

Kertas Asli/Original Article

Immunofluorescence (IF) Pattern, Autoantigens of Anti-neutrophil Cytoplasmic Antibodies (ANCA) and Their Clinical Associations

[Corak Imunopendar, Autoantigen Antineutrofil Antibodi Sitoplasma dan Hubungan Klinikalnya]

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ABSTRACT

Antineutrophil cytoplasmic antibodies (ANCA) are autoantibodies directed against primary granules of neutrophils and monocytes' lysosomes. In general, c-ANCA is strongly associated with vasculitic disorders mainly in ANCA-associated systemic vasculitis (AAVS). p-ANCA have been identified in several diseases such as primary (AAVS) and secondary vasculitis such as collagen vascular diseases, rheumatoid arthritis and inflammatory bowel diseases given the term 'ANCA-associated disease.' The objective of this study was to determine the rate of ANCA positivity by indirect immunofluorescent (IF) and enzyme linked immunosorbent assay (ELISA) and its association with AAVS and ANCA associated diseases. Serum from patients with history suspicion of systemic vasculitis were tested for ANCA by IF. Those samples positive for ANCA by IF were further tested for antibodies against myeloperoxidase (MPO) and proteinase 3 (PR3) using the ELISA. Clinical data from medical records were obtained and analyzed. Of 468 samples, a total of 110 were positive for ANCA by IF. IF results showed a p-ANCA pattern in 96 patients and c-ANCA in 14. Of 110 IF positive ANCA, 45 patients were positive by ELISA. Seventeen were positive for MPO-ANCA, 9 were PR3-ANCA positive and 19 were both MPO and PR3 positive. Only 2 patients were classified AAVS ie Wegener granulomatosis and the other with microscopic polyangiitis. The remaining patients ($n = 108$) may be classified as ANCA associated diseases. Our study showed that p-ANCA (87.3%) was the more common ANCA pattern and 40.9% of IF positive samples were positive for PR3- and MPO-ANCA.

Keywords: Antineutrophil cytoplasmic antibodies; p-ANCA, c-ANCA; myeloperoxidase; Proteinase 3

ABSTRAK

Antineutrofil antibodi sitoplasma (ANCA) merupakan antibodi terhadap granul primer di dalam lisosom neutrofil dan monosit. Secara amnya, c-ANCA berkaitan dengan penyakit radang salur darah terutama radang salur darah sistemik berkaitan ANCA (AAVS). p-ANCA diidentifikasi di dalam beberapa penyakit seperti penyakit radang salur darah jenis primer (AAVS) dan sekunder iaitu penyakit salur darah kolagen, sendi rheumatoid dan penyakit inflamasi usus yang dirumuskan sebagai penyakit berkaitan ANCA. Untuk memgetahui kadar positif ANCA melalui ujian imunofluoresen secara tidak langsung (IF) dan ujian menggunakan enzim imunosorben (ELISA) dan hubungannya dengan penyakit AAVS dan penyakit berkaitan ANCA. Serum pesakit yang disyaki mempunyai penyakit radang salur darah sistemik disaring dengan ujian IF. Ujian ELISA dijalankan ke atas sampel ANCA yang positif dengan ujian IF untuk mengesan antibodi terhadap spesifisiti antigen ANCA iaitu "myeloperoxidase" (MPO) dan "proteinase 3" (PR3). Data daripada rekod pesakit dicatit dan dianalisa. Daripada 468 sampel yang diterima, sebanyak 110 serum didapati positif dengan ujian IF. Keputusan IF menunjukkan p-ANCA seramai 96 pesakit dan c-ANCA seramai 14 orang. Daripada jumlah 110 positif IF, 45 pesakit positif dengan ujian ELISA iaitu 17 orang adalah MPO, 9 orang PR3 dan 19 orang mempunyai kedua-dua MPO dan PR3. Dua pesakit dikategorikan sebagai penyakit AAVS iaitu "Wegener's granulomatosis" dan "Microscopic polyangiitis". Selebihnya ($n = 108$) adalah di dalam kategori Penyakit Berkaitan ANCA. Kajian ini menunjukkan p-ANCA (87.3%) merupakan "pattern" IF yang sering dijumpai dan sebanyak 40.9% sampel positif IF yang mengandungi antibodi terhadap PR3- dan MPO-ANCA.

