

Detection of Antiplatelets Antibody Specially GPIIb/IIIa using MIAPA Method in Baghdad Patients with ITP

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Abstract: Immune thrombocytopenic purpura (ITP), is an autoimmune disease that caused by sensitization of platelets by auto-antibodies leading to increase platelet destruction mainly by spleen macrophages. **Methods:** This study has determined and characterized the antiplatelet glycoproteins in Adult patients with ITP. Fifty patients, who were hospitalized with clinical signs of ITP in national center of hematology during 6 months, went under our clinical studies in a research project. ELISA and MAIPA (Monoclonal Antibody Immobilization of Platelet Antigens) methods were used to determine serum anti-platelet antibodies level. **Results:** The anti-platelet antibodies level above mean + 3SD of control group was assumed as positive. The platelet counts ranged between $0 \times 10^9/L$ and $115 \times 10^9/L$. Among the patients 52% of them were anti-platelet antibodies positive with ELISA method.. Antibody against platelet GPIIb/IIIa using MAIPA method were 28%, **Conclusion:** MAIPA is the specific method for the detection of antibody against glycoprotein antigens, it has the advantage of differentiating between immune and non-immune thrombocytopenia.

Keywords: Anti-platelet antibody, gpIIb/IIIa, ELISA, MAIPA

1. Introduction

Immune thrombocytopenia purpura (ITP) is an autoimmune disease characterized by loss of self tolerance leading to the production of auto antibodies directed against platelet antigens⁽¹⁾

The autoantibodies produced against platelet glycoproteins are able to bind to platelet membranes, initiating pathways that result in dysfunction and destruction of platelets and clinical sings. These include petechiae, ecchymosis, and bleeding in some patients⁽²⁾. Around 85% of platelet autoantigens lie on the platelet GPIIb/IIIa, GPIb/IX or GP Ia/IIa complexes, and 15% of them are found on other membrane glycoproteins⁽³⁾

The pathophysiology is complex and the concepts surrounding the mechanisms of thrombocytopenia have shifted from the traditional view of increased platelet destruction mainly by the splenic macrophages and mediated by platelet autoantibodies to associated impaired platelet production and T cell-mediated effects. Platelet autoantibodies are involved both in peripheral destruction and impaired platelet production. They may impair megacaryocyte development, induce apoptosis, impede platelet release, or promote intramedullary phagocytosis^(4,5)

However, despite many studies on antiplatelet antibodies, characterization of binding and estimation of anti-platelet auto antibodies remain poor⁽⁶⁾ and many questions remain unanswered⁽¹⁾. The aim of our study is to determine and characterized the anti-platelet antibodies in adult patient with ITP with MIPA method and procedures.

2. Material and Method

This study was carried out at the National Center of Hematology Al Mustansiriyah University / Baghdad. Fifty patients attended to this center were diagnosed, registered and followed up by specialists. The study was performed during the period from October 2012- March 2013. All the patients were diagnosed as Chronic ITP on proper therapeutic Protocol of prednisolone and Immuran, with history of disease from few months to several years. Fifty apparently healthy subjects with normal platelet count, age and gender matched to the patients group were included in this study.

Five ml of blood sample was obtained from each patient by vein puncture, three ml was poured to non-heparinized plain tube and then centrifuged at 3000 RPM for 5 min to separate serum then labeled and store at $-80^{\circ}C$ until use.

Platelets Preparation

Blood from group O donor in to EDTA were taken. Centrifuged at 1000 rpm for 10 min in bench top centrifuge. Then Removed the top 3/4 of the Plasma rich platelets (PRP) from the top of tube and transfer to 10 ml conical centrifuge tube. PAS/EDTA(PH=7) buffer were added to 10 ml and centrifuged at 4000rpm for 5 min. Decanted supernatant and re-suspended in 2 ml buffer.

