Clinical Significance of Positive Platelet Immunofluorescence Assay in Adult Immune Thrombocytopenia

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Abstract Immune thrombocytopenia (ITP) is a relatively common hematologic disorder manifested by low platelet count due to immune-mediated platelet destruction and/or suppression of platelet production. This study aim was to evaluate characteristics and clinical presentation of adult patients with ITP and exploring the clinical value of platelet antibodies assay in proposed cases in Iranian population. In this prospective case series 46 adult patients with ITP and platelet count of $<100 \times 10^9/L$, referred to the Taleghani Medical Center, Tehran, Iran between 2007 and 2009 were evaluated. There were 26 females and 20 males (1.3:1) with mean age of 38.9 ± 19.7 years. The platelet autoantibodies were measured by means of indirect platelet suspension immunofluorescence test. According to our results, 7 patients (15.2 %) displayed a positive platelet antibody assay. There was a significant negative correlation between platelet count and antibody level ($r = -0.59$; $p < 0.001$). Additionally, a positive correlation between platelet count and patients' age ($r = 0.302$; $p = 0.042$) was detected. 20 patients (56.5 %) were symptomatic at presentation and the most common bleeding signs were petechia, purpura and epistaxis. Results indicated no significant correlation between increased platelet antibody level and bleeding manifestations except for hematuria ($r = 0.435$; $p = 0.02$) and epistaxis ($r = 0.382$; $p = 0.015$). Disclosure of platelet autoantibodies and the consequential thrombocytopenia associated to some extent

but not completely with the propensity to bleed which necessitates more factors to be evaluated.

Keywords Immune thrombocytopenia · Platelet autoantibodies · Platelet immunofluorescence test

Introduction

Immune thrombocytopenia (ITP) is a relatively common hematologic disorder manifested by low platelet count due to immune-mediated platelet destruction and/or suppression of platelet production. The abbreviation ITP which originally stood for idiopathic thrombocytopenic purpura is now used for ITP. A large proportion of ITP cases are categorized as primary since they occur in the absence of any clinically apparent alternative etiologies of low platelet count [1, 2]. According to recent studies, the estimated annual incidence is 100 new cases per 1 million persons, with adults accounting for about half of that [3].

The clinical features of ITP in adults are quite distinct from those seen in childhood. Adult ITP predominantly affects women between the ages of 30 and 60 years while there is a gender balance with increasing age [4]. Although ITP in adult patients often has an insidious onset with no prodromal illness; it typically tends to follow a chronic course. Moreover, the clinical status of ITP is heterogeneous. Signs and symptoms range from common asymptomatic patterns or mild mucocutaneous bleeding through to serious hemorrhage [5].

According to the American Society of Hematology (ASH) guidelines, the diagnosis of ITP remains one of exclusion and principally is based on medical history, physical examination and ancillary laboratory data such as complete blood count and peripheral blood smear. This

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guideline does not recommend bone marrow examination in patients with the typical features of ITP, irrespective of the age of the patient [6]. Further diagnostic studies are generally not recommended as the routine work-up in patients suspected of ITP [6]. Considering the fact that a diagnosis of exclusion bears inherent difficulties, [5, 7] detection of antibodies directed against platelet epitopes may have a higher degree of diagnostic value. However, the importance of platelet antibodies assay in the clinical course of ITP has not been clearly established [8].

Up to now various methods have been applied for detection of anti platelet antibody in ITP patients. ELISA, flow cytometry, monoclonal antibody immobilization of platelet antigen (MAIPA), enzyme-linked immunospot assay, lymphocytotoxicity test (LCT) and solid phase red cell adherence assay (SPRCA) are some of these applied methods for detection of anti platelet antibody [9–15]. It has been shown that flow cytometry assay using donor platelets as target cells can detect autoantibodies in 70 % of patients with ITP and it is more sensitive than SPRCA assay (50–65 % sensitivity) for antibody detection in these patients [9]. However, SPRCA method with 100 % specificity has high specificity for anti platelet antibody detection [9]. MAIPA is another specific method for detection of platelet antibody that can differentiate immune from non-ITP [10]. Flow cytometry assay is faster and more sensitive than modified MAIPA [14].

