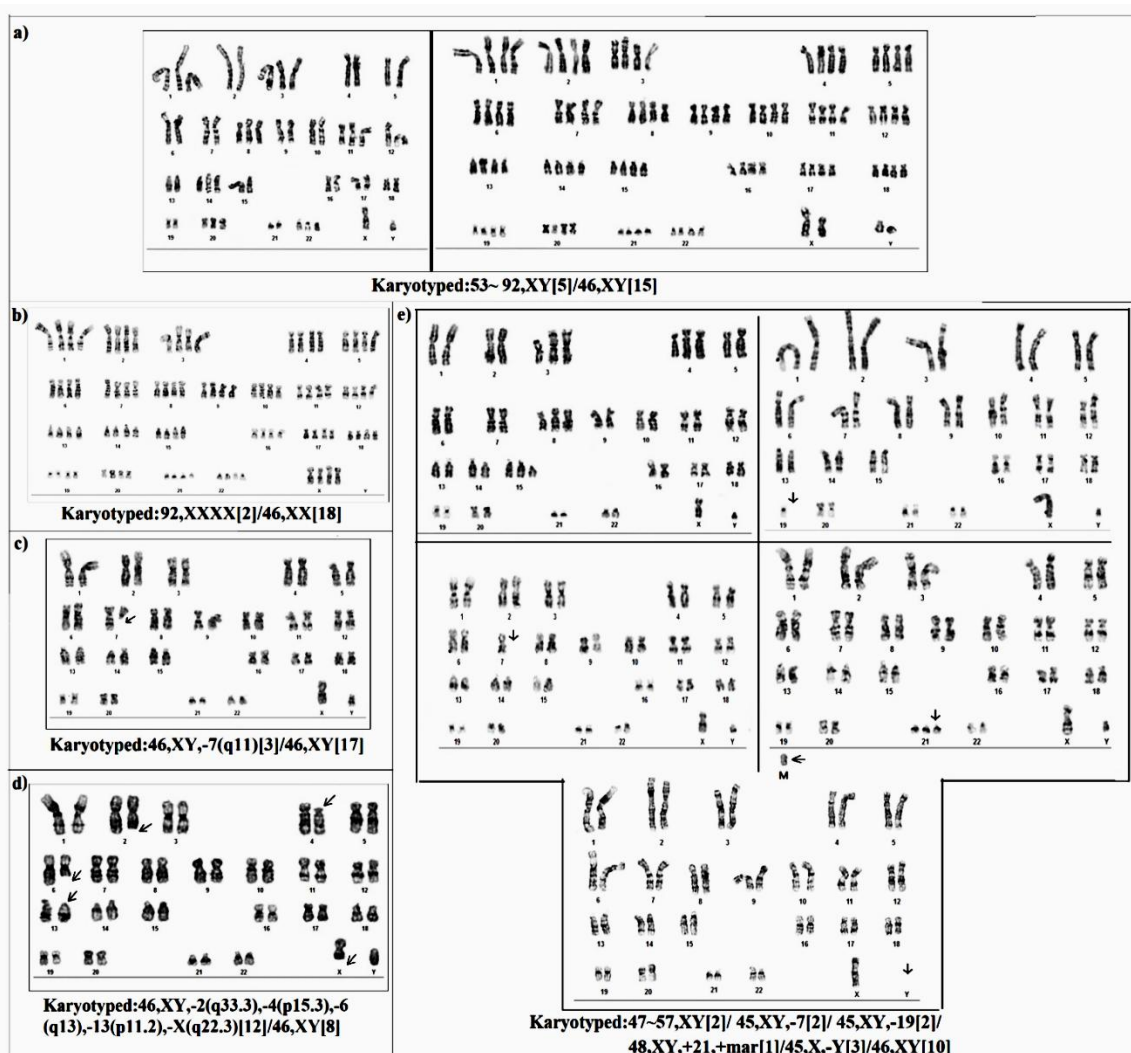

Figure 4: *Showing the cytogenetic abnormalities in 100 BM Samples of AAA patientsi.e. a) 53~92,XY[5]/46,XY[15], b) 92,XXXX[2]/46,XX[18], c) 46,XY,-7(q11)[3]/46,XY[17], d) 46,XY,-2(q33.3),-4(p15.3),-6(q13),-13(p11.2),-(q22.3)[12]/46,XY[8] and e) 47~57,XY[2]/ 45,XY,-7[2]/ 45,XY,-19[2]/ 48,XY,+21,+mar[1]/45,X,-Y[3]/ 46,XY[10].*