Kata kunci: Antineutrofil antibodi sitoplasma; p-ANCA; c-ANCA; Myeloperoxidase; Peroxidase 3

INTRODUCTION

ANCA are predominantly immunoglobulin G (IgG) autoantibodies directed against constituents of primary granules of neutrophils and monocytes' lysosomes. ANCA

were first described as diffuse granular cytoplasmic immunofluorescence staining (c-ANCA) on ethanol-fixed neutrophils in association with glomerulonephritis, vasculitis and Wegener's granulomatosis (Bosch et al. 2006). Proteinase 3 (PR3) was subsequently identified as

the principal target antigen for these ANCA. At the same time, ANCAs reacting to myeloperoxidase (MPO) and resulting in a perinuclear immunofluorescence staining pattern on ethanol-fixed neutrophils (p-ANCA) which were found in patients with microscopic polyangiitis, its renal-limited variant, pauci-immune glomerulonephritis and less frequently, in Wegener's granulomatosis (Savage et al. 2000).

Laboratory tests for ANCA are used when a systemic vasculitis or renopulmonary syndrome is suspected clinically. Today, Wegener's granulomatosis (WG), microscopic polyangiitis (MPA) and the Churg-Strauss syndrome (CSS) are commonly referred to as the ANCA-associated systemic vasculitides (AASV) or primary systemic small vessel vasculitides. In general, c-ANCA is strongly associated with vasculitic disorders mainly in ANCA-associated systemic vasculitis (AASV). p-ANCA have been identified in several diseases such as primary (AASV) and secondary vasculitis such as collagen vascular diseases, rheumatoid arthritis and inflammatory bowel diseases given the term 'ANCA-associated disease.' (Jenette et al. 1994; Seo & Stone 2004).

The overall incidence of AASV is approximately 10-20 per million populations and the majority of data on the incidence and prevalence of AASV has come from studies in the United Kingdom, France, Norway, Sweden and Germany. In these populations AASV is slightly more common in men (1.5:1.0) and the peak onset is in those aged 65 to 74 years. AASV is rare in childhood (Lane et al. 2005).

Despite advances in ANCA testing techniques, histopathology remains the gold standard for diagnosis in most cases. When the biopsy specimen is not diagnostic, ANCA assays provide an important adjunct to diagnosis (Seo & Stone 2004).

The "International Consensus Statement on Testing and Reporting of ANCA" has been developed to optimize the usefulness of ANCA testing and to ensure more uniformity in the laboratory results that are issued. It stipulates that all sera should at least be tested by indirect immunofluorescent (IF) examination of normal peripheral blood neutrophils and, where there is positive IF, an enzyme-linked immunosorbent assays (ELISAS) should be performed for antibodies against proteinase 3 (PR3) and myeloperoxidase (MPO) (Savage et al. 1999).

In Malaysia, routine tests for ANCA employed either IF or ELISA. Thus, the aims of this study were: 1) to determine the frequency of ANCA positivity using two different methods ie. IF for pattern and ELISA for specific target to PR3 and MPO; 2) to describe the clinical features of ANCA associated diseases.

MATERIAL AND METHODS

STUDY POPULATION

This was a cross-sectional study comprising of 468 consecutive sera from patients in Selayang Hospital,

Malaysia with suspicious systemic vasculitis from June 2005 until July 2006 were tested for ANCA by IF test. Samples were tested at the Institute for Medical Research. Those samples positive for ANCA by IF were further tested for antibodies against myeloperoxidase (MPO) and proteinase 3 (PR3) using the ELISA test. The study was approved by the Research and Ethical Committee of the Medical Faculty, Universiti Kebangsaan Malaysia (UKM).

