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Original Article

Anti-globulin test positivity indicates advanced disease in Indian CLL patients

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Abstract: Autoimmune Hemolytic Anemia (AIHA) occurs in 10% to 25% of Chronic Lymphocytic Leukemia (CLL) while Direct Antiglobulin Test (DAT) positivity seen in 35% of cases. The prevalence and prognostic significance of DAT positivity is not well documented especially in Indian population. The present study was undertaken to know prevalence and prognostic significance of DAT positivity in CLL in India by associating it with stage and CD 38 expression. The study included fifty-eight newly diagnosed and untreated cases of CLL staged according to Binet and Rai system. Complete hemogram, DAT and immuno-phenotyping by flow cytometry was done to diagnose CLL and to assess CD 38 expression. Student's t test and Chi square test was used to calculate difference between means. *p* value ≤0.05 was considered significant. Results-DAT positivity was found in 27.58% cases. A positive association was seen between DAT and advanced Rai and Binet stage (P = 0.024 and P = 0.014 respectively). A positive association was also seen between DAT and CD 38 (P = 0.008). The study concluded that DAT positivity in Indian CLL patients is high as compared to West. As DAT correlated with advanced Rai/Binet stage, as well as CD 38 positivity, it can be considered as a surrogate marker for advanced disease and used to select patients needing close follow up especially at places where molecular and flow cytometric set up for prognostication is not available.

Keywords: Chronic lymphocytic leukemia, direct antiglobulin test, autoimmune haemolytic anemia, prognosis, CD 38, stage

Introduction

Chronic lymphocytic leukemia (CLL) is associated with immune dysregulation and autoimmune phenomena. Of these, autoimmune hemolytic anemia (AIHA) is the most common, occurring in 10% to 25% patients of CLL during the course of their disease [1, 2]. The pathogenic antibodies responsible for approximately 90% of cases of AIHA are produced by non-malignant B cells producing polyclonal high-affinity immunoglobulin IgG directed against red blood cells [3, 4]. Patients with anemia due to immune mechanisms have a better outcome than those in whom these features are due to bone marrow infiltration by the disease [5, 6]. This highlights the importance of determining the origin of the anemia in CLL patients for both prognostic and therapeutic reasons.

The most frequently used method to identify AIHA is Direct Antiglobulin Test (DAT) which may be positive during the course of CLL in up to 35% of cases [1]. However, not all patients with a positive DAT develop AIHA and is currently no method to predict which patients with CLL will become DAT-positive and which DAT positive patients will develop AIHA [7, 8]. DAT being a simple, cheap and easily available investigation, it may be interesting to see if it can be further utilized to follow up or prognosticate these patients. Traditionally, CLL is prognosticated using Rai and Binet's staging systems. Though simple and useful, both the systems do not predict the subsequent evolution of the disease and fail to address its dynamic nature. Another useful marker for CLL prognosis is CD 38 expression but this requires a flow cytometric set up which may not be available everywhere [9, 10]. Some studies have predicted a negative outcome in patients with a positive DAT [1, 11]. In a study by Quinquenel et al on 378 CLL patients, DAT positivity was found to be associated with reduced overall survival in patients

with stage A [12]. Similarly, Dearden et al studied 783 patients with CLL and concluded that DAT-positivity at the time of diagnosis was associated with more advanced stage of disease and reduced overall survival [13].

The prevalence of a positive DAT in patients with CLL in Indian subcontinent is not well investigated and there is lack of data on its prognostic significance in these patients who may have different disease biology than their Western counterparts. Also, there is no study which has correlated a positive DAT with CD 38, a well-known marker used in current times for predicting adverse prognosis. So this study was undertaken to estimate the prevalence of positive DAT at diagnosis in Indian patients with CLL and to study the prognostic significance of DAT positivity by comparing it with stage of the disease and CD 38 expression on malignant cells.

Materials and methods

Case selection

The study included fifty-eight study subjects. Inclusion criteria was newly diagnosed cases of CLL which fulfilled requirements according to the International CLL workshop guidelines [14]. Patients on prior chemotherapy, steroid medications, recent transfusion, known auto immune diseases and those showing negative CD 23 and/or CD 5 expression were excluded.