3.1.3. Results of Cytogenetic Analysis of 100BM Samples

The age range of the patient was 2-85 years. The median age of the patients was 52 years (n=100).Between them 52.00% (n=52) were male and 48.00% (n=48) were female. Out of these patients, 24.00% (n=24) came out with very severe aplastic anaemia (VSAA), 51.00% (n=51) came out with severe aplastic anaemia (SAA), and 25.00% (n=25) came out with non-severe aplastic anaemia (NSAA).Out of 100 BM five (5.00%) cases showed cytogenetic abnormalities i.e. a)53~92,XY[5]/46,XY[12], b)92,XXXX[2]/46,XX[18],c)46,XY,-7(q11) [3] /46,XY[17],d)46,XY,-2(q33.3),-4(p15.3),-6 (q 13),-13(p11.2),-X(q22.3) [12]/46, XY[8], e)47~57,XY[2]/45,XY,-7[2]/45, XY,-19[2]/ 48,XY,+21,+mar[1]/45,X,-Y[3]/46,XY[10].Rest of the 95 (95.00%) patients showed normal karyotype [i.e. 46,XY for male and 46,XX for female] .

3.2. Results of Stress Cytogenetics Study

Between 170 AA patients 99 patients was <50 years old. Hence, with their PVB samples Stress Cytogenetics with MMC was performed. The age range of the patient was 2-49 years. The median age of the patients was 23 years (n=99). The median age of the patients positive for Stress Cytogenetics was 6 years (n=9). Between them, 69.69 % (n=69) were male and 30.30 % (n=30) were female. Out of these patients, 41.41% (n=41) came out with VSAA, 45.45% (n=45) came out with SAA, and 13.13 % (n=13) came out with NSAA. Break, gap, radial, tri-radial and higher order radial structures were observed under the microscope (100X magnification). Nine patients (9.09%) were found to be positive for stress cytogenetics, suggestive for Fanconi anaemia (figure 5). Among these nine cases six (66.67%)normal and did not had any phenotypical abnormality but rest of the three (33.33%) showed congenital abnormalities.

Table 2: *Showing the phenotypical abnormalities found in the Stress Cytogenetics positive patients*

Sl. No.	Age/ Sex	Develo pment al delay	Microce phaly	Gastroint estinal system	Hypogon adism	Skin pigment ation and café-au-lait spots	Malform ation of thumb, radius and ulnar bone	Complica tions in eye, ear, heart, genitalia, central nervous system	Abnorm ality in Kidney and urinary tract	Histor y of paren tal consa nguini ty
FA Patient No.1	7Y/F	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)	(+ve)
FA Patient No.2	4Y/M	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)
FA Patient No.3	7Y/M	(+ve)	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)	(+ve)
FA Patient No.4	14 Y/M	(+ve)	(-ve)	(+ve)	(-ve)	(+ve)	(+ve)	(+ve)	(-ve)	(+ve)
FA Patient No.5	6Y/M	(+ve)	(+ve)	(-ve)	(-ve)	(+ve)	(+ve)	(-ve)	(+ve)	(+ve)
FA Patient No.6	5Y/F	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)
FA Patient No.7	3Y/F	(-ve)	(-ve)	(+ve)	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)	(+ve)
FA Patient No.8	8Y/F	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)	(+ve)
FA Patient No.9	2Y/M	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)	(+ve)	(-ve)