CLINICAL DATA

Complete clinical data and laboratory results were documented. Diseases were classified either as primary systemic small vessel vasculitides (Wegener's Granulomatosis (WG), microscopic polyangiitis (MPA), Churg-Strauss syndrome) according to Chapel Hill Consensus definition or other diseases (such as systemic lupus erythematosus, rheumatoid arthritis, inflammatory bowel disease and other disease process).

METHODS

Immunofluorescence All serum samples were tested for ANCA using the kit from Immuno Concepts (USA) for the semi quantitative detection of anti-neutrophil cytoplasmic antibodies (ANCA) in human serum. Diluted patient samples were incubated with human neutrophils, which are fixed on glass microscope slides, to allow specific binding of ANCA. If ANCA are present in the serum, the autoantibodies bind to neutrophilic antigens. After washing to remove non-specific antibodies, the antigen-antibody complex is incubated with anti-human IgG conjugated to fluorescein. Fluorescence patterns were defined as c-ANCA when giving a diffuse granular cytoplasmic staining and as p-ANCA when giving a diffuse or peripheral nuclear staining of the neutrophils on ethanol fixed cells.

Enzyme-linked immunosorbent assay (ELISA) All IF ANCA positive samples were assayed subsequently, by ELISA (BINDAZYME™, Binding Site, UK), to determine the precise antigen specificity of the autoantibody present (anti-MPO and anti-PR3). The microwells were pre-coated with the MPO/PR3 antigen. The calibrators, controls and diluted patient samples were added to the wells. After washing the wells to remove all unbound proteins, purified peroxidase labelled rabbit anti-human IgG (γ chain specific) conjugate was added. The conjugate bound to the captured human autoantibody and the excess unbound conjugate is removed by a further wash step. The bound conjugate is visualized with 3,3'-5,5' tetramethylbenzidine (TMB) substrate which gives a blue reaction product, the intensity of which is proportional to the concentration of autoantibody in the sample. Phosphoric acid is added to each well to stop the reaction. This produces a yellow end point colour that is read at 450 nm. Results more than 3.5 U/mL and 9.0 U/mL were considered positive to PR3 and MPO respectively.

RESULTS

ANCA RESULTS

A total of 110 out of 468 (23.5%) serum samples were positive for ANCA by IF. Of these, 96 samples showed a p-ANCA IF pattern (Figure 1) and 14 showed a c-ANCA pattern (Figure 2). Only 45 out of 110 samples (40.9%) tested positive by ELISA. 17 samples were positive to MPO-ANCA, 9 to PR3-ANCA and 19 to both MPO- and PR3-ANCA.

