ISSN:1306-3111
e-Journal of New World Sciences Academy
2012, Volume: 7, Number: 2, Article Number: 1B0031

NWSA-MEDICAL SCIENCES
Received: December 2011
Accepted: April 2012
Series : 1B
ISSN : 1308-7312

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ASSESSMENT OF ANTI-NEUTROPHIL CYTOPLASMIC ANTIBODY POSITIVITY IN VARIOUS DIAGNOSTIC GROUPS

ABSTRACT

In present study, anti-neutrophil cytoplasmic antibody (ANCA) and its' relationship with clinic situations were investigated retrospectively in samples which were referred by miscellaneous diagnosis. All 1668 sera were first evaluated by indirect immunofluorescence technique (IIF). Then, samples with positive florescence were evaluated to determine specific antigens by enzyme-linked immunosorbent assay (ELISA). Positive staining was found in 320 (19.2%) of 1668 serum samples by IIF technique. Samples in which specific antigens were found by ELISA were obtained from 49 patients. Of the 49 ANCA positive patients, anti-myeloperoxidase antibody was found in 42.9%, whereas anti-proteinase-3 antibody in 49%. Both anti-myeloperoxidase and anti-proteinase-3 antibodies were detected in 8.1% of these patients. Identifying presence of ANCA as well as determining antibodies directed against specific antigens will improve its' diagnostic significance.

Keywords: Autoantibody, Diagnosis, Indirect Immunofluorescence, Anti-neutrophil Cytoplasmic Antibody, Enzyme-Linked Immunosorbent Assay

ÇEŞİTLİ TANI GRUPLARINDA ANTİ-NÖTROFİL SİTOPLAZMİK ANTİKOR POZİTİFLİĞİNİN DEĞERLENDİRİLMESİ

ÖZET

Bu çalışmada, çeşitli tanılarla gönderilen örnelerde anti-nötrofil sitoplazmik antikorların (ANCA) klinik durumlarla ilişkisi retrospektif olarak araştırıldı. Toplam 1668 serum önce indirekt immunofloresan (IIF) tekniği ile (Euroimmun, Lübeck, Almanya) çalışıldı. Pozitif floresan veren örnekler spesifik antijenleri belirlemek için enzyme-linked immunosorbent assay (ELISA) ile (Euroimmun, Lübeck, Almanya) test edildi. Tüm serum örneklerinin 320'sinde (%19.2) IIF tekniği ile pozitif boyanma saptandı. ELISA testi ile 49 hastada spesifik antijen belirlendi. ANCA pozitif bulunan 49 hastanın %42.9'unda anti-MPO antikoru, %49'unda anti-PR3 antikoru bulundu. Hastaların %8.1'inde ise hem anti-PR3 hem de anti-MPO antikoru birlikte mevcuttu. Örneklerde ANCA varlığının saptanması, bunun yanısıra spesifik antijenlere karşı oluşan antikorların belirlenmesi ilgili hastalıkların tanısında bu testin önemini artıracaktır.

Anahtar Kelimeler: Otoantikor, Tanı, İndirekt İmmunofloresan, Anti-nötrofil Sitoplazmik Antikor, Enzyme-Linked Immunosorbent Assay

1. INTRODUCTION (GİRİŞ)

Anti-neutrophil cytoplasmic antibodies (ANCAs) are autoantibodies that formed against antigens in cytoplasmic granules of neutrophils and lysosomes of monocytes [1, 2, 3 and 4]. Primarily, they are markers for small vessel vasculitis [2]. Granulomatosis with polyangiitis (Wegener's), Microscopic polyangiitis (MPA) and Churg-Strauss syndrome (CSS) are termed as ANCA-related vasculitis according to widely accepted classification [5].

ANCA is classified according to patterns which are displayed on neutrophils by indirect immunofluorescence (IIF) technique and target antigens which are identified by enzyme-linked immunosorbent assay (ELISA) [5 and 6]. If perinuclear fluorescence with nuclear enlargement is seen, it is termed as perinuclear ANCA (p-ANCA), whereas cytoplasmic or classical ANCA (c-ANCA), if there is a diffuse cytoplasmic granular staining pattern. Occasionally, perinuclear staining without nuclear enlargement or homogenous cytoplasmic fluorescence can be seen. Alternatively, both cytoplasmic and perinuclear staining can be seen together. Such conditions are termed as atypical ANCA [7 and 8].

Main target antigens for ANCA are proteinase 3 (PR3) and myeloperoxidase (MPO). These antigens are generally detected in Granulomatosis with polyangiitis (Wegener's), MPA and CSS. Target antigens other than these includes bactericidal permeability increasing protein (BPI), cathepsin G, elastase, lactoferrin and lysozyme [6, 8, 9 and 10].