(+ve)-Present; (–ve)-Absent

Remainder 161 patients were of AAA category. In Stress Cytogenetics positive cases, six out of nine cases (66.66%), was found to be born out of consanguineous marriage. Breaks and radial structure were not found in control samples (figure 5). SCpositive patients did not report any CA.
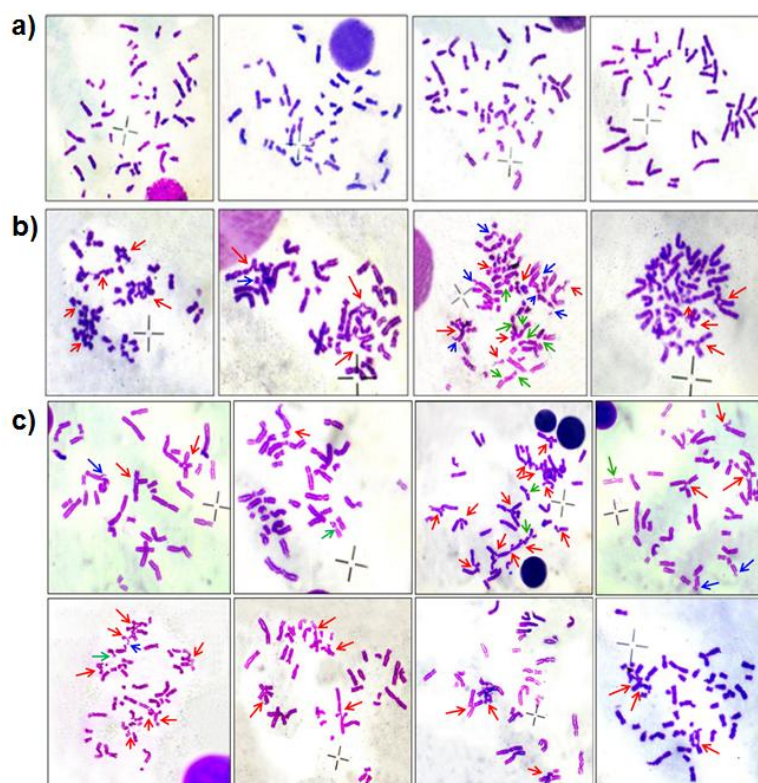
Figure 5: *Showing the representative picture of the metaphases obtained from the SCculture; a) metaphase cells from the negative control cases, where virtually no break or radial structure was found, b) metaphase cells from the positive control cases, where very high number of break, radial, bi-radial, tri-radial structures were observed, c) metaphases from the nine SCpositive patients, having a high number of break, radial, bi-radial, tri-radial structures like positive controls. The picture of the metaphases found from other 90 AA cases that were negative for SCand was like negative controls hence were categorized as acquired aplastic anaemia. [Radial (red), break (blue), gap (green)]*
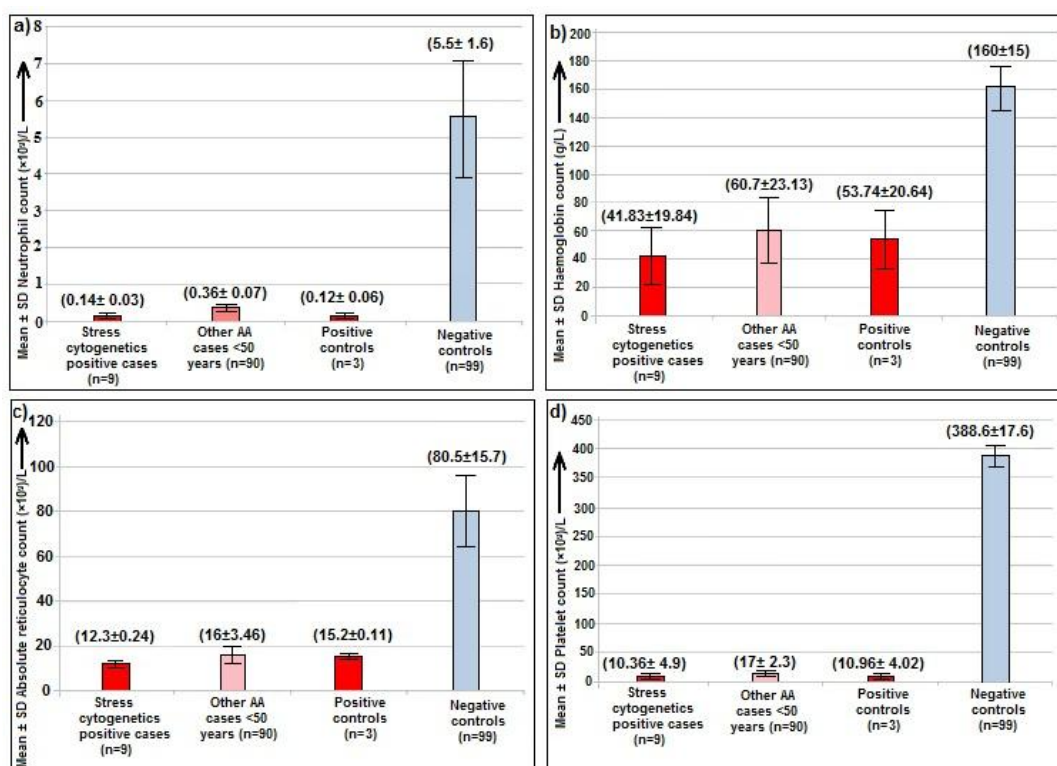