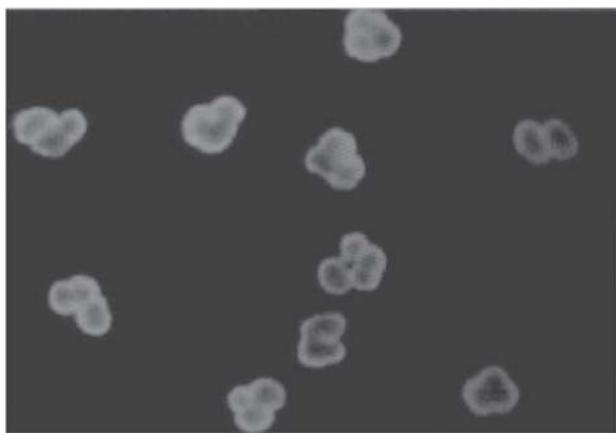


FIGURE 1: p-ANCA IF pattern on ethanol-fixed neutrophils

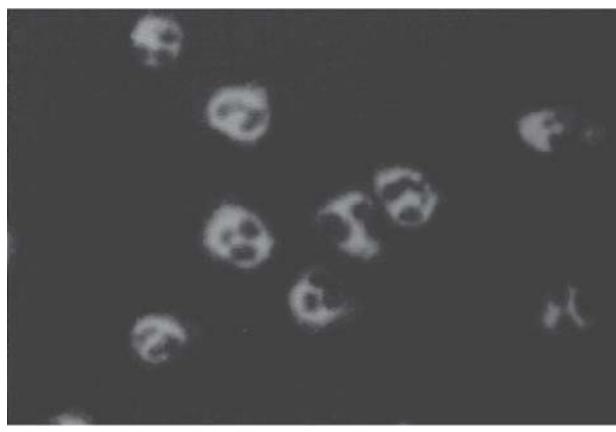


FIGURE 2: c-ANCA IF pattern on ethanol-fixed neutrophils

DEMOGRAPHICS OF ANCA POSITIVE SAMPLES

Females comprised 74.5% of the study population and males 25.5%. Out of 110 patients, 57.3% were Malays, 27.3% were Chinese, 14.5% were Indian and 0.9% other races. The mean age of the study group was 38 years.

ANCA POSITIVE SAMPLES ACCORDING TO DIAGNOSIS

Out of 110 patients with a positive ANCA, 40 patients were diagnosed as systemic lupus erythematosus (SLE). Two patients were diagnosed as ANCA-associated systemic vasculitis (AASV) ie. Wegener granulomatosis and microscopic polyangiitis. Table 1 showed the clinical diagnosis among positive ANCA patients.

TABLE 1. Diagnosis of ANCA positive patients

Disease	Number of patients
SLE	40
CLD (consists of)	
1) Autoimmune hepatitis	8
2) Hepatitis B virus infection	8
3) Primary biliary cirrhosis	5
4) Hepatitis C virus infection	3
5) Unknown aetiology	9
Total CLD patients	33
Rheumatoid arthritis	10
Drug induced hepatitis	7
Scleritis	4
Nephrotic syndrome	3
Scleroderma	3
Mixed connective tissue disease	2
Non-specific diagnosis	2
ITP	1
Pulmonary hypertension	1
Crohn's disease	1
Wegener's granulomatosis	1
Microscopic polyangiitis	1
Malignancy	1
Total (Bold numbers)	110

ANCA RESULTS AND OTHER AUTOIMMUNE TESTS

Most positive ANCA patients ($n = 49$) also had other autoimmune tests done. 49 patients were anti-nuclear antibody positive. The association between ANCA and other autoantibodies were shown in Table 2.

DISCUSSION

It is important that the main antigen involved in the c-ANCA pattern is PR3, and the main antigen producing the p-ANCA pattern is MPO (Savage et al. 1999). However, our findings indicate 57/96 (59.4%) of p-ANCA and 8/14 (57.1%) of c-ANCA positive samples were negative for MPO and PR3. This finding suggests that antibodies targeting antigen other than MPO and PR3 may be involved.

Human neutrophils contain at least three types of granules, each of which contains a variety of constituent proteins: azurophilic granules contain PR3, MPO, bactericidal permeability increasing protein/BPI, elastase/Elast, cathepsin G/Cath G; secondary granules contain lactoferrin/LF, lysozyme; and tertiary granules contain gelatinase. Antigens within any of these granules are potential targets for an ANCA response. 'Minor' antigens involved all the above granules except PR3 and MPO (Roos et al. 1990). Talor et al. (2007) examined antibodies to minor antigens such as anti-BPI, anti-Elast, anti-Cath G and anti-LF using enzyme immunoassay (EIA) in patients with ANCA.

