Etiology and Survival of Aplastic Anemia: A Study Based on Clinical Investigation

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Background: Management of aplastic anemia is etiology driven, whether constitutional or acquired. Age, gender, and severity of disease also play crucial role in the survival of aplastic anemia. Since, inadequate data are available from India, the present study was conducted with the aim to evaluate the etiology and survival of aplastic anemia. **Methods:** Three hundred patients were enrolled between May 2007 and April 2010. Severity analysis and chromosomal breakage study was performed and patients were followed up to calculate the survival rate. **Results:** Only 9.4% of the cases demonstrated the evidence of constitutional disease. Patients with acquired disease showed a significantly higher odd ratio for hepatitis. Overall survival was found to be independent of the gender and inherited

etiology. Phenotype resembling to constitutional disease was present in only 22.22% (6/27) of patients. Similar ratio of the constitutional and acquired disease in both the age groups was observed. **Conclusion:** Irrespective of the age and phenotype, chromosomal breakage study should be mandatory for all patients with aplastic anemia. Hepatitis as a preceding event may be associated with the cause of aplastic anemia. Young age and less severe disease were strongly associated with better survival. Lack of tertiary care facility in the country, time lag between diagnosis and treatment, and unaffordability to abide the treatment cost could be the major contributory factors for poorer survival. J. Clin. Lab. Anal. 26:452–458, 2012. © 2012 Wiley Periodicals, Inc.

Key words: chromosomal breakage study; constitutional aplastic anemia; haploinsufficiency; mitomycin-C

INTRODUCTION

Aplastic anemia (AA) is a rare, heterogeneous disorder, with two- to three-fold higher annual incidence rates in Asian countries (1). The disease is characterized by failure of hematopoiesis and leads to bone marrow aplasia and pancytopenia. The counts of all the blood cell types go down drastically making the patient more vulnerable to opportunistic infections (2). AA has been classified as acquired aplastic anemia (AAA), and constitutional aplastic anemia (CAA). The AAA and CAA (Fanconi anemia (FA), Dyskeratosis Congenita, and Swachman Diamond syndrome) account for 80% and 20%, respectively. About 65% AAA is considered to be idiopathic,

where the causative agent of the disease is unknown (3). In a subset of cases, a drug or infection can be identified that precipitates the bone marrow failure/AA, although it is not clear why only some individuals are susceptible (4). The only known primary cause is

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Received 3 May 2012; Accepted 2 August 2012

DOI 10.1002/jcla.21546

Published online in Wiley Online Library (wileyonlinelibrary.com).

autoimmune disorder where the T cells attack other cell types. However, exposure to toxins like benzene, chloramphenicol, quinine, and even radiations are known to cause AA.

Various infectious agents can cause AA, yet neither of these is significantly associated as preceding episodes to AA. Some viral infections, notably Hepatitis and Epstein–Barr virus have been associated with AA. Human parvovirus B19 has been linked with pure red cell aplasia but not with severe aplastic anemia (SAA). Some cases of AA have been reported with human Immunodeficiency virus (HIV) infections. Despite varying degrees of peripheral cytopenia, whether, anemia results from a direct effect by the virus on the bone marrow or from a host immunologic response, is unclear (5). The treatment strategy, clinical course, and prognosis of AA is, to a large extent, based on the etiology, i.e., whether constitutional or acquired. The fact that it leads to wider range of chromosomal breaks and aberrations with chromosomal breakage study (CBS), is strongly indicative of a genetic basis of the disease. There are several candidate genes but no established diagnostics procedure (6, 7). The cost effectiveness of the CBS makes it the first-line investigation for differential diagnosis of AA in developing countries like India (8). To rule out the associated etiology, CBS was performed for all the patients of the present study.

AA patients have varied clinical symptoms; while some patients may have mild symptoms with a stable clinical course that necessitate little therapy, others present with life-threatening pancytopenia. The fatality rate of SAA is high. The outcome of SAA patients is not only influenced by age and severity of the disease, but also by the choice of the initial treatment (9). Treatment, whether by allogeneic stem cell transplantation or immunosuppressive therapy, has dramatically improved the prognosis over the last 25 years, and more than 75% of patients can be expected to have long-term survival with either therapy (10). Although bone marrow transplants have increased the survival rate of patients with AA, it leads to several transplant-related complications and most people do not have access to this therapy because of socioeconomic status. Majority of patients with SAA do not survive more than a year due to lack of early diagnosis and treatment (11).

