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DNA Aneuploidy Study for Early Detection of Chromosomal Abnormality in Patients with Aplastic Anemia: Prognostic and Therapeutic Implications

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Abstract. *Background: Undetected aneuploidy exists in a large percentage of patients with aplastic anemia at the time of diagnosis, which may not be identified by conventional cytogenetics. The presence of aneuploidy at any time in the clinical course implies poor prognosis in such patients. This warrants a need for the early detection of chromosomal abnormalities for prognosis and delineation of therapeutic modalities. Patients and Methods: Fifty patients with aplastic anemia and 30 controls were studied with an aim to determine the role of aneuploidy as an indicator of chromosomal abnormalities. DNA aneuploidy analysis was carried out by flow cytometry using Mod Fit-LT V3.0 software, whereas chromosomal analysis was performed by conventional cytogenetics. Results: DNA aneuploidy was present in 14% of cases and chromosomal abnormalities were found in 4% of cases of aplastic anemia at the time of diagnosis before therapy. Overall, DNA aneuploidy was detected in 36% of cases by flow cytometry, whereas the cytogenetic method revealed chromosomal abnormalities in 14% of cases of aplastic anemia. Flow cytometric analysis showed hypodiploidy in one patient at the time of diagnosis who developed monosomy 7 during follow-up. All patients with hypodiploidy had short survival and they did not respond to therapy. Conclusion: The present study demonstrates the role of flow cytometry in the early detection of chromosomal abnormalities in patients at a time when they remain undetected by conventional cytogenetics. The presence of DNA aneuploidy in patients with aplastic anemia may be an early indicator of subsequent overt cytogenetic abnormality, associated with poor response to immunosuppressive therapy and a lower survival.*

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Aplastic anemia (AA) is a potentially life-threatening failure of hematopoiesis characterized by pancytopenia and hypocellular bone marrow. It has a variable course and may transform in some patients into paroxysmal nocturnal hemoglobinuria (PNH), myelodysplastic syndrome (MDS), or acute myeloid leukemia (AML). A proportion of patients with AA may have or develop cytogenetic abnormalities in due course, which may herald the conversion into MDS or AML. Once the survival of AA was shown to improve with immunosuppressive therapy (IST), observation of clonal evolution raised questions as to whether the development of secondary MDS or AML is a part of the extended natural history of the disease or is related to the therapies applied (1). Cytogenetic abnormalities most frequently encountered in MDS secondary to AA involve particularly aneuploidy of chromosomes 6, 7 and 8 (1). The evolution rates seem to be in the range of 10-15% in 10 years, but there are no predictive clues as to which patients are at greatest risk for this complication (1). Cytogenetic abnormalities in association with AA have been reported fairly infrequently and may be underestimated (1). Cytogenetic abnormalities in AA at initial diagnosis are uncommon. These abnormalities are late events and they are stereotypically aneuploid rather than structural chromosomal alterations (2). The evolution of aneuploidy can be an early phenomenon in leukemogenesis, which in course may affect proliferation, differentiation and cell death. There is a strong possibility that a proportion of cases of AA may be in fact preleukemia. Chromosomal abnormalities are relatively accessible findings with which to diagnose pre-leukemics, however small abnormal clones may remain undetected (3). This is perhaps because of poor chromosome morphology and the tendency for abnormal clones to proliferate poorly (4). Flow cytometry (FCM) can

rapidly measure the cellular DNA content of large numbers of cells and estimate ploidy that is independent of cell proliferation (5-7). It has been studied in patients with myelodysplastic syndrome (8, 9) acute lymphoblastic leukemia (10, 11), chronic myeloid leukemia (12), adult acute leukemia (13), multiple myeloma (14) and prostate cancer (15). FCM analysis of DNA aneuploidy is an alternative tool to conventional cytogenetics for the early detection of karyotypic changes resulting in abnormal DNA content. For over 100 years, aneuploidy, an abnormal balance of DNA content, has been widely accepted as a biomarker of malignancy (16). The detection of aneuploidy in DNA histograms obtained by FCM studies of clinical samples from cancer patients is of major biological and clinical importance.

In the present study, we looked for DNA aneuploidy as a novel tool to evaluate its role in early detection of aneuploidy in patients with AA and their propensity to develop cytogenetic abnormalities, dysplasia or malignancy. The study was planned to assess the clinical course of patients with aneuploidy as compared to those without aneuploidy. We also investigated whether the appearance of aneuploidy in aplastic anemia patients influences the response to treatment or *vice-versa*.

