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Title page: ANTIPHOSPHOLIPID ANTIBODIES AND ITS CLINICAL ASSOCIATION WITH NON-HODGKIN LYMPHOMA

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ABSTRACT

Introduction: The association of antiphospholipid antibodies (APA) with Non-Hodgkin Lymphoma (NHL) has been reported and complete remission was associated with disappearance of APA. APA are autoantibodies which include anticardiolipin antibody (ACA), anti-beta2-glycoprotein-I (β2 GPI) antibodies and lupus anticoagulant (LA).

Objective: The purpose of this study was to determine the prevalence of APA and its clinical association among NHL patients in our institution.

Methodology: A study was conducted in Hospital University Science Malaysia (HUSM) between June 2003 and July 2004.Fifty-three selected NHL patients were tested for ACA and anti-β2 GPI at presentation using ELISA technique. They were followed-up over a median period of 6 months to detect the occurrence of thromboembolism (TE) and bone marrow recovery following chemotherapy using full blood counts.

Results: APA was found in 23 out of 53 NHL patients, with ACA 35.8% and anti-β2 GPI antibodies 18.9%. The incidence of elevated APA was increased after 40. years old (91.3%). However, positivity for APA was not associated with gender, survival, histology or stage of lymphoma. There were three patients who developed TE and all of them were ACA positive. Positive APA was found to correlate with thrombocytopenia at presentation (p= 0.032) and associated with poor platelet recovery following chemotherapy (p=0.001).

Conclusion: APA was common among NHL patients especially after 40 years old. Although no statistical association was found between APA positive NHL and thrombosis, there was a tendency for TE to occur in APA positive NHL.

Key words: Antiphospholipid antibodies; anti-beta2-glycoprotein-I antibodies; anticardiolipin antibodies; non-Hodgkin Lymphoma.

Introduction

Thromboembolism (TE) and autoimmune phenomena are often seen in patients with NHL. Among all the malignant tumors, lymphoma patients are the fourth most likely population at risk to significant morbidity and mortality related to thrombosis[1]. The association of APA has been reported in several cases of patients with NHL, with or without thromboembolic complications[2]. In previous studies, complete remission was associated with disappearance of APA after treatment[3].

APA are autoantibodies with specificity towards negatively charged phospholipids that include ACA, anti-β2 GPI and LA[4]. The association of APA with recurrent venous or arterial thrombotic events, thrombocytopenia and fetal loss has been well established and is known as antiphospholipid syndrome (APS)[5]. In one study, increased incidence of vascular thrombosis among APA positive cancer patients (22%) was reported[6].

According to Malaysia National Registry 2002, lymphoma ranked seventh amongst male cancer and eleventh amongst the female cancers in Malaysia. Lymphoma constituted 3.7% of all cancers in Malaysia. There was male preponderance of 3:2. Ratio of Hodgkin to NHL is 1:9. In NHL, the male to female ratio was 1.4:1. The peak incidence of both sexes was in the age group 60-69 years[7].

The present study was designed to explore the prevalence and clinical associations of elevated APA titers (ACA and anti-ß2 GPI) in patients affected by NHL in our institution. It is hoped that this study will clarify the clinical descriptions of APA in NHL. Because of the potential APA test to influence therapy, routine testing for APA can be justified in NHL patients if statistically and clinically found to be significant.

Patients and methods

The study was conducted prospectively during one-year period. The participants were patients with active disease either newly diagnosed or relapsed non-Hodgkin lymphoma, admitted to medical and pediatric wards in Hospital University Science Malaysia for treatment. The study was conducted between Jun 2003 and July 2004.