TABLE 2. Association between ANCA and other autoantibodies

Autoimmune tests	ANCA pattern		ANCA antigen specificity		
	P-ANCA no (%)	C-ANCA no (%)	MPO no (%)	PR3 no (%)	MPO & PR3 no (%)
Antinuclear antibody (n = 49)	49 (100)	0	7 (14)	5 (10)	9 (18)
Anti-double stranded DNA (n = 21)	21 (100)	0	3 (14)	3 (14)	3 (14)
Rheumatoid factor (n = 17)	15 (88)	2 (12)	0	2 (12)	2 (12)
Antimitochondrial antibody (n = 16)	14 (88)	2 (12)	2 (22)	3 (19)	5 (36)
Anti-SSM (n = 18)	18 (100)	0	5 (28)	2 (11)	1 (56)
Anti-RNP (n = 8)	8 (100)	0	0	2 (25)	2 (25)
Anti-cardiolipin antibody (n = 6)	6 (100)	0	1 (17)	0	0
Anti-sm (n = 4)	4 (100)	0	0	0	1 (25)
Lupus anticoagulant (n = 2)	2 (100)	0	0	0	0

They found that IF positivity with negative MPO and PR3 ELISA sera showed the most varied reactivity to the minor antigens. Our results implied that ANCA testing by IF would also detect other ANCA minor antigens based on lack of association between IF and ELISA for MPO and PR3.

ANCA are serological markers for a significant subset of patients with primary systemic small vessel vasculitis known as ANCA-associated small vessel vasculitides (AASV), which include Wegener's granulomatosis, microscopic polyangiitis and Churg-Strauss syndrome. In this study, only two patients with positive ANCA were diagnosed with AASV ie. one patient with Wegener granulomatosis and another with microscopic polyangiitis. Other patients with positive IF ANCA fall under the category of ANCA associated diseases.

Our study showed that 40 patients (36.4%) with positive ANCA were diagnosed with SLE. In another study done in Malaysia by Fauzi et al. (2004), they reported ANCA was present in 24.8% of SLE patients. They found that there was no association between ANCA positivity and disease activity, thus routine testing for ANCA in lupus patients is therefore not recommended. While ANCA may be detected in SLE, its detection does not provide any additional diagnostic or prognostic data. (Molnar et al. 2002).

In this study, there were 33 patients with chronic liver disease (CLD). Savige et al. (2005) reported that ANCA are prevalent in CLD patients (70% patients with chronic autoimmune hepatitis and 90% patients with primary sclerosing cholangitis). These are usually p-ANCA and with multiple antigen specificities.

Amongst the rheumatoid arthritis (RA) patients (n = 10), 8 patients were positive for p-ANCA and 2 patients were positive to c-ANCA. However none of them had any antigen specificities to MPO or PR3. The lack of association between IF result and ELISA among RA patients in our study may suggest that the ANCA are targeting 'minor' antigens instead. In a study carried out by Birkan et al. (2004), they found that the prevalence of ANCA was 18% and only 8 out of 85 patients with RA tested were positive for MPO. They concluded that ANCA occurred in a minority of RA patients.

Analysis of association between ANCA results and other autoantibodies showed that among 96 patients with p-ANCA, 49 had antinuclear antibody (ANA). In primary systemic small vessel vasculitides, ANA were shown to occur in up to 30% of patients, and these IF pattern vary and may mask a p-ANCA (Savige et al. 2000). In this study, interference by ANA is eliminated as the IF test for p-ANCA is done using fixation of cells in formalin.

We evaluated all requests for ANCA testing from Selayang Hospital and noted several issues. Out of 468 requests for ANCA tests, only two were categorized as AASV and 108 patients were only ANCA-associated diseases. The prevalence of AASV in Selayang hospital was considered very rare (0.43%). In both cases of AASV, the immunofluorescence and ELISA tests were positive for ANCA. This highlighted the importance of ANCA testing in the diagnosis of AASV. We noted that most of the requests for ANCA testing were not to diagnose AASV but to rule out autoimmune diseases. If tests are ordered for patients with a high suspicion for AASV, the number of positive results will be meaningful.

In conclusion, the prevalence of primary systemic small vessel vasculitides in Selayang hospital was considered rare. Our study showed that p-ANCA 96/10 (87.3%) was common for ANCA pattern and only 45/110 (40.9%) of IF ANCA positive samples were positive for PR3- and MPO-ANCA.

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