Anti-platelet antibodies detection

ELISA plate was coated with 100 μL of platelet lysate (200 $\mu g/ml$) and incubated at $4^{\circ}C$ overnight. The following day plate was centrifuged at 1500 rpm for 5 minutes. After washing, 100 μL of diluted patients and controls sera (1:10 diluted in PBS with 1% BSA) were added and the plate was

incubated for an hour at 37°C. The unbound anti-platelet antibodies were removed by washing four times with TBST. Hundred µL Goat anti human HRP-conjugated antibody (1: 30 000 diluted in PBS), the optional dilution was modified after experimented more than one dilution, was added to each well and incubated at RT for an hour. The plate was washed five times with washing buffer (TBST) then 100 µL of substrate [Tetramethylbenzidine (TMB) sigma] was added and the plate incubated for 30 min in a dark place. The reaction was stopped by adding 100 µL of 3 M HCL, and the OD was measured at 620 nm.

Monoclonal Antibody Immobilization of Platelet Antigens (MAIPA)

ELISA plate was coated with 100 µL of Goat anti mouse IgG [final concentration 5 ug/ml diluted in Carbonate Bicarbonate Buffer (CBB) pH 8.6 in each well and incubated overnight at 4°C. After washing with Tris Buffer Saline (TBS), the plate was blocked with 200 µL of 3% BSA in PBS and incubated for an hour at room temperature (RT). Hundred µL of washed O negative platelets suspension containing 100 x 10⁹ /L were added to the tubes which were blocked with 50 µL 3% BSA and incubated for an hour with 100 µL of patients and controls serum for an hour at 37°C. Forty µL of each monoclonal mouse anti platelet glycoproteins [IIb/IIIa, Ib/IX and IIa/Ia with final concentration of 20 µg/ml diluted in PBS were added to the individual tubes containing mixture of washed platelets and patients or controls sera and incubated at 37°C for 30 minutes. The unbound human anti platelet-glycoproteins were removed by washing three times with (TBST washing buffer). Hundred µL of lysis buffer were added to each tube and incubated for 30 minutes at 37°C. After centrifuging all tubes at 1500rpm for 30 minutes at 4°C, 100 µL of supernatant was added to each well which were coated with anti-mouse antibody and incubated at 4°C for 90 minutes. The plate was washed four times with washing buffer and 100 µL of Goat antihuman HRP conjugated antibody (1: 30 000 diluted in PBS) was added to each well and incubated at RT for an hour. The plate was washed five times with washing buffer. 100 µL of substrate (TMB) was then added and the plate incubated for 30 min in a dark place. The reaction was stopped by adding 100 µL of 3 M HCL, and the OD was measured at 650 nm.

Calculation of the result

The mean controls absorbance was used as normal value. We also used the mean controls with three standard deviation (mean+3SD) as the cut-off. The anti-platelet antibodies level of each sample above the cut off was assumed as antibody positive.

Statistical Analysis

All data were analyzed using the SPSS, version 22 software. Statistical analysis included descriptive statistics (frequency tables), T-Test, and Spearman correlation (r). P value which is . 0.05 was considered statistically significant.

3. Result

In this project the goal of our study is to determine anti-platelet GPs antibody in Adult with ITP. 50 patients and 50

healthy individuals were included in our study controls. 72% of patients were female and 28% Male and their age ranges between (17 – 68) years. The clinical signs were variable including 15 individuals only with petechiae, 11 of them not only with petechiae, but also with purpura and 8 of them with ecchymosis signs, and finally 4 of them were found out with severe bleeding from gums .patient were treated with prednislone and some of them with Immuran.

Table 1 shows more details about Patients and control characteristic.

Table 1: Patient and control characteristic

Parameter	Patients	control
Number	50	50
Age(mean)	40	40
Gender		
Male : No.(%)	14 (28)	15 (30)
Female: No.(%)	36 (72)	35 (70)
Platelets count x10 ⁹ /l (mean)	52	235
Antiplatelets antibody (mean)	0.43	0.17

Table 2 shows more details about platelet count in patients.

Table 2: The platelet count in patients

Platelets count x10 ⁹ /l	Number
< 10 x10 ⁹ /l	4
10 – 30 x10 ⁹ /l	9
31 – 90 x10 ⁹ /l	28
>90 x10 ⁹ /l	8

The determination of anti-platelet antibodies:

The results with ELISA indicated that anti-platelet antibodies in sera of all patients and controls, The mean controls absorbance was used as normal value. We also used the mean controls antibody level with three standard deviation (mean+3SD) as the cut-off. The anti-platelet antibodies level of each sample above the cut off was assumed as antibody positive ⁽²⁾.