This study was approved by the ethical committee, School of Medical Sciences, University Science of Malaysia. Each patient's serum was examined for the presence of anticardiolipin antibodies (IgM & IgG) and anti-ß2 GPI antibodies (IgM & IgG) by commercially available enzyme-linked immunosorbent assay (ELISA). All cases were followed-up for at least 6 months to look for the development of thromboembolism and bone marrow recovery following the treatment with conventional chemotherapy according to the type of NHL. Peripheral cell count is a good indicator of marrow recovery and blood were taken randomly after chemotherapy when the counts were expected to normalize. The expected recovery time is 3 to 4 weeks following the first day of chemotherapy or just before the patient entered the next cycle of chemotherapy. Duration for 6 months follow-up was chosen in view of patients were expected to complete the treatment at that time or possibly already achieved some remissions.

The study group consisted of 53 NHL cases, regardless of the age, ethnicity and histological findings. Diagnosis was made based on histopathological examination (HPE) findings from lymph node or tissue biopsy. The histological classification was based on Working Formulation and were staged according to Ann Arbor staging system. Complete staging by CT scan and bone marrow biopsy were carried out.

Inclusion criteria for study group

1- All NHL cases (pediatric & adult).

Exclusion criteria for study group

1- Patients with chronic infection eg. Hep C, HIV and syphilis.

2- Patients with active infection receiving treatment (antibiotics).

3- Patients on drugs that had been documented to be related with development of APA

The cases were approached and consents obtained for blood taking. Blood samples were collected by venipuncture into three containers, one containing 1/10 volume 3.8% sodium citrate for D-dimer and the other is 1/10 volume 0.5M sodium EDTA for Direct Coombs test and 2cc plain bottle for immunoassay (ELISA). Direct Coombs test and D-dimer were analyzed on the same day of blood collection. Blood in plain bottle were allowed to clot naturally and the serum separated after centrifugation. After centrifugation, the serum was stored at –34°C until batch analysis. Test for D-dimer was done to look at the possibility of occult TE that might occur in these cases. Direct Coombs test was

carried out to see any evidence of autoimmune hemolytic anemia or other underlying autoimmune activity related to NHL.

The immunoassay was intended for in-vitro measurement of IgG/ IgM autoantibodies against cardiolipin and β2 GPI in human serum, as an aid in the diagnosis of antiphospholipid syndrome. These antibodies concentrations were measured by a commercially available ELISA technique produced by The Binding Site Ltd-UK. Microwells are pre-coated with cardiolipin or ß2 GPI. Calibrators, controls and patient samples are added to the wells and autoantibodies recognizing cardiolipin bind during the first incubation. After washing the wells to remove all unbound proteins, peroxidase labeled rabbit anti-human IgG antibody conjugate was added. The conjugate binds to the captured human antibody and the excess unbound conjugate was removed by further wash step. The bound conjugate was visualized with tetramethylbenzidine (TMB) substrate that gives a blue reaction product, the intensity of which is proportional to the concentration of autoantibody in the sample. Phosphoric acid was added to each well to stop the reaction. This produces yellow end point colour, which was read at 450nm.Interpretation of results was done by semiquantitative methods (according to the manufacturer's recommendations).

The D-Di test® (Diagnostica Stago, France) is a rapid latex agglutination slide test using mouse monoclonal antibodies for the qualitative and semi-quantitative determination of D-dimer in plasma. The latex particles provided in

the test are coated with mouse anti-human D-dimer monoclonal antibodies. Test sample containing D-dimers when mixed with the latex particle suspension make the particles to agglutinate. Citrated plasma was used for testing. Samples were centrifuged for 10minutes at 2500g and supernatant were collected. Undiluted plasma and 1:1 dilution plasma were mixed with one drop of latex suspension on a glass slide and the slide was gently rocked for 2 minutes. Interpretation of the test was done as recommended by the manufacturer. Agglutination indicates positive results.