This study was conducted to elucidate the clinical significance of platelet autoantibodies in adult patients with ITP. This study aimed to determine demographic characteristics and presenting manifestations of Iranian patients with ITP as well as to explore the laboratory assays which might be relevant to diagnosis and management of ITP.

Methods and Materials

Study Design

This prospective case series characterized newly presenting adults (aged 14 years or above) with ITP attending the ...University Hospital in ..., ..., between 2007 and 2009. ITP was defined as the presence of isolated thrombocytopenia with platelet counts of equal or less than $100 \times 10^9/L$, normal or increased percentage of bone marrow megakaryocytes (if available), absence of splenomegaly and exclusion of other potential causes of thrombocytopenia [6].

A total of 46 adult patients who fulfilled the proposed criteria, were included in the study. The patients' charts were evaluated considering a thorough medical history and physical examination in addition to the other parameters such as patients' age, gender and the bleeding signs and symptoms at presentation. In order to exclude the

secondary etiologies contributed to thrombocytopenia and/or increased risk of bleeding, we analyzed the laboratory data involving complete blood counts (both differential and reticulocyte count), liver and renal function tests, thyroid profile, anti-nuclear antibody, HIV test and bone marrow findings if available. Also, patients were excluded if they were under pharmacotherapy.

The study protocol was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences and patients signed informed consent before enrollment.

Methods

We measured the level of platelet autoantibodies in the sera of patients (Phase II assays) as well as the platelet count. Specimens were collected at onset of diagnosis and prior to initiation of therapy.

Circulating platelet autoantibody was detected by indirect- platelet-suspension- immunofluorescence-test (IP-SIFT) based on Rose et al. [13] method. This method (second generation) was the only available test in Iran at the time of our study. Fluorescein- labeled anti- immunoglobulin's (DAKO, Denmark) including anti-IgG, anti-IgM and anti-IgA, were used as secondary antibodies. Subsequently, immunofluorescence data were interpreted by a Leica fluorescence microscope. In this study we analyzed the results based on the semi quantitative estimation and sera with the platelet antibody levels above the cut-off point of 1:16 were considered as antibody-positive.

Statistical Analysis

Statistical analysis of data was performed using SPSS software version 15.00 for Windows. For all continuous variables, mean and standard deviations (SD) were calculated. Independent sample *T* test and χ^2 test were applied to compare continuous (with normal distribution) and categorical variables between the two groups, respectively. The Pearson's correlation test was used to explore the relationship between continuous variables such as platelet counts and platelet antibody levels. Also, Spearman's correlation test was applied to find the correlation between continuous and categorical variables such as platelet counts and bleeding signs. *P* value of less than 0.05 was considered as significance level.

Results

Patients Characteristics

A total of 46 patients with ITP were studied, among which 26 (56.5 %) were female resulting in a female to male ratio

Table 1 Characteristics of antibody-positive and antibody-negative ITP patients

Variable	Ab-positive group (n = 7)	Ab-negative group (n = 39)	p-value
Mean age, (years)(Mean $\pm$ SD)	40.3 $\pm$ 25*	38.62 $\pm$ 19	0.839, T-test
Gender, (Males/Females)	3/4	17/22	0.65, χ^2 test
Platelet count ($\times 10^9/L$) (Mean $\pm$ SD)	10.42 $\pm$ 11.35	48.74 $\pm$ 28.6	0.001, T-test
Platelet antibody titer (Mean $\pm$ SD)	1/4.17 $\pm$ 8.042	–	<0.001, T-test

* $P < 0.05$ **Fig. 1** Correlation between antibody titer and platelet count in ITP patients. (Spearman $r = -0.59$; $p < 0.001$)

of 1.3:1. The mean age of the patients was 38.9 ± 19.7 years ranging from 14 to 86 years.