We have conducted a contemporary population-based survival study stratified by age, gender, severity, preceding infectious episodes, and inherited etiology, which has not been done in India before. To the best of our knowledge, the present study is the largest and first report of its kind from India whereby an attempt has been made to evaluate the etiology and survival of AA.

MATERIALS AND METHODS

Patients

The study was designed to enroll all the biopsy-based confirmed AA patients who have been catered between May 2007 and April 2010 for their differential diagnosis to Indraprastha Apollo Hospital, and Medanta—The Medicity, two of the referral centers for tertiary care diagnosis. Cases were considered as 300 unrelated patients with the diagnosis of AA (males 230; females 70; age ranges, 1–52 years; median age, 17 years). An informed consent was taken for each and every patient before being subjected to a common questionnaire to draw the sketch of the clinical symptoms and habitat. Human peripheral blood samples were taken as per protocols approved by the Ethical Review Board of the hospitals, Departmental Research Committee of the AIB, Amity University, UP, and as reflected in the guidelines of the medical ethics committee, Ministry of Health, Government of India.

Exclusion and Inclusion Criteria

Registered cases were identified by regular contacts with hematologists or other physicians for analysis and required to meet at least two of the following criteria, a neutrophil count $\leq 500/\text{mm}^3$, a platelet count $\leq 20,000/\text{mm}^3$, a reticulocyte count $\leq 20,000/\text{mm}^3$ along with bone marrow biopsy specimen with hypocellularity compatible with the disease, showing no significant fibrosis or neoplastic infiltration with hematopoietic cells representing less than 30% of residual cells. The biopsies were reviewed by hematologists to determine final eligibility. Severity of the disease at diagnosis was assessed using the criteria of Camitta et al. (12) Patients who received chemotherapy, immunotherapy, or radiotherapy were excluded. To avoid false negative results, a specific criterion was designed in the present study—a minimum 15 days transfusion free interval was mandatory at the time of sample collection. A total of 300 cases of AA were identified and confirmed as potential subjects for the study over a period of 3 years. CBS was failed for 13 patients, and resampling could not be done as patient deceased meanwhile.

Chromosomal Breakage Studies

CBS was performed on both the patient and control population. For the performance of CBS, we used control population of patients that consisted of patients hospitalized for conditions not related to AA, matched by sex and age group. To be suitable, controls had to be independent of drug uses or occupational exposure and any acute infections, e.g., pneumonia, acute abdominal emergencies like appendicitis, trauma, and other selected conditions like

TABLE 1. Hematological Profile of AA Cases at Diagnosis

Parameter	HGB (g/dl)	PLT (10 ³ /μl)	RBC (10 ³ /μl)	WBC (10 ³ /μl)	Neutrophil (10 ³ /μl)
N	253	238	185	206	58
Range	1.8–14.4	0.2–125	0.7–11.5	0.5–22	0.06–2.7
Mean	6.61	15.95	2.17	3.46	0.8
Standard Deviation	2.43	15.82	1.07	2.62	0.59

cataract surgery. A 3–4 ml of peripheral blood sample was collected in heparinized vacutainer (BD Vacutainer®, Becton Dickinson, Franklin Lakes, NJ) for each patient and control. A peripheral blood culture stimulated with phytohemagglutinin for 72 hr was set up. The clastogen mitomycin-C (MMC) was added 48 hrs before the termination at a concentration of 200 ng/ml of culture. Also a peripheral blood routine cytogenetic culture was set up, to see the structural and numerical chromosomal aberrations, for each patient to rule out any congenital chromosomal abnormality. Termination of culture using colchicine, harvesting, and slide preparations were done as per the standard protocol.

CBS was done on MMC-treated slides stained with Giemsa stain, whereas the routine culture slides were processed for GTG (G-bands by Trypsin using Giemsa) banding. A minimum of 50 metaphases per treatment were analyzed. The MMC-induced chromosomal breakages were then compared to healthy controls. Achromatic areas less than a chromosome width, i.e., gaps were excluded in the calculation of chromosomal breakage frequency. Achromatic areas more than a chromosomal width were scored as breaks. Single chromatid breaks, isochromatid breaks, and acentric fragments were scored as one break each, while dicentric, ring chromosomes, and chromosomal breaks were scored as two breaks each (13).