Patients and Methods

The study group comprised 50 consecutive patients with aplastic anemia and 30 age- and sex-matched control individuals who were free from any hematological disease. The diagnosis of AA was made on the basis of complete blood counts (CBC), bone marrow aspiration and biopsy. Severity of AA was defined according to the criteria of Camitta *et al*. (17). Patients who had received anti-lymphocyte globulin (ALG)/anti-thymocyte globulin (ATG), steroid or IST in the past were excluded from the study. All the patients with AA were put on cyclosporine (CsA) (5 mg/kg/day) as no one was able to afford ATG/ALG or bone marrow transplantation (BMT). The patients were followed up periodically and the dosage was adjusted according to clinicohematological parameters at every month. The DNA aneuploidy analysis and bone marrow examinations (aspiration and biopsy) were carried out at diagnosis and then at six month intervals whereas cytogenetic analysis was performed yearly. All the patients with AA were evaluated for the presence of a PNH clone using flow cytometry and sucrose lysis test. Informed written consent was obtained from patients and controls. The study protocol was approved by the Institutional Ethics Committee.

Cytogenetic analysis was performed by a conventional method using peripheral blood (18). Analysis of the karyotype was performed according to the International System for Human Cytogenetic Nomenclature (ISCN) (19), as amended in 1991 (20). A chromosome index (CI) was calculated by dividing the mean or modal chromosome number (obtained from the analyzed metaphase) by 46. Chromosome abnormalities were defined according to the accepted criteria (21) – namely, two or more metaphases containing the same additional chromosome or structural rearrangement and three or more metaphases showing loss of the same chromosome. A karyotype was only conclusively reported as diploid where only normal metaphases were found, hypodiploid where a clonal abnormality characterized by partial or complete chromosome loss was seen, and hyperdiploid when there was a clonal abnormality due to additional chromosomal material.

DNA aneuploidy analysis in peripheral blood lymphocytes was carried out according to the method described by Krishnan (7). Samples were analyzed on a Becton Dickinson flow cytometer (Becton Dickinson India Private Limited, Gurgaon, Haryana, India) with Mod Fit-LT V3.0 software (Verity Software House, Topsham, ME, USA) using 488 nm excitation wavelength. Cells acquired by FCM were plotted in side-scattered light (SSC) *versus* forward-scattered light (FSC), fluorescence-2 area (FL-2A) *versus* fluorescence-2 width (FL-2W) and FL-2A *versus* count. The threshold was adjusted to have the first peak at 200 channel of FL-2W plot and the cell population was gated from the plot of FL-2A *versus* FL-2W for analysis. The gated population was analyzed using the broadened rectangular model of the Mod Fit-LT V3.0 software, with a constant coefficient of variation of less than 6 and residual χ^2 value less than 5. This modal was also fitted with single cut debris subtraction and aggregate subtraction. Ten thousand cells were analyzed per sample. Human peripheral blood lymphocytes from hematologically normal individuals were used as the diploid reference standard.

According to an international convention on nomenclature for DNA cytometry (22), only the samples with at least two separate G0/G1 peaks were considered DNA aneuploid. The degree of DNA aneuploidy, *i.e*. DNA index (DI), was obtained by dividing the mean channel number of the aneuploid G0/G1 peak by the mean channel number of the diploid G0/Gl peak. DI=1.0 refers to a diploid cell population, whereas DI≠1.0 was defined as DNA aneuploidy (DI<1.0 was referred to as DNA hypodiploidy and DI>1.0 as DNA hyperdiploidy). The degree of DNA content aberration was expressed by the DNA index.

Response to immunosuppressive therapy in patients with aplastic anemia was defined according to Bacigalupo *et al*. (23).

Statistical analysis. The findings were expressed as the mean±standard deviation. The differences between the groups were compared with the use of Student's *t*-test. Fisher's exact test was used as appropriate. Pearson's coefficient of correlation (r) was used to calculate the concordance between the two methods, *i.e*. flow cytometry and conventional cytogenetics. A value of $p<0.05$ was considered statistically significant.