The clinical signs were variable including, petechiae, purpura (41 %), epistaxis (41 %), hematuria (11 %), GI bleeding (9 %) and bleeding from gums (26 %) and conjunctiva (7 %). There was no record of intra cranial hemorrhage or hemoptysis.

Findings

Peripheral platelet counts ranged from less than $1 \times 10^9/L$ up to $100 \times 10^9/L$ with the mean of $42.91 \pm 30.03 \times 10^9/L$.

The platelet antibody was not demonstrable in 33 (71.7 %) patients, while the antibody titer of 1:8 detected in 6 (13 %) and the titer of 1:16 and 1:32 reported in 5 (10.9 %) and 2 (4.3 %) patients, respectively. Considering the antibody level of 1:16 as the cut-off point, 7 (15.2 %)

Table 2 Distribution of clinical bleeding signs in antibody-positive and antibody-negative ITP patients

Bleeding signs	Ab-positive patients (n = 7)	Ab-negative patients (n = 39)	p-value
Overall bleeding diathesis, n (%)	6 (85.7)	20 (51.3)	0.091, χ^2 test
GI bleeding, n (%)	2 (28.6)	2 (5.1)	0.104, χ^2 test
Epistaxis, n (%)	6 (85.7)	13 (33.3)	0.015, χ^2 test
Gingival bleeding, n (%)	4 (57.1)	8 (20.5)	0.65, χ^2 test
Hematuria, n (%)	3 (42.9)	2 (5.1)	0.02, χ^2 test
Petechiae, purpura, n (%)	5 (71.4)	14 (35.9)	0.091, χ^2 test
Conjunctival bleeding, n (%)	2 (28.6)	1 (2.6)	0.056, χ^2 test

of the patients showed a positive platelet antibody while, 39 (84.8 %) patients had a negative assay.

The main characteristics of antibody-positive and antibody-negative ITP patients are illustrated in Table 1.

There was a statistically significant negative correlation between platelet count and antibody titer in ITP patients ($r = -0.59$; $p < 0.001$) (Fig. 1).

Evaluation of clinical signs revealed that 26 (56.5 %) patients were symptomatic at presentation. There was no significant correlation between antibody levels and the overall bleeding tendency. However, considering each sign separately, epistaxis and hematuria correlated with the platelet antibody titers ($r = 0.382$; $p = 0.015$) and ($r = 0.435$; $p = 0.02$), respectively.

The clinical bleeding signs in ITP patients according to the results of platelet antibody analysis have been outlined in Table 2.

No significant correlation was detected between the platelet antibodies and patients' gender ($p = 0.65$).

Clinical Outcome and Follow Up

For each patient, the follow-up period started right after the initial diagnosis and they were followed for about 12 months. Among 39 patients with negative serum platelet antibody, 13 patients missed the follow up and 17 patients never require therapy. Of the nine patients who treated with corticosteroids, five cases responded to therapy, while the others failed the treatment and three of them underwent splenectomy.

Out of the seven patients with positive platelet antibody assay, three patients missed the follow up. Among the others, three patients treated with corticosteroids, while two cases

had complete response and splenectomy was performed for the nonresponder patient. Also, none of the patients underwent bone marrow examination for ITP diagnosis.

Discussion

The pathogenic effect of platelet auto antibodies in ITP has been clearly established. Furthermore, a positive antibody assay provides strong evidence for the presence of ITP. This study determined demographic characteristics and presenting manifestations of Iranian patients with ITP.

The platelet antibodies involved in ITP most often direct toward certain platelet membrane glycoproteins, either the GP IIb/IIIa or GP Ib/IX complexes. Nevertheless, some patients shows autoantibodies against multiple platelet antigenic targets [16].

Numerous methods have been devised for detection of platelet antibodies since Harrington et al. [17] and Shulman et al. [18] demonstrated the autoimmune pathophysiology of ITP [9–15]. The current techniques are based on detection of immunoglobulins on platelets, either by direct assays on patients' platelets or through an indirect test on normal platelets after exposure to patients' sera.