The DCT or direct antiglobulin test is a technique that can be used to detect for the presence of immunoglobulins or complement that have been bound to the red cells in vivo. This test was done by adding antihuman globulin to red cells of the patient's blood suspended in saline. The presence of agglutination in the samples after spinning indicate positive DCT, indicating the presence of surface immunoglobulin or complement component that have been bound to the red cells. Polyspecific antihuman globulin (AHG) contain antibody to human IgG and the C3d component of human complement and may also react weakly with IgA and IgM molecules due to anti-light chain activity. The reagent used was ID-card "DC-Screening II" produced by Diamed consisting of polyspecific AHG reagent. Patients with auto-immune haemolytic anaemia (AIHA) may give positive results.

For full blood count (FBC), the determination of haemoglobin level (Hb), Total white blood cell (TWC) count and platelet count was done by using automated Hematological Analyzer, Sysmex KX-21N.

All data were analyzed using the statistical package programmed for social sciences (SPSS) software version 10 11.0 for windows. The proportion was calculated as 95% confidence interval (CI). The differences of various parameters, between APA positive and negative patients were evaluated using Chi square or Fisher's exact test when applicable with level of significance, $p < 0.05$. The degree of association was presented by odd ratio (OD) and its 95% CI and relative risk (RR) and its 95% CI as appropriate

Results

There were 53 cases altogether, including 5 paediatric patients and 48 adult patients.

Patient's characteristics:

Patient's characteristics are summarized in Table 1. The median age of the group was 41.5 year (range 3-80 year). Early disease was defined as stage I or II while advanced stage as stage III and IV. At the time of the last contact, 7 patients were dead. Causes of death were attributed to complications of sepsis.

Detection of APA:

All 53 patients were tested for the presence of 4 types of antiphospholipid antibodies (IgM and IgG anticardiolipin antibodies, anti-IgG and anti-IgM β2 Glycoprotein I antibodies). Twenty-three patients out of 53 (43.4%) showed a positivity for at least one of the APA. The proportion of APA in patients with NHL was calculated as 95% confidence interval is 43.4% (95%CI:30.3%, 57.2%). Ten patients (18.9%) were positive for anti-β2GP1 antibodies and 19 patients (35.8%) were positive for ACA. Five patients (9.4%) were positive for both antibodies. Details are given in Table 2 and the summary of laboratory data of 23 patients with positive APA is shown in table 3.

Association between APA positivity and haematological parameters:

The summary of haematological data of all subjects is shown in table 4.

The association between APA positivity and available haematological parameters were tested by using x^2 (Chi square) test and the degree of association was presented by odd ratio (OR) and its 95%CI and relative risk (RR) and its 95%CI as appropriate. We found that from crosstabs descriptive statistical analysis (SPSS version 10.0), there was significant association between positive APA and development of thrombocytopenia at presentation (p=0.032) and poor platelet recovery post chemotherapy (p value=0.001). However, there was no significant association of APA positivity and other laboratory parameters such as Coombs test, D-dimer, hb level and TWC count (p value>0.05).

Association between APA and age, gender, histology, stage of Lymphoma and patient's survival.

The results and statistical analysis are shown in table 5.

There were a greater proportion of men than women among 23 patients with elevated APA. The median age of the 24 patients with positive APA was 48.5 year. From statistical analysis by Fisher exact test, there was a significant association between patient's age and APA positivity (p value = 0.002). However, there was no association between APA positivity and sex, stage of disease, histology, survival and bone marrow infiltration noted.

Association of APA and thrombosis

TE events were observed in three out of 53 patients (5.7%), all of them were positive for APA. Three TE events that were observed are pulmonary

embolism (diagnosed by pulmonary scan angiography), ishaemic heart disease (anteroseptal ishaemia by ECG) and deep vein thrombosis (right superficial femoral vein thrombosis diagnosed by Doppler ultrasound). Thrombosis occurred in two patients with high grade NHL and one with low grade NHL. All three patients with TE were positive to anticardiolipin antibodies with weak to moderate positivity. None are positive to anti-β2 GPI antibodies. The clinical and laboratory data of these patients are summarized in Table 6. The association between APA positivity and occurrence of thromboembolism was tested by using Fisher exact tests and the degree of association was presented by relative risk (RR) and its 95%CI as appropriate. We also tested with simple logistic regression and the same results as analysed by Fisher exact test was obtained. We did not find any significant differences in occurrence of thromboembolism among the subjects with either positive or negative for APA (p value=0.076).