Results

The mean age of the patients with AA was 29.06±15.24 years (n=50, range 13-71 years, with a median age of 22 years) while that of controls was 30.93±14.93 years (n=30, range 14-64 years, with a median age of 24 years). The male to female ratio of patients with AA was 3.5 to 1. The median follow-up of patients was 18 months (range 6 to 25).

The flow cytometric and cytogenetic results from individual samples are listed in Table I. Aneuploidy (DI≠1.0) was detected by FCM analysis in 14% of cases of AA at the time of diagnosis, whereas no controls had aneuploidy (Figure 1). Six patients out of seven (85.7%) were hyperdiploid, with a mean DI of 1.12±0.03 (range

Table I. *Flow cytometric and cytogenetics results from 50 patients with aplastic anemia.*

No.	Age (years)/Gender	At the time of diagnosis			During the follow-up			Status
		DI	C I	Karyotype	DI	C I	Karyotype	
1	15 / M	1	1	46 XY	1	1	46 XY	Alive
2	52 / M	1	1	46 XY	1.09	1	46 XY	Dead
3	15 / F	1	1	46 XX	1	1	46 XX	Alive
4	71 / M	1	1	46 XY	1.22	1	46 XY	Dead
5	22 / F	1	1	46 XX	0.91	1	46 XX	Dead
6	13 / M	1	1	46 XY	1	1	46 XY	Alive
7	15 / M	1	1	46 XY	1	1	46 XY	Alive
8	20 / M	1	1	46 XY	1	1	46 XY	Alive
9	65 / F	1.08	1	46 XX	1.32	1.02	47 XX, +8	Alive, RA
10	21 / M	1	1	46 XY	1	1	46 XY	Alive
11	25 / M	1	1	46 XY	1.10	1	46 XY	Alive
12	18 / M	1	1	46 XY	1	1	46 XY	Dead
13	22 / M	1	1	46 XY	1	1	46 XY	Alive
14	18 / F	1	1	46XX	1	1.02	47 XX, +8	Alive
15	27 / M	1.09	1	46 XY	1.05	1	46 XY	Alive
16	15 / M	1	1	46 XY	1	1	46 XY	Alive
17	24 / F	1	1	46 XX	1	1	46 XX	Alive
18	17 / F	1	1	46 XX	1	1	46 XX	Alive
19	35 / M	1	1	46 XY	1.08	1	46 XY	Alive
20	16 / M	1	1	46 XY	1	1	46 XY	Alive
21	45 / M	1	1	46 XY	1.20	1	46 XY	Alive
22	34 / M	1	1	46 XY	1	1	46 XY	Alive
23	52 / M	1.11	1	46 XY	1.18	1.02	47 XY, +6	Alive, RA
24	35 / M	1	1	46 XY	1	1	46 XY	Alive
25	34 / M	1	1	46 XY	1	1	46 XY	Alive
26	31 / M	1	1	46 XY	1.15	1	46 XY	Dead
27	15 / M	1	1	46 XY	1	1	46 XY	Alive
28	23 / M	1	1	46 XY	1	1	46 XY	Alive
29	24 / M	1	1	46 XY	1	1	46 XY	Alive
30	17 / F	0.90	1	46 XX	0.95	0.97	45 XX, –7	Dead, AML
31	40 / F	1	1	46 XX	1	1	46 XX	Alive
32	22 / M	1	1	46 XY	1	1	46 XY	Alive
33	18 / M	1	1	46 XY	1	1	46 XY	Alive
34	58 / M	1.18	1.02	47 XY, +21	1.21	1.02	47 XY, +21	Dead, RAEB
35	20 / M	1	1	46 XY	1	1	46 XY	Alive
36	28 / M	1	1	46 XY	1	1	46 XY	Alive
37	19 / M	1	1	46 XY	1	1	46 XY	Alive
38	51 / F	1.13	1	46 XX	1.28	1.02	47 XX, +6	Dead
39	33 / M	1	1	46 XY	1.25	1	46 XY	Dead
40	19 / M	1	1	46 XY	1.07	1	46 XY	Alive
41	16 / M	1	1	46 XY	1	1	46 XY	Alive
42	46 / M	1	1	46 XY	1	1	46 XY	Alive, RA
43	40 / M	1	1	46 XY	1.05	1	46 XY	Alive
44	20 / F	1	1	46XX	1	1	46 XX	Alive
45	23 / M	1	1	46 XY	1	1	46 XY	Alive
46	65 / M	1.12	1.02	47 XY, +8	1.34	1.02	47 XY, +8	Dead
47	20 / M	1	1	46 XY	1	1	46 XY	Alive
48	14 / M	1	1	46 XY	0.89	1	46 XY	Dead
49	19 / M	1	1	46 XY	1	1	46 XY	Dead
50	46 / F	1	1	46 XX	1	1	46 XX	Dead

DI: DNA index, CI: chromosome index (mean or modal chromosome number/46), RA: refractory anemia, RAEB: refractory anemia with excess blast cells, AML: acute myeloid leukemia.