Out of 50 patients, 52% had anti-platelet antibody positive with mean platelet count of 35x10⁹/L. using t- test , it was statistically shown that there was a significant differences between ITP patients and control group (p= 0.001) Table 3 shows the anti- platelet antibodies in ITP patients. Figure 1 shows anti platelet antibody in groups and when we use the sperman correlation between antiplatelets antibody showed there was significant correlation (r= - 0.66, p= < 0.001)

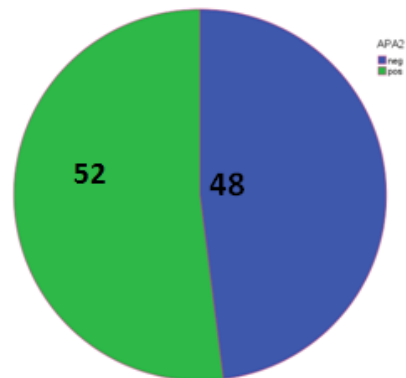


Figure 1: Shows the anti- platelet antibodies in groups .

Table 3: Compare between ITP patients & healthy control in relation to Total anti platelets antibody mean .

p-value	Control n=50	ITP Patient n=50		Parameter
<0.001**	0.17 ± 0.05	0.43 ± 0.28	Mean± SD	Total Anti platelet antibody
* (P<0.05), ** (P<0.01), NS: Non-significant.				

Anti gpIIb/IIIa IgG antibody

Using MAIPA method, antibodies in patients' sera were analyzed against three most important platelet glycoproteins (GPIIb/IIIa) result shown Out of 26 (52 %) ITP patients were positive total antiplatelet antibodies there were 8 (30.7%) positive Anti gpIIb /IIIa IgG antibody.

4. Discussion

ITP is an autoimmune disease characterized by platelet destruction, which can result in increased bleeding and haemorrhage^[6,7]. For further characterization function of auto antibodies against platelet glycoproteins in ITP patients, our study has indicated, first of all the determination of anti-platelet antibodies using ELIZA method, then the detection of anti gpIIb/IIIa antibodies by MAIPA.

During our study period, 50 adult patient with ITP clinical symptoms were studied. The result of our clinical studies indicated that major signs were petechia and purpura with minor signs of severe bleeding. The age variety was between 17 to 68 years. The platelet count showed that more than 26% of patients had platelet count < 30 × 10⁹/L, 74% had a persistent platelet count < 150 × 10⁹/L, but only 20% had a count < 20 × 10⁹/L.

Fifty normal voluntary adults were used as the normal controls and the main result of anti-platelet antibodies + 3SD (Mean + 3SD) was used as the cut off, and ITP patients who their anti-platelet antibodies were above the cut off line placed with patients with antibody positive. It was found that 52% out of 26 patients in our assay were anti-platelet antibodies positive with the main platelets count 35 × 10⁹/L which show a good correlation between decreasing circulated platelets and increasing antibodies, compare with anti-platelet negative patients. In some cases there were not relations between anti-platelet antibodies and platelet account. This could be due to the acute phase of disease which their platelets absorbs antibodies and removes these platelet sensitivities from circulation by phagocyte system. In the other hand, detection of auto antibodies on platelet surface due to platelet structure is not simple^[8]. However, using the platelet lysate as source of platelet antigens, 52% of patients were antibodies positive. It is suggested that in the platelet lysate perhaps, the conformational structure of platelet glycoproteins was changed, resulting in not expression of more platelet antigens [9]. We employed MAIPA technique to detect anti-platelet glycoproteins. The results showed that 28% of patients were anti-GPIIb/IIIa positive. The correlation between this antibody and antibodies against whole platelets was 0.13.

Hamidpour M. and college's reported that 48% out of 47, ITP patients were anti-GPIIb/IIIa positive. Finally, the pathogenesis and diagnoses of ITP has proven complex and concurs with several issues such as: genetic predisposition, underlying autoimmune repertoire, inhibition of platelet production, perturbations of cell mediated effector and effector pathways, sequestered harbors within lymphoid organs, and responsiveness to intervention^[10].

5. Conclusion

MAIPA is the specific method for the detection of antibody against glycoprotein antigens, could be used to differentiate between immune and non-immune thrombocytopenia

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