Figure 6:*The graphical representation of the mean and standard deviation of the (a) neutrophil count, (b) haemoglobin count, (c) absolute reticulocyte count and (d) platelet count, which are responsible for pancytopenia in SC positive patients (n=9), other AA patients (n=90), positive control (n=3) and negative controls (n=99).*

The Mean (±) standard deviation of neutrophil count, haemoglobin count, absolute reticulocyte count and platelet count are graphically represented in Stress Cytogenetics positive patients (n=9), other AA patients (n=90), positive control (n=3) and negative controls (n=99) (figure 6). The data of Stress Cytogenetics positive cases and the positive control are almost consistent with each other. The counts of other 90 AA cases, which are not sensitive to MMC, are moderately higher than MMC sensitive and positive control groups. The counts of the negative controls are significantly higher than the other three groups.

4. RESULTS OF STATISTICAL ANALYSIS

a) Results of the Statistical Analysis of Cytogenetic Study

Chi-square (X^2) statistic was performed with the data of 170 AA patients. Since the calculated value of X^2 (0.8532) is less than the table value (5.99) [df = 2, at 5% significance level] and the p-value at a 5% significance level was 0.652, hence the hypothesis (H_0) "cytogenetic analysis is not essential in AA patients" was accepted.

b) Results of Statistical Analysis of Stress Cytogenetics

Chi-square (X^2) statistic was performed with the data of 99 AA patients (<50 years). Since the calculated value of X^2 (9.4285) is greater than the table value (3.84) [df= 1, at 5% significance level] and the p-value was 0.002 at 5% significance level, the hypothesis (H_0) "Stress Cytogenetics in phenotypically normal AA patients (<50 years) is not required in ruling out FA" was rejected. This statistical analysis is supporting the fact that Stress Cytogenetics in phenotypically normal AA patients (<50 years) is vital in ruling out FA.

5. DISCUSSION

This study is a milestone in its kind from the Eastern India. Aplastic anaemia (AA) is defined by pancytopenia with hypo-cellular marrow and no abnormal cells.[18]In Eastern India, virtually the research on AA is at the budding phase and this particular research is the first ever documentation from Eastern India focusing on the cytogenetic profile AA patients.

5.1. Cytogenetics Analysis

After 130 years of discovery of AA, still, there is a gloomy part between cytogenetic abnormality and its implication in the diagnosis and treatment. Cytogenetic analysis of the bone marrow should be attempted although this may be difficult in a very hypo-cellular BM and often insufficient metaphases are obtained from the failed marrow culture which could undervalue the real frequency, predominantly at the phase of diagnosis.