Statistical Analysis

Statistical analysis was performed using SPSS 13.0 and Graph Pad Prism 5.0 software. Quantitative variables

were described as means, medians, and standard deviations. Categorical variables were expressed as absolute and relative frequencies. Survival probabilities were estimated using the Kaplan–Meier method, and comparisons between curves were based on the log-rank statistic. Univariate logistic regressions were used to investigate the association between potential risk factors and disease occurrence. The odds ratios (OR) and 95% confidence intervals (CI) were estimated. The differences were considered significant when *P* value was less than 0.05.

RESULTS

A total of 300 subjects were enrolled for the study. As per the hematological profile, most of the patients had pancytopenia at the time of diagnosis (Table 1). The male to female sex ratio was 3.2:1. The subjects were categorized in <21 years and ≥21 years age groups. More than three-fourth of the patients were of <21 years of age (Table 2). The overall survival (OS) for AA disease at 1, 2, and 3- years was 82%, 46%, and 32%, respectively. There were differences in survival when age was taken into account. The 1-year survival was 90.0% and 42.10% in the <21 and ≥21 age groups, respectively. The survival rate was found to be significantly associated with the age of patients ($P \leq 0.001$). The data were incomplete for 2nd and 3rd year survival for ≥21 age group as some patients were lost to follow-up. The 1-year survival rate was higher in the younger age group, i.e., <21 years (Log-Rank Mantel–Cox = 57.23, $P \leq 0.001$) (Fig. 1). Although survival rates in males were higher than in females, no statistically significant differences were observed. The differences in survival rate of male and female patients at 1, 2, and 3 years were 0.59 ($P = 0.56$), 0.25 ($P = 0.80$), and 0.53 ($P = 0.60$), respectively. (Table 3).

Infection as Prediagnostic Event

We have also tried to shed light on infectious etiology of AA. A total of 166 patients had evidence of infection.

TABLE 2. Correlation of Age, Sex, and Etiology with Severity of Aplastic Anemia

Acquired										Constitutional			
<21 Years	Total	MAA	SAA	VSAA	Total	MAA	SAA	VSAA	Total				
M	155	21/99	75/99	3/99	99	3/13	10/13	0	13				
F	54	16/51	34/51	1/51	51	5/5		0	5				
M + F	209	37	109	4	150	8	10	0	18				
≥ 21 Years	Total	MAA	SAA	VSAA	Total	MAA	SAA	VSAA	Total				
M	75	17/56	37/56	2/56	56	1/5	4/5	0	5				
F	16	-	0	-	0	0	0	0	0				
M + F	91	17	37	2	56	1	4	0	5				
Total	300	54	146	6	206	9	14	0	23

Fig. 1. Survival curve for the younger and older age group.

TABLE 3. Correlation of Age, Sex, Severity, and Etiology with Overall Survival of Aplastic Anemia

Percent survival	Etiological parameter		P value
	<21 years (N = 92)	≥21 years (N = 19)	
1 year	90.0	42.1	<0.001
2 year	47.8	NA	-
3 year	30.4	NA	-
	MAA (N = 24)	SAA (N = 62)	
1 year	95.8	77.4	0.035
2 year	79.2	33.9	<0.001
3 year	62.5	22.6	<0.001
	Acquired (N = 96)	Constitutional (N = 24)	
1 year	86.5%	75.0%	0.19
2 year	47.9%	NA	-
3 year	31.3%	NA	-
	Male (N = 82)	Female (N = 29)	
1 year	84.1	79.3	0.56
2 year	47.6	44.8	0.80
3 year	32.9	27.6	0.60

NA, not available.

The evidence of viral hepatitis was 41.15% and 7.41% of the patients affected with AAA and CAA, respectively (Table 4). AAA patients showed a significantly higher OR for hepatitis (OR = 8.74; CI, 2.0275–37.6919; $P \leq 0.0036$).

Etiology

Differential diagnosis was successfully done for 287 patients tested using CBS while cell culture failed for 13 patients. Twenty-seven (9.4%) patients showed constitutional etiology whereas 260 (90.6%) patients were classified as acquired. The patients with constitutional etiology exhibited more than 3.5- to 5.0-fold increase in chromosomal breaks, when compared to controls. The magnitude of

TABLE 4. Frequency of Infection as Prediagnostic Event of Aplastic Anemia

Type of infection	Frequency	
	Acquired N (%)	Constitutional N (%)
Hepatitis	107 (41.2)	2 (7.4)
Mononucleosis	19 (7.3)	1 (3.7)
Pneumonia	14 (5.4)	1 (3.7)
Malaria	12 (4.6)	0
Typhoid	10 (3.9)	0
None	98 (37.7)	23 (85.2)
Total	260	27

acquired and constitutional etiology was similar in both male and female as well as in both the age groups, i.e., <21 years and ≥21 years (Table 2). Interestingly, the breakage study was positive in a patient with fifth decade of her age. The 1-year OS was found to be independent of the etiology ($P = 0.19$) (Table 3). Only 22.2% (6/27) patients of constitutional disease had phenotypic resemblance to FA.