Discussion

In NHL patients, whether autoimmune antibodies contribute to pathogenic relevance is still controversial. There was one study to determine the autoimmune phenotype of 64 NHL patients by using Coombs test, platelet autoantibodies, antiphospholipid antibodies, anti-nuclear antibodies and LA as autoimmune markers. They found 39 % of patients displayed one or more autoimmune marker positivity. They observed median survival was longer in autoimmune marker negative patients than in the positive counterpart. No significant association between this autoimmune marker positivity with sex and remission rates[8]. We found 60.3% of NHL patients exhibit one or more autoimmune markers. It is difficult for us to compare the median survival between positive and negative autoimmune markers of NHL patients in our study because of the short follow-up period.

The relationship between lymphoproliferative disorder and autoimmunity is well documented. A Study by Zuckerman et al, noted the prevalence of ACA was 39% in non-Hodgkin lymphoma[6]. Patient with certain autoimmune disorders such as systemic lupus erythematosus have several fold increased risk for development of malignant lymphoma[9]. A four-year prospective study from the Italian Registry reveals that hematological malignancies can develop during follow-up in patients with positive for APA. They found four out of 360 of their APA patients had developed NHL[10]. In our study, APA was detected in 43.4% of NHL cases; which is slightly higher to the previous studies reported. A

prevalence of 16.6% anti-β2GP-1 was reported in NHL patients[2]. In this study we found 18.9% were positive for this antibody. The conditions associated with alloantibody development, particularly infectious diseases, were not identified during sample collection, thus suggesting that APA activities in these subjects are indeed autoantibodies in relation to underlying NHL.

There have been several studies published which have estimated the prevalence of APA in NHL. Stasi et al explored ACA and LA among recently diagnosed high-grade NHL patients and reported a prevalence of 35.7%, whereas Genvresse et al and Zuckerman et al reported a prevalence of 26.6% and 39% of APA in their study respectively[3,2,6]. In addition to NHL, other conditions associated with the development of auto-antibodies have to be considered to explain the slightly high prevalence of APA in our study compared to their study. High prevalence of APA in our study is unlikely due to infection or due to certain drugs because none of the subjects were reported to have any clinically overt infectious diseases or on any drugs that can cause false positive APA during sample collection. However we cannot rule out the hypothesis of a possible relationship between APA positivity and some drugs or unknown infectious agent.

Our results showed that there was a significant association between APA positivity and patient's age (p value <0.05). Majority of patients that had elevated APA are from age group more than 40 years old. As with other

antibodies, the prevalence of APA increases with age, especially in elderly patients with coexistence chronic diseases.

Ten percent of patients with Hodgkin and non-Hodgkin lymphoma are at risk of thromboembolism[1]. In our case, the venous thromboembolic event is about 5.7%. We observed three patients developed TE during follow-up and all of them were positive for ACA.This is an important observation although the findings did not reach statistical significance.

Although APS has been described primarily with elevated IgG antibodies but it also occurs with elevated IgM antibodies[11]. Therefore multiple tests for APA should be used since patients may be negative according to one test yet positive according to another. Levels of IgG ACA greater than 40 units have been linked to increased risk of thromboses[10]. However one study suggested that the presence of persistent positivity regardless of the APA titer identifies patients at risk of thromboembolic events[12]. Antiphospholipid antibody mediated thrombosis tends to reccur[13]. However the information on the recurrence TE in APA positive NHL is limited. There was one case-control study looking at the risk of thrombosis in healthy patients with ACA and they found a significant association between elevated ACA titers with DVT and PE but not with ischemic stroke[14]. However a few prospective studies have shown an association between APA and first episode of venous thrombosis, first myocardial infarction and recurrent stroke [11]. In our study, ishaemic heart disease occurrence in one of

the patients may be directly or indirectly related to APA. Our observation in this study is important since all the three patients who developed TE are positive for ACA.