Table II. *Aneuploidy within various age groups.*

Age group (years)	DNA aneuploidy	Mean DI	*p*-value
<20 (n=3)[a]	16.6%	0.97±0.04	[ab]0.42, [ac]0.02, [ad]0.0005
20-30 (n=3)[b]	16.6%	1.02±0.09	[bc]0.14, [bd]0.004
30-40 (n=4)[c]	22.2%	1.13±0.08	[cd]0.06
>40 (n=8)[d]	44.4%	1.23±0.08	

Table III. *Aneuploidy with severity of disease.*

Group	DNA aneuploidy	Mean DI	*p*-value
NSAA (n=3)[a]	16.7%	1.02±0.09	[ab]0.5, [ac]0.0003
SAA (n=7)[b]	38.9%	1.06±0.10	[bc]0.0002
VSAA (n=8)[c]	44.4%	1.26±0.05	

NSAA: Non-severe aplastic anemia; SAA: severe aplastic anemia; VSAA: very severe aplastic anemia.

1.08-1.18) (Figure 2), whereas one patient (14.3%) was hypodiplod with a DNA index of 0.90. Cytogenetic analysis was performed on the same sample analyzed by FCM in all the patients. Chromosomal abnormalities were present in 4% of cases of AA at the time of diagnosis. Trisomy 8 was detected in one patient and trisomy 21 in another. Both patients had a hyperdiploid pattern by FCM analysis with a DI of 1.12 and 1.18 respectively. During the follow-up, aneuploidy was detected by FCM analysis in an additional 11 patients, including 9 with hyperdiploidy (DI range 1.05-1.34) and 2 with hypodiploidy (DI of 0.89 and 0.91 respectively). In contrast, conventional cytogenetics discovered chromosomal abnormalities in 5 patients during the follow-up: trisomy 8 was seen in two patients, trisomy 6 in another two and monosomy 7 in one patient. Out of these 5 patients, 4 had already shown aneuploidy by FCM analysis at the time of diagnosis, but in one patient who had trisomy 8, aneuploidy was not detected through FCM analysis. One patient who showed a hypodiploid pattern at the time of diagnosis through the FCM analysis later demonstrated chromosomal abnormality in the form of monosomy 7. There was a very good agreement (r=0.91) between these two methods for 6 cases of aneuploidy. Aneuploidy was more often found in elder patients than in younger ones. There was a significant difference between the DI of different age groups (Table II). Patients with very severe aplastic anemia (VSAA) were statistically significantly more likely to have aneuploidy than the others ($p<0.01$) (Table III). PNH was present in 10% of patients with AA and was subclinical in all. The incidence of aneuploidy in AA/PNH patients was 40%. The mean serum LDH level (560.6±353 U/l) was significantly higher ($p<0.03$) in patients who had aneuploidy in comparison to patients without aneuploidy (404±176.7 U/l).

Table IV. *Response to immunosuppressive therapy (cyclosporine).*

Response parameter	Patients with aneuploidy (n=18)	Patients without aneuploidy (n=32)	*p*-value
Complete response	5 (27.8%)	3 (9.4%)	0.02
Partial response	3 (16.7%)	19 (59.4%)	
Total response	8 (44.5%)	22 (68.8%)	
No response	10 (55.5%)	10 (31.2%)	

All the patients were evaluated for the response to IST *i.e.* cyclosporine (CsA) at the end of the study (Table IV). Thirteen patients died during the study, of whom 10 had aneuploidy by FCM analysis, including three with hypodiploidy and seven with hyperdiploidy. All patients with hypodiploidy had short survival and did not respond to therapy. Four patients with AA developed MDS (three refractory anemia and one refractory anemia with excess blast cells) within the 12 months of follow-up. Aneuploidy (hyperdiploidy) was present in two patients with refractory anemia (RA) and one with refractory anemia with excess blast cells (RAEB). Cytogenetic analysis detected trisomy 8 and trisomy 6 abnormalities in two patients with RA. The patient with RAEB had trisomy 21 chromosomal abnormality and died during the follow-up. One patient with monosomy 7 died of acute leukemia (Table I).