Among diseases, p-ANCA is most commonly seen in MPA and CSS. MPO is main target antigen for p-ANCA. Furthermore, it can be detected in non-vasculitic conditions such as cancers, infection, inflammatory bowel diseases (IBD) and autoimmune diseases [5, 6, 8 and 11]. When p-ANCA is detected in these diseases, it is generally directed against neutrophil constitutions other than MPO. Target antigens for p-ANCA other than MPO are elastase, lactoferrin, lysozyme, BPI, cathepsin G and ß-glucuronidase [6, 8, 10, 12 and 13]. c-ANCA is mostly directed against to PR3 and also rarely to MPO, or both. ANCA positivity is detected in 70–90% of patients with granulomatosis with polyangiitis (Wegener's), while it was seen in 30% of the patients with MPA. Apart from ANCA-related vasculitis, atypical c-ANCA pattern is mostly seen during the course some infectious diseases at IIF. This atypical ANCA is generally directed against BPI [2, 8 and 14].

Early diagnosis is important in systemic small vessel vasculitis [3 and 4]. Renal failure develops soon in most patients with granulomatosis with polyangiitis (Wegener's) and MPA but not diagnosed yet or appropriately treated. Mortality and morbidity is high, even with optimal treatment [15 and 16]. Diagnosis in granulomatosis with polyangiitis (Wegener's), CSS and MPA is primarily based on histology; but, histology is not always conclusive [5 and 17]. ANCA is used to facilitate diagnosis and to monitor disease activity in small vessel vasculitis. Apart from small vessel vasculitis, ANCA is also recommended in terms of providing information for differential diagnosis in ulcerative colitis, Crohn's disease, autoimmune liver diseases, systemic lupus erythematosus, primary sclerosing cholangitis and Felty's syndrome [12].

2. RESEARCH SIGNIFICANCE (ÇALIŞMANIN ÖNEMİ)

International Consensus Statement on Testing and Reporting ANCA recommends analyzing serum samples of all patients with suspected

small vessel vasculitis by IIF and to test positive serum samples by antigen specific ELISA [7].

In the present study, it was aimed to assessed ANCA positivity in various diagnostic groups.

3. MATERIALS AND METHODS (MATERYAL VE METOD)

ANCA positivity patterns were retrospectively investigated on 1668 blood sample which were referred to Serology Laboratory of Erciyes University, Medicine Faculty with various diagosis between January 2007 and December 2008. Firstly, all serum samples were assayed by using formalin and ethanol-fixed human neutrophils by IIF technique (Euroimmun, Lubeck, Germany) according to manufacturer's instruction. Each slide contained positive and negative controls. ANCA patterns were defined as c-ANCA, p-ANCA and p and c-ANCA according to fluorescence pattern that was observed. Samples with positive fluorescence were assayed by ELISA (Euroimmun, Lubeck, Germany) for most common antigens, MPO and PR3. Serum samples which were positive for none of these antigens were re-evaluated by ANCA ELISA profile test (Euroimmun, Lubeck, Germany) for more uncommon antigens, cathepsin G, lactoferrin, elastase and BPI. All data of patients who were considered as ANCA positivity pattern were collected and recorded from patient' files.

3.1. Statistical Analysis (İstatiksel Analiz)

Statistical analysis were performed by using SPSS (v. 11.0; SPSS Inc, Chicago, IL, USA). Chi-square test was used for data analysis.

4. FINDINGS (BULGULAR)

ANCA positivity patterns were evaluated in 1668 blood samples by using IIF technique. Of these, no fluorescence staining was detected in 1348 samples and these were considered as negative. IIF test was interpreted as positive in 320 samples. Among positive samples, none of the specific antigens including PR3, MPO, cathepsin G, lactoferrin, elastase and BPI were detected in 252 samples. These samples were considered as either anti-nuclear antibody (ANA) positivity or undefined antigen positivity other than our ELISA-ANCA panel. Of 320 samples which were positive by IIF, PR3 or MPO antigen or both were found in 68 samples (4.1%). MPO or PR3 antigen were found alone in 28 samples (1.7%) and 32 samples (1.9%), respectively, whereas both MPO and PR3 antigen were concurrently detected in 8 samples (0.5%). The same patients were included in one or a few examples. Samples in which specific antigens were found by ELISA were obtained from 49 patients. Of 49 patients with positive ANCA, there were 28 female (57.1%) and 21 male (42.9%). Mean age was 47.57±18.96 (ranging 1 to 77 years) years. No significant difference was found between men and women regarding all three ANCA groups (3 groups; MPO, PR3 or MPO and PR3) ($p>0.05$). When we compared MPO and PR3 ANCA (2 groups; MPO or PR3) regarding gender, a significant difference was found in favor of PR3 in men