The sensitivity and specificity of these techniques in detection of anti platelet antibody is different. Flow cytometry assay with 70 % sensitivity and SPRCA method with 100 % specificity have been determined as the most sensitive and specific techniques, respectively for antibody detection in ITP patients [9, 10, 14].

The LCT sensitivity and specificity were reported 94.73 and 100 %, respectively in a study in Thai patients [15]. That study showed lower sensitivity and specificity of LCT and SPRCA in detecting platelet antibodies than that of flow cytometry [15].

In addition to the routine and currently in use methods, recently a novel assay based on peptide aptamer technology has been developed for the detection of anti-platelet antibodies. This assay sensitivity is about 90 % and gives better results than the MAIPA method for detecting antibody in Neonatal alloimmune thrombocytopenia [19].

In the current study, autoantibody levels were measured by IPSIFT in 46 adult patients with ITP. Some literature in the 1970s have reported a sensitivity of about 90 % for direct platelet-associated IgG assays in ITP patients. Whereas, recent studies using antigen-specific assays, demonstrated platelet antibodies in around 50–60 % of the ITP cases [2]. In our study, positive platelet antibody was detected in 7 patients (15.2 %) which is statistically close to the findings of similar studies carried out by Phase II platelet antibody assays [20].

Possible factors influencing the detection rate include limited sensitivity of the indirect antibody assay, along with technical problems associated with the test; presence

of alternative platelet destruction mechanisms which are not attributed to autoantibody such as direct T-cell cytotoxicity [21] and complement mediated lyses [22]. Furthermore, some patients suspected of having ITP satisfying ASH diagnostic guidelines may not have primary ITP. Hence, there is need for additional evaluations and modifications to develop diagnostic criteria that can be used as a routine for confirm diagnosis of ITP [23, 24].

The ITP phenotype is heterogeneous varying from mild bleeding through frank hemorrhage. We observed that the majority of studied patients (56.5 %) were symptomatic at presentation and the most common bleeding signs were petechia, purpura and epistaxis (Table 2). There was no significant correlation between antibody titer and the overall bleeding risk ($p = 0.091$). However, a positive correlation was demonstrated between increased platelet antibody level and the clinical signs including epistaxis ($r = 0.382$; $p = 0.015$) and hematuria ($r = 0.435$; $p = 0.02$), compared to the patients with negative antibody assay. These findings suggest that platelet autoantibodies initiate antigen shedding and inhibit specific glycoprotein functions, leading to bleeding tendency in some ITP patients [25].

According to our findings, there was a statistically significant negative correlation between platelet count and antibody titer in ITP patients ($r = -0.59$; $p < 0.001$), (Fig. 1). This is in agreement with previously reported data [26].

A possible explanation might be the fact that antibody-coated platelets are destroyed prematurely by reticuloendothelial system. The resulting shortened circulatory life span of platelets along with the immune mediated ineffective thrombopoiesis, contributes to decreased platelet count in ITP patients [2]. Likewise, some studies addressed elevated platelet counts following immunosuppressive therapy in most ITP patients [27].

Comparing with the previous published data there was a considerable decrease in the number of ITP patients with positive assay in our study which may be explained, at least in part, by the relatively low sensitivity of the implicated assay [9].

The severity of thrombocytopenia associated to some extent but not completely with the propensity to bleed which necessitates more factors to be evaluated [28, 29]. On the other hand, increased platelet antibodies level is only one of several factors involved in development of the clinical status of ITP. Further evaluations will be needed to modify the current clinical guidelines for diagnosis of ITP as well as to improve the diagnostic modalities in order to specifically target the pathogenic mechanisms of ITP.

Conclusion

In conclusion, it has been suggested that detection of platelet antibody might help to predict the course of ITP. In

fact, patients with positive antibody assay have been reported to have more bleeding symptoms and lower platelet counts. In our series, the increased antibody level was correlated with thrombocytopenia. However, no significant difference was observed in bleeding manifestations between the two groups of ITP patients except for hematuria and epistaxis.

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