Discussion

Aplastic anemia is rare in Western Europe and the United States (3-6 cases per million population per year). Its incidence in Southeast Asia and China is 3-4 times higher (24). Important known causes of AA are benzene, hepatitis and nonsteroid anti-inflammatory drugs (NSAIDs) (24). Drugs such as phenylbutazone, indomethacin, diclofenac sodium and clopidogrel are known to cause AA (24). However, in most cases of AA (65%), the cause is not obvious (idiopathic) (25). Fifty patients with AA and 30 controls were included in this longitudinal case-control study. The median follow-up was 18 months (range 6 to 25 months). The median age of patients was 22 years, with a range of 13–71 years. This is similar to reports from countries both from Western Europe and Southeast Asia where AA is most common between the ages of 15-25 years (24). In the present study, a possible association of NSAIDs with the disease was found in 6% of cases.

The diagnosis of AA is based on the presence of pancytopenia in peripheral blood and hypocellular bone

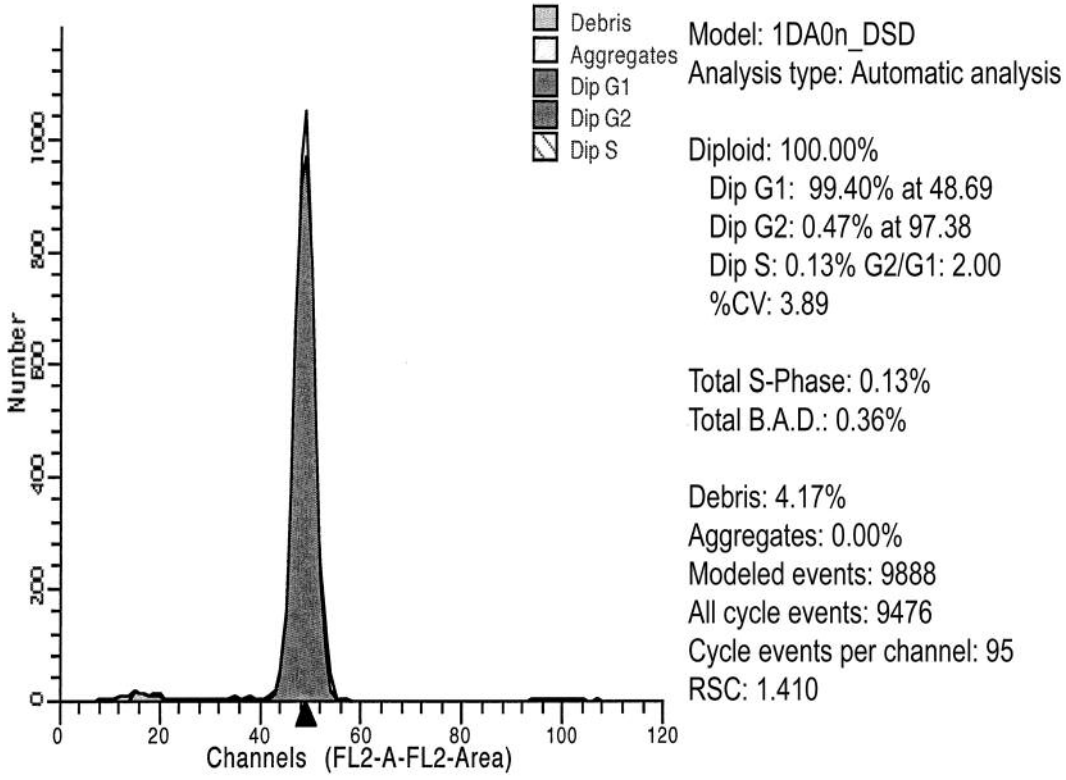

Figure 1. *DNA histogram of control.*

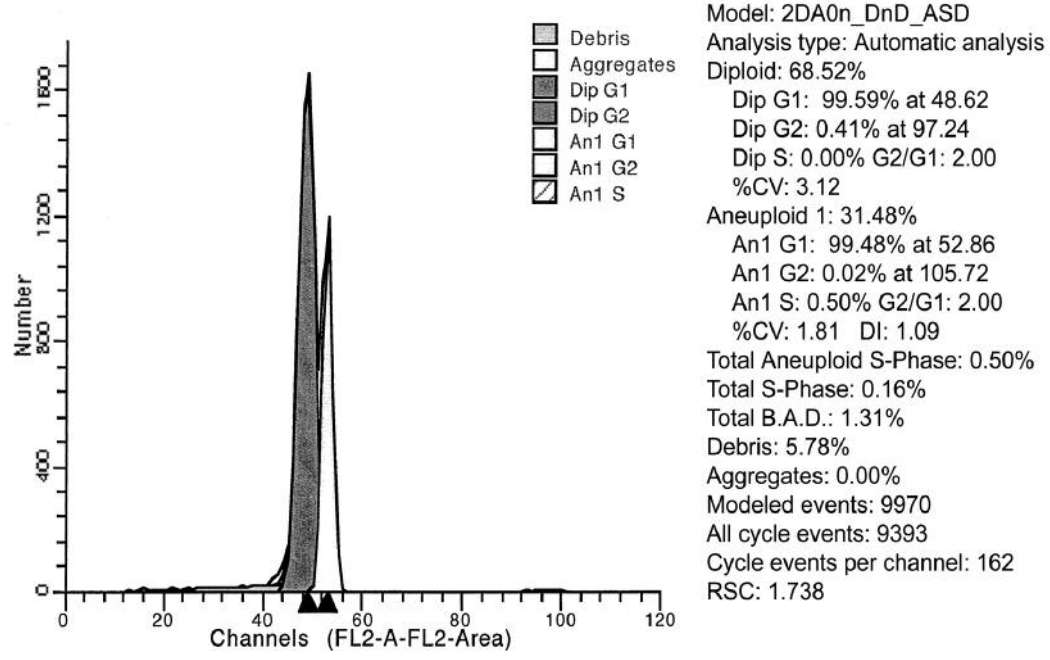

Figure 2. *DNA histogram of patient (NK, 19-year-old male) at the time of diagnosis.*

marrow. However, other conditions such as hypoplastic myelodysplastic syndrome (HMDS) and PNH may also represent similar observations. Hence, differentiation of these conditions from AA may be difficult. It is imperative to exclude the presence of HMDS and PNH, since the therapeutic decision may differ in these conditions. Moreover, the natural course of disease in patients with AA is variable. Following IST, about 9.6% and 6.6% patients of AA are seen to develop MDS and AML in 10 years of follow-up, respectively (24). Occurrence of PNH in patients with AA is well known and may arise following IST. In various studies, 9-57% patients of AA developed PNH clone during long-term survival (24).