Severity

A total of 229 cases were evaluated for the severity of the disease according to Camitta's criteria. (12) They were classified as moderate aplastic anemia (MAA, $N = 63$), SAA ($N = 160$), and very severe aplastic anemia (VSAA, $N = 6$). Among 27 cases of CAA, we could assess severity in 23 patients only. Of these 23 cases, majority of the patients were in severe category (14/23, 60.9%) while 39.1% (9/23) of CAA cases were of moderate severe category. Amongst 260 cases of AAA, we could assess severity in 206 patients only. Of these 206 cases, majority of the patients were in severe category (146/206, 70.9%) while 26.2% (54/206) of AAA cases were of moderate severe category. There were only six cases of very severe category and all of them were in AAA. When compared with the age groups, of 168 cases of <21 years of age, the proportion of SAA in acquired and CAA groups was 72.67% and 55.56%, whereas with ≥21 years age group it was 66.07% and 80%, respectively (Table 2). The major chunk of MAA of <21 years of age group was 88.9% in CAA category while in AAA category it was 68.5% only.

The survival prospects were much poorer for SAA as compared to MAA (Fig. 2). The observed survival rates for MAA at 1, 2, and 3 years were 95.8%, 79.2%, and 62.5%, while for SAA 77.4%, 33.8%, and 22.5%, respectively (Table 3). The differences in survival rate of MAA and SAA patients at 1, 2, and 3 years were 2.01 ($P = 0.035$), 3.75 ($P = 0.001$), and 3.53 ($P = 0.001$), respectively (Fig. 2).

Fig. 2. Survival of MAA and SAA patients.

DISCUSSION

We hereby report a prospective research targeted to extensively study the survival assessment of patients affected with AA. The objective was to find out the association of various epidemiological and etiological factors viz. age, sex, severity, inherited etiology, infections, etc. with the OS of AA patients. The strength of our study lies in relatively larger size of the study population, which has allowed us to calculate precise up-to-date estimates of the etiology, and stratify these by age, gender, and severity. Furthermore, the extensive follow-up data contained in this dataset had enabled us to accurately calculate the survival rates. AA is a disorder with a highly variable natural history and etiology. While some patients with AA have very low proliferation rates, with pancytopenia, other may be asymptomatic and live for years with little or no treatment (14). In present study, the OS is different from the previously published reports (15).

We observed higher number of males than females. This is in agreement with the results of several population-based studies (15, 16). The skewed sex ratio in the present study, might have affected by the male dominant society of India. We did not find any marked differences in the severity of AA when the gender of the patient was considered. In contrast to analysis done on 174 patients of AA at Manchester Royal infirmary, Manchester (17) in present study, we could not find any significant difference in the OS of the patients when gender was taken into account.

It is well reported that AA is mainly a disease of young adults (15). The present data of age distribution at diagnosis of AA are consistent with the previous findings (18). In our study, higher frequency of disease was observed for the patient with <21 years of age. The findings of present study are in agreement with one of the recent publications from the Indian subcontinent but the number of patients studied was remarkably less (18). The age of the patient at the time of diagnosis was found to be inversely associated

with the OS, which is similar to the findings of previous studies (10, 19). Although, the constitutional etiology is more common in younger patients, yet the positive break-age study in a patient at fifth decade of life suggests, the symptoms may arise at the older age also. Similar findings have been reported by other authors also (10, 16, 18).

The severity analysis showed that more than two-thirds of cases were of SAA, which is similar to other studies (10). The higher frequency of SAA cases, in the present study might be a reason for steep decline observed in OS of the AA. The observed survival rates for MAA and SAA at 1, 2, and 3 years supports the earlier reports of superior survival rates of MAA over SAA (20, 21). In present study, 70% of the patients presented as SAA at the time of diagnosis. Inadequate supportive care and the late presentation to the center in the course of the disease could be the major contributory factor for poorer survival of patient affected with SAA. Current survival rates are in conflict with some previous studies that have reported 70–90% 5-year survivals (22) and 51% at 15 years. However, this data were from the patient treated with the immunosuppression or transplantation (10, 23). The lack of tertiary care center in the country, the time lag between diagnosis and treatment, unaffordability to hematopoietic stem cell transplant, and nonavailability of bone marrow/stem cell transplant donor might be the major contributory factor to the very short survival recorded in the present study.