None of our 9 NHL patients with anti-β2 GPI antibodies developed a thrombosis during follow-up. Previously Genvresse et al has reported two cases of arterial thrombosis associated with ACA and anti-β2GP-1 antibodies in patients with NHL[15]. The lack of occurrence of thrombosis in anti-β2 GPI positive NHL patients in this study may be explained by their low anti-β2 GPI antibody titers or only one marker (anti-β2 GPI) which was detected positive.

We observed a significant association between the incidence of APA and development of thrombocytopenia at presentation (p=0.032) and post chemotherapy. (p= 0.001). This indicates that cell recovery after chemotherapy may be affected particularly platelet count in APA positive NHL patients. We also found that NHL patients with positive APA are five fold at risk (Relative risk = 5.2) to develop thrombocytopenia during any course of the disease. This suggests that APA positivity may be one of the contributory factors in complicating the thrombocytopenia in patients with NHL. Autoimmune thrombocytopenia in lymphoproliferative disorder showed a poor response to glucocorticoids[16]. More systematic studies should be addressed because so far no such study to relate between APA positivity and peripheral cells recovery post chemotherapy.

In our study, bone marrow infiltration and other causes of thrombocytopenia for example active infection and DIC were excluded.

D-dimer levels are typically elevated in the plasma of patients with acute thrombosis. D-dimer assays have been advocated to exclude underlying TE on the basis of a normal test results. Although D-Dimer assays have high negative predictive values for the diagnosis of deep vein thrombosis, their accuracy in patients with cancer is uncertain[17]. We found 37.7% were positive for D-dimer at presentation but none of them had symptomatic thrombosis. Looking to our three patients with TE, one patient was negative for D-dimer. One of the most clear-cut reasons for this patient to have negative D-dimer in the presence of TE is a false normal result, due to low concentration of D-dimer for the cut-off value to be reached. A negative D-dimer test result in patients with cancer does not reliably exclude deep vein thrombosis because the negative predictive value is significantly lower in these patients than in patients without cancer[17].

B cell lymphomas and leukaemias are the malignancies most frequently associated with autoimmune haemolytic anaemia[16]. In this study we found that 50.9% of our NHL patients had positive direct Coombs test (DCT). A positive test results must be correlated with clinical or laboratory evidence of haemolysis since positive DCT can occur in many conditions. High positive DCT was observed in hospitalized patients[18]. In this study we observed only one case

of autoimmune haemolytic anemia that developed concurrently with NHL. However, this patient was negative for APA.

We have identified a few limitations in our study. One potential limitation of our study is the fact that we did not perform LA test. As LA and elevated ACA occurred concurrently in 30% of cases, therefore the combined use of the two methods should be advocated[3]. More sample size should be considered with longer follow-ups to observe for the TE complications in future. However APA was prevalent among our NHL patients (43.4%) and in subjects more than 40 years old. Although statistically there is no significant between APA positivity and occurrence of TE, we observed that all three patients with thromboembolic events were positive for ACA.

In conclusion, the association between TE and APA remains uncertain from this study. Our results were compatible with a wide range of effects including substantial decreased and increased in TE. A larger sample size would enable a definite conclusion regarding this relationship. However we proposed that it is still beneficial to detect these antibodies in selected NHL patients particularly with TE events. This could improve the diagnosis and aid in the management of TE, for example to monitor for the risk of recurrence. Poor recovery of platelet count post chemotherapy or during follow-up in the absence of bone marrow infiltration may indicate an underlying co-existing APA

particularly in older NHL patients. Hence, confirming its presence could help in the patient's management especially in cases with severe thrombocytopenia.

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