Various attempts have been made to find related conditions such as HMDS and PNH in patients with AA at the time of initial diagnosis. The presence of splenomegaly and increased serum LDH has been suggested to favour the diagnosis of HMDS. Response to IST in AA varies and depends upon age, severity of disease and the presence of PNH (26-28). It is important to know which patients with AA are more likely to undergo transformation, since this may have a great implication for prognosis and therapy. Patients with AA who have aneuploidy at the time of diagnosis may not be chosen for IST, but for BMT with an HLA-matched sibling donor at the outset. Furthermore, patients with AA on IST, if aneuploidy develops, may be regarded for BMT in order to prevent them from transforming into MDS/AML. Allogeneic BMT is currently the treatment of choice for young patients with AA. This has a lower risk of relapse and late clonal disorders as compared to patients treated with IST.

There are various ways to assess clonality in patients with AA, such as X-chromosome inactivation patterns and cytogenetic analysis. Aneuploidy detected by conventional cytogenetics is cumbersome, time consuming and limited by the requirement for cells with a relatively high proliferation rate; it also has limited sensitivity. Flow cytometric analysis of DNA aneuploidy provides the maximum amount of genetic information when a satisfactory karyotype might not be available, or where failure of an abnormal cell population to proliferate might give an incomplete cytogenetics picture. It is conceivable that numerical chromosomal aberrations are associated with differences in cellular DNA content. Hence, in addition to conventional cytogenetics, we used flow cytometry, as it allows fast and automated estimation of aneuploidy status (abnormal DNA content). It is also free from all encumbrances of karyotyping such as a small number of recognizable metaphases, minimal chromosome spreading, poor banding quality and condensed or fuzzy appearance of chromosome.