Based on various previous reports, some viral infections, notably infectious agents such as hepatitis viruses and Epstein–Barr virus (mononucleosis) have been associated with AA (5). Several authors believe it is difficult to find a significant association between infectious disease and AA because study subjects are usually exposed to multiple drugs and multiple factors (24, 25). In the present study, patient were documented for history of infection as prediagnostic event as elicited in the administered questionnaire. An intriguing association between AA and hepatitis was found in the present study. Higher frequency of Hepatitis in the patient affected with AAA suggests role of Hepatitis as prediagnostic event in development of AA. Whether the hepatitis associated with AA is autoimmune or results from an undiscovered virus or toxin remains unclear.

The presence of significantly higher number of chromosomal breaks in patients with CAA differentiates it from AAA. In the present study, irrespective of age groups, constitutional disease such as FA was seen in 9.4% of all the patients. Similar to our result, the proportion of CAA has been reported from the various parts of country that ranges from 11.3% to 13.8% (18, 26). On contrary to our result, relatively higher proportion of CAA has been reported from one of the tertiary health care center in India (27), which could probably be because of the chance where most of the reported patients were

having phenotypic resemblance to CAA, namely FA. The possible hypothesis for the significantly higher number of patient with AAA in the present study, is that in last few years, there could be a steep increase in the idiopathic AAA due to various environmental factors that might have altered the ratio of inherited to acquired disease.

One potential weakness of this study is the issue of diagnostic validity in the dataset. In other words, do people recorded as having AAA really have this condition? As the CBS was the only test used for differential diagnosis in the present study, the results of higher number of AAA cases might have affected by the limitations of this test. Interpretation of the results of the CBS may be complicated by somatic mosaicism that has been attributed to gene conversion events, back mutations, or compensatory deletions/insertions leading to selective advantage of the gene-corrected lymphocytes. Lymphocyte mosaicism can develop in individuals initially found to be sensitive to MMC. These individuals may have a falsely normal MMC test. FA heterozygote cannot be detected by the MMC test as due to haploinsufficiency their results fall within the normal range (22). Thus, the patient categorized as AAA could be in the category of CAA. In this scenario, the patients should further be evaluated by molecular tests like gene expression and/or mutation analysis (28). However, we could not confirm the findings of CBS by the molecular study, which is a potential limitation of this study. Apart from this, while analyzing the data, we could not adjust the OR or *P* value for any confounding factors.

All three factors, viz. the presence of phenotype resemblance to constitutional disease in only 22.22% of the CBS positive patient, the similar ratio of the constitutional and acquired disease in both the age groups and the clinical presentation in fifth decade of age strongly suggests that, irrespective of their age and phenotype, all the patients with AA should be tested for CBS. The young age and less severe disease are strongly associated with the better survival. Hepatitis infection may lead to AA. The present study would help to understand the increased cases and risk factor of AA in the Indian population. The findings also suggest that there is evidence of improved survival in AA globally, but disparate situation in India. The lack of tertiary care facility in the country and unaffordability to abide the treatment cost could be the major contributory factors for this poor survival. There is clearly need of policy that ensures the availability of proven therapeutic opportunity for patients such as bone marrow/stem cell registry for transplantation and cost-effective drug to improve on the situation. The findings also suggest further studies to identify the involved genetic risk factor/s, if any, which can subsequently be used to design molecular drug/s for the disease. These studies are underway in our laboratory. To the best of our knowledge, the present study could be one of the largest and first reports of its

kind from India whereby an attempt has been made to evaluate the etiology and survival of AA.

ACKNOWLEDGMENTS

We would like to thank Dr. Padam Singh (Chief statistical advisor, Medanta, The Medicity) and Ms. Bhavna Alapati (Biostatistician) for their assistance. We would also extend our gratitude to Dr. Pooja Sharma and Dr. Aseem Kumar Tiwari for their valuable suggestions on parts of the article and analytical help in experimental planning. The authors are grateful to Ms. Rajani Chauhan for technical assistance.

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