It was previously assumed that the presence of an abnormal cytogenetic clone is suggestive of MDS and not of AA. But now various studies have already reported that abnormal cytogenetic clones may be present in 4-15% of patients with otherwise typical AA at diagnosis.[2-6,17,24-27]Due to differences in diagnostic criteria, patient population, treatment protocol and the frequency of follow-up BM examinations, acquisition of an abnormal karyotype in AA are frequent, but estimates have been variable between published studies.[7,8]A study in the USA established the fact that AA patients with the presence of cytogenetic abnormalities have a higher risk of developing leukaemia. It showed 4% of SAA patients with clonal cytogenetic abnormalities in unstimulated BM cells in the published study.[2]

Although circumstantial shreds of evidence have documented the response to IST in AA patients with cytogenetic abnormalities, still the clinical relevance of chromosomal abnormalities has not been studied in an organized manner. Few institutionally based studies are present.[28,29]But there was no well-designed population-based study depicting the role of cytogenetics in AA has been done till date from Eastern India. A study was done on 1501 patients in North India, which concentrates only on epidemiology, clinico-haematological profile and management of AA.[30]Another study showed five (11.9%) AA patients with cytogenetic abnormalities, like tri(12), tri(8), mon(7), del(7q) and t(5;12).[1]

The percentage of cytogenetic abnormality in this study is almost consistent with published Western data. Certain South-Indian hospital-based studies are also there, depicting the present situation and clinico-haematological profile of AA, but they did not focus on the cytogenetic aspect.[31-33] In Western India, there are few studies that focused on epidemiology, pathogenesis, diagnosis and the treatment procedure of AA.[34,35]A review article from western India raised a query of the relation between the evolution of the disease and cytogenetic abnormalities in paediatric AA patients.[36]

Finally, coming to the Eastern part of the country, only a review article was published

before the present study, which suggests that a cytogenetic examination of the BM is useful to exclude hypoplastic MDS.[37] Another study on clinico-haematological analysis of AA among children was performed in the northern districts of West Bengal but did not focus on the cytogenetic analysis.[28] Other than these studies, there was no instance of any specific study in India till our research period, that emphasized on the cytogenetic aspect of AA solely.

In the present study, to find out the cytogenetic abnormalities in AA patients', cytogenetics analysis of 170 cases were performed by the modified version of the Moorhead protocol.[38] All the cultures were successfully karyotyped. The study was started with the PVB sample followed by BM & PVB paired samples. After performing a cytogenetic analysis of 51 PVB and 19 BM & PVB paired samples, only two cytogenetic abnormalities were found. The cytogenetic study was further continued only with the BM samples and cytogenetic analysis

of 47 BM samples was done. At this stage of the sample's strength 117, 1.7% cytogenetic abnormality was found. Henceforth, it is concluded that cytogenetic study as a routine examination in otherwise archetype AA is not essential until the BM aspiration and biopsy is showing blasts, dysplasia, pre-leukemic changes, fibrosis, etc.[39]

However, some random transient chromosomal abnormalities can appear, and abnormalities usually disappear after successful therapy. Such cytogenetic abnormalities will not modify the treatment strategy in classical AA patients (except monosomy 7). The study was further continued with 53 BM samples of AA patients and five more cytogenetic abnormalities were detected. The overall percentage of cytogenetic abnormality has been raised to 4.11%. The individual percentages of cytogenetic abnormality were only 1.96% in PVB, 5.26% in BM & PVB paired samples and 5% in BM samples.

Table 4: *Showing the percentages of cytogenetic abnormalities of AA patients in different studies*

Study	Number of patients (N)	Cytogenetic abnormalitiesn (%)
Appelbaum et al.1987[2]	176	7(4)
Mikhailova et al.1996[4]	34	3(9)
Geary et al.1999[10]	13	5(38)
Keung et al. 2001[3]	30	2(6.7)
Gupta et al. 2006[27]	81	10(12)
Gupta et al. 2013[1]	42	5(11.9)
Heuser et al. 2014[40]	38	3 (7.9)
Yoshizato et al. 2015[25]	439 (All)	20 (8.5)
	256 (NIH)	9 (3.8)
Babushok et al. 2015[41]	22	2 (9.1)
Lee et al. 2010[42]	127	6 (4.7)
Dutta et al.[39]	117	2(1.7)
Present Study	170	7(4.11)

In this study, a unique chromosomal abnormality was found from the PVB samples (i.e. 45,XY,rob(14;21)(q10;q10)[20]) never reported before in AA patients. But the cytogenetic study from the BM of the same patient could not be performed due to the 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. Few articles have been reported on the clonal haematopoiesis in AAA[43] whereas, some have summarized the burden of somatic mutations and other genetic lesions by whole exome sequencing[44]. Even they did not report any such chromosomal abnormality in chromosome 14 and chromosome 21. That is why it was considered as unique translocation with respect to the disease AA.