In the present study, DNA aneuploidy was present in 14% of cases and chromosomal abnormalities were found in 4% cases of AA at the time of diagnosis. Two cases (4%) with cytogenetic abnormality have also shown DNA aneuploidy at diagnosis. Overall, DNA aneuploidy was detected in 36% of cases by flow cytometry whereas the cytogenetic method revealed chromosomal abnormalities in 14% cases. The incidence of cytogenetic abnormalities in AA has been variably reported among various studies. The difference may be perhaps due to differences in diagnostic criteria, patient populations, treatment protocols and the frequency of follow-up bone marrow examinations. In a National Institutes of Health series of 122 patients treated with intensive immunosuppression, 14 developed cytogenetic abnormalities, with a risk of about 21% at 10 years (29). In an Italian study, cytogenetic abnormalities were reported in 18 out of 69 patients, where only 7 had cytogenetic abnormality at initial diagnosis (30). Similar reports have been published from other parts of the world including Japan (31) and Pakistan (32). It is noteworthy that conversion of normal to abnormal karyotype occurs at a constant rate after initial diagnosis, with around half of the cases developing within the first 30 months (33). Although few studies have been carried out on flow cytometric analysis of DNA content in myelodysplastic syndromes (8, 9, 34, 35), no published report on flow cytometric analysis of DNA aneuploidy in patients with AA is available in the literature. Our study revealed that the incidence of aneuploidy increased with the age of patients and was statistically significant (Table II). The aneuploidy also matched the severity of disease. We found a significantly higher aneuploidy rate SAA (38.9%) and VSAA (44.4%) groups as compared to NSAA (16.7%). Previous studies have shown that cytogenetic abnormalities are more common in patients with SAA (30, 36) as compared to those with NSAA. It is noteworthy that patients with aneuploidy had a greater propensity to develop MDS (16.7%, median time 10 months) than patients without aneuploidy (3.1%).

Patients with AA were put on cyclosporine (CsA) (5 mg/kg/day). All three patients who were hypodiploid belonged to the non-responder group. The difference in response to therapy between various groups was statistically significant (Table IV). The response to IST in patients with AA is known to depend upon age, severity, presence of PNH and dysplasia. Our results have shown (Table IV) that response (CR+PR) to CsA in AA patients having aneuploidy was 44.5% as compared to 68.8% in patients without aneuploidy and the difference was statistically significant (p=0.02). Hence it is conceivable that the presence or absence of DNA aneuploidy at the time of initial diagnosis in patients with AA can be taken as an indicator for response to CsA and this may also apply in cases of standard IST, *i.e.* ATG + CsA. DNA aneuploidy could even be useful at a stage when karyotypic abnormalities have not appeared. The relationship of PNH with aneuploidy in AA patients could not be ascertained in the present study due to its small sample size. However, patients with PNH-positive AA are reported to respond to IST better (28).

DNA aneuploidy in AA may also be useful in terms of prediction of survival. AA patients showing hypodiploidy (n=3) did not respond to therapy and died within twelve months. It is important to note that many of the patients who had aneuploidy either had a cytogenetic abnormality or developed one later in the course of their disease. Although the response to CsA in patients with aneuploidy was lower than in those without aneuploidy, the development of cytogenetic abnormalities in the course of their disease did not further add negatively to the continued response observed in these patients. Patients of AA with aneuploidy develop karyotypic abnormalities in due course of time. Hence, DNA aneuploidy may be regarded as an early indicator of clonal abnormality and it may influence the therapeutic decision. Patients with AA had aneuploidy at the time of diagnosis may not be chosen for IST but for BMT. The significance of DNA aneuploidy would be more clear in patients with AA as they are followed up for longer periods of time. Even if AA with cytogenetic abnormality is recognized as a disease entity, it is difficult to distinguish it from HMDS by morphological and cytological findings. The evidence of DNA aneuploidy in a substantial number of AA patients may prompt the recognition of a distinct disease entity, bone marrow failure with clonal abnormality, rather than AA itself.

We conclude that the presence of DNA aneuploidy in patients with AA may be a novel tool for early detection of chromosomal abnormality, poor response to IST and a lower survival rate. Studies involving larger number of patients with longer follow-up are needed to further establish the role of DNA aneuploidy in early detection of chromosomal abnormality, disease progression and response to IST in patients with AA.

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