Informed consent was taken from all patients and ethical approval for the study was taken from the Institutional Ethics Committee for Human Research.

Sampling and investigations

3 mL peripheral venous blood sample was collected in EDTA vial under complete aseptic conditions and used for *Hemogram* (Hemoglobin (Hb), Total Leucocyte Count (TLC), Platelet Count (PLT) by Beckman Coulter LH 500); *Direct Antiglobulin Test* (Column agglutination technique using polyvalent sera by Bio-Rad laboratories Pvt Ltd); and *Immunophenotyping (IPT)* by flow cytometry.

IPT

Done from lysed whole peripheral blood (PB) (lyse and wash protocol) using dual laser 5 color flow cytometer (Beckman coulter cytomics FC 500). Analysis was done using FCS express software version 3.0.

Four tube antibody panels were employed tagged to flourochromes FITC, PE, ECD, PE-Cy5 and PE-Cy7 respectively: tube 1-Kappa/lambda/CD 38/CD 19/CD 45; tube 2-CD 5/CD 23/CD 19/CD 45/CD 20; tube 3-FMC 7/CD 10/CD 19/CD 45; and tube 4-CD 45/CD 4/CD 8/CD 3. Matute's score of > 3 was used for diagnosis [15, 16]. A cutoff value of 30% was used to define expression of CD 38 (**Figure 1**) [17, 18].

Statistical analysis

It was performed using MS EXCEL and SPSS software version 20. Differences between groups were evaluated using Student's *T*-test for quantitative and Chi-square test/Fishers exact *T*-test for qualitative variables. *P* value less than 0.05 was considered significant.

Results

Clinical profile

Fifty-eight diagnosed cases of CLL were evaluated in this study. The mean age was 65.4±8.3 years. There were 42 males and 16 female patients. Lymphadenopathy was present in 16 patients while splenomegaly in 10 patients. Out of 58 patients, 20 (34.48%) were in Rai stage 0, 10 (17.24%) were in Rai stage I/II and 28 (48.27%) were in Rai stage III/IV. Also, 30 (51.72%) patients were in Binet stage A and 28 (48.27%) patients were in Binet stage C.

Hematological profile

The mean Hb was 8.8±2.3 g/dL. The mean TLC was 78±104 × 10^9/L and Absolute Lymphocyte Count (ALC) was 68.9±95 × 10^9/L. The mean PLT was 111.2±63.3 × 10^9/L.

DAT results

DAT positivity was found in 16/58 patients (27.58%). **Table 1** shows the comparative analysis of DAT positive and negative CLL patients. A positive association was seen between DAT and advanced clinical Rai and Binet stage as 14/16 (87.5%) positive DAT cases were seen in Stage III/IV Rai and Binet C (P = 0.024 and P = 0.014 respectively). A positive correlation was also seen between DAT (10/16, 62.5%) and CD 38 expression (P = 0.008). Thus in this study