It was 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[45] 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.[46] Although the role of loss of Y chromosome in marrow failure is not very clear. Out of 100 BM five (5%) cases showed cytogenetic abnormalities i.e. a) 53~92,XY[5]/46,XY[15], b) 92,XXXX[2] /46, XX[18], c) 46,XY,-7(q11)[3]/ 46,XY[17], d)46, XY,-2(q33.3),-4(p15.3),-6(q13),-13(p11.2), X(q22.3)[12]/46,XY[8],e)47~57,XY[2]/45,XY,-

7[2]/45,XY,19[2]/48,XY,+21,+mar[1]/45,X,-Y[3]/46,XY[10].

Hyperploidy and deletion were found to be a common event in the bone marrow. Abnormal chromosome 7 was reported in two cases, i.e. del(7q) and mon(7). Persistent mon7 is a poor prognosis as compared to tri8.[27]Laboratory findings suggested that aneuploid clones expand in an anomalous cytokine milieu rich with G-CSF. The existence of a short G-CSF receptor isoform provides a signal of proliferation, but not differentiation.[47]The presence of even small amount of mon7 clones in the BM, as detected by FISH (but not by routine cytogenetics), is a marker of poor prognosis for a response to IST.[48]Del(Y) and mon(19) were also found in the BM sample. The role of chromosome 19 in marrow aplasia is unknown to date. There are also shreds of evidence of detectable clonal mosaicism and its relationship to ageing and cancer.[49,50]

5.2. Stress Cytogenetics Analysis

Fanconi anaemia (FA) is an autosomal recessive chromosomal instability syndrome characterized by congenital abnormalities, progressive BM failure and cancer predisposition.[51,52-59] Many international researches have been made on the FA patients using DNA cross-linking agents. The chromosomes of the FA patients are susceptible to DNA cross-linking agents.[52] Documentation from Southern Brazil found 41.17% confirmed FA cases.[60]

Although, research works on FA patients with atypical phenotypes are common.[55] A study in Serbia showed the percentage of DNA cross-linker-induced aberrant cells was more than 26 times higher in FA patients compared to non-FA patients.[51] In India there are few documentations on FA patients.[29,31,61-68] Above mentioned studies considered both kinds of AA patients, irrespective of their phenotypical condition for stress cytogenetics.

In the present study, to discriminate between inherited and acquired AA, the gold standard stress cytogenetic using Mitomycin C was performed.[51-59]This study also supports the observation of DNA cross-linker-induced chromosomal breakage is very high in FA patients. Our study also justifies the fact of the high percentage (66.66%) of consanguinity is found among FA patients. Between these six patients five patients belong to Muslim community. These may be due to the fact that first or second degree consanguineous marriage

is a common incidence in their religion. Maximum but not all patients of FA present with some typical physical abnormalities. It was observed that a considerable percentage (9.09%) showed positivity for Stress Cytogenetics suggestive for FA, although among nine cases six patients were phenotypically absolutely normal.[19,69]

There was no such difference in the clinico-haematological profile of AA patients with or without abnormal cytogenetics. Sometimes the absence of typical phenotypical features makes the clinicians confuse. Therefore, it is the utmost treatment requirement to perform Stress Cytogenetics in AA patients who are < 50 years old even if they are phenotypically normal. More studies are needed from West Bengal as well as from the other parts of the country to come to any specific conclusion. To estimate the variety and frequency of the mutations in the FA patient population of West Bengal whole genome sequencing should be performed.

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