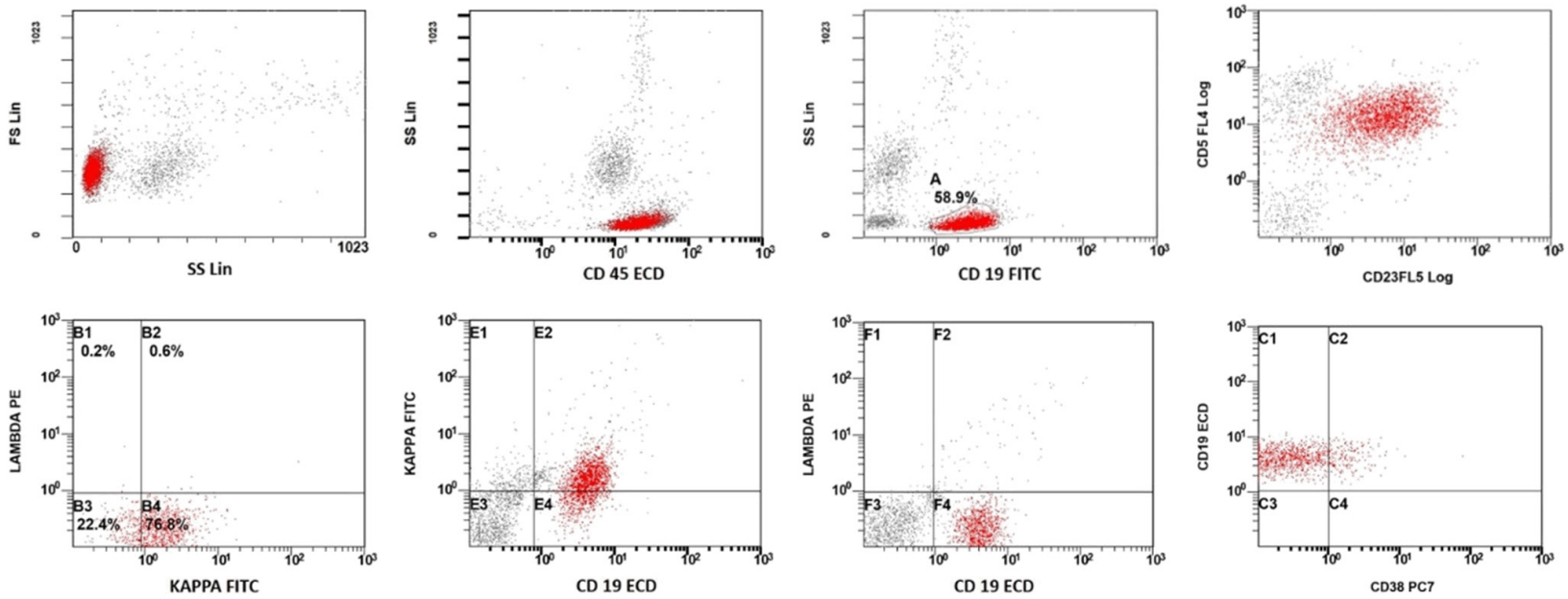

Figure 1. A case of CLL showing kappa light chain restriction in CD 19 positive cells along with heterogeneous expression of CD 38.

Table 1. Comparative analysis of DAT positive and negative CLL patients (n = 58)

Variables	DAT negative	DAT positive	*p*-value
No. of cases	n = 42 (Total = 58)	n = 16 (Total = 58)	
Percentage out of total cases	72.4%	27.5%	
Age > 65 years	24 (57.1%)	12 (75%)	0.671
Sex: M/F	32/10	10/6	0.646
Hb < 11 g/dL	30 (71.4%)	16 (100%)	0.148
TLC > 50 × 10^9/L	16 (38.1%)	8 (50%)	0.683
ALC > 30 × 10^9/L	24 (57.1%)	10 (62.5%)	1.000
PLT < 100 × 10^9/L	14 (33.3%)	10 (62.5%)	0.218
Lymphadenopathy	14 (33.3%)	2 (12.5%)	0.381
Splenomegaly	8 (19.0%)	2 (12.5%)	1.000
CD 38	4 (9.5%)	10 (62.5%)	0.008*
Rai 0	20 (47.6%)	0	0.024*
I/II	8 (19.0%)	2 (12.5%)	
III/IV	14 (33.3%)	14 (87.5%)	
Binet A	28 (66.6%)	2 (12.5%)	0.014*
C	14 (33.3%)	14 (87.5%)	

Hemoglobin (Hb), Total Leucocyte Count (TLC), Platelet Count (PLT), Absolute Lymphocyte Count (ALC), Male (M), Female (F), Cluster of differentiation (CD). **P* value less than 0.05 was significant.

Table 2. Comparison of DAT positivity in CLL in literature [1, 3, 8, 11-13, 19-22]

Serial number	Ethnicity	Number of CLL patients studied	DAT positivity in CLL patients (n, %)
1. Hamblin TJ (1986)	England	195	15, 7.7%
2. Lischner M (1988)	Israel	79	6, 7%
3. Mauro FR (2000)	Rome	1203	52, 4.3%
4. Kyasa M (2003)	United states	132	6, 4.5%
5. Dearden C (2008)	United kingdom	637	89, 14%
6. Xu W (2009)	Chinese	123	34, 27.6%
7. Moreno C (2010)	Spain	960	43, 4.5%
8. Ricci F (2013)	Italy	146	20, 14%
9. Abbas SA (2015)	Pakistan	60	14, 23.3%
10. Quinquenel A (2015)	France	376	56, 14.8%

CLL: Chronic lymphocytic leukemia, DAT: Direct antiglobin test.

we observed that those patients who had a positive DAT at diagnosis were mostly in advanced stage of the disease. Also the malignant cells in more than half of DAT positive patients expressed CD 38, a marker associated with poor prognosis and shortened survival. No correlation of a positive DAT however was found with advanced age, gender, low Hb, high TLC count, high ALC, low PLT and organomegaly.

Discussion

DAT positivity was seen in 16/58 (27.58%) patients in this study. This is higher than reported by most western studies, but comparable to Asian countries [1, 19]. The reported frequency of positive DAT in CLL at diagnosis ranges from as low as 4.3% in western literature to as high as 23.3% in Pakistani and 27.6% in Chinese population [1, 11, 19]. These differences have been attributed to more immune disturbances in Asian population by some authors [11]. In addition, advanced disease at presentation may be an additional factor. **Table 2** shows the comparison of DAT positivity in CLL patients in literature [1, 3, 8, 11-13, 19-23].

In the present study, 14/16 (87.5%) DAT positive cases were seen in Stage III/IV Rai and

Binet C with a positive correlation with advanced Rai and Binet stage (P = 0.024 and P = 0.014 respectively) (**Table 1**). Abbas et al studied 60 patients of CLL and found 10/14 DAT positive cases to be in advanced Rai stage III (P = 0.005) [11]. In a multivariate analysis by Dearden et al, stage of disease was found to be an independent predictor of DAT result, with Binet stage C patients more likely to be DAT positive than stage A progressive or B patients (P = 0.004) [13]. Similar findings have been documented by Xu et al who found 42% of DAT positive patients in Binet stage C (P≤0.001) [1]. However, Ricci et al found no such association as majority (70%) of their DAT positive patients presented in Binet stage A [3]. Our findings are in accordance with majority of the previous studies.

In our study, a positive correlation was found between positive DAT (10/16, 62.5%) and CD 38 positive CLL cases (P = 0.008) (**Table 1**). Though an association between autoimmune cytopenia and high expression of CD 38 has been reported, however on extensive literature search we could not find any study correlating positive DAT with CD 38 expression [23]. Such an association further addresses the fact that DAT positivity at diagnosis can also be a predictor of adverse outcome in CLL patients. Our findings can be explained by monoclonal autoantibody production by malignant CLL cells and the ability of malignant B-CLL cells to present purified Rh protein to autoreactive T-helper cells, driving an autoimmune response against erythrocytes, provides the biological mechanism of DAT positivity [3, 24]. These malignant cells may be expressing high levels of CD 38 providing a link between CD 38 and DAT positivity in our setting.

In the present study, no correlation was found between DAT positivity and other clinical and biological prognostic markers i.e. advanced age, gender, low Hb, high TLC count, high ALC, low PLT and organomegaly (**Table 1**). Our findings are similar to most of the studies in relation to advanced age and gender [1, 3, 13]. In one of the study, a positive correlation has been reported between low Hb, low PLT and autoimmune cytopenias but we did not find any study correlating positive DAT with low Hb, high TLC count, high ALC, low PLT and organomegaly [22].

The limitation of the study was its small sample size. These preliminary findings need to be validated in a larger set of patients. However, based on these results, we recommend that patients with a positive DAT at presentation need to be followed up closely. This is especially relevant for those centers where flow cytometric prognostication may not be available.

Conclusion

To conclude, DAT positivity in CLL in our patients appears relatively higher than West. Also, a positive DAT may be a surrogate marker for advanced clinical disease. Being an inexpensive and simple investigation, DAT may be used for identifying patients needing a closer follow up and aggressive treatment. This is the first study in literature to correlate DAT positivity with CD 38 expression. The study's limitation was its small sample size.

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The manuscript has been read and approved by all the authors, the requirements for authorship as stated earlier in this document have been met, and each author believes that the manuscript represents honest work.

Disclosure of conflict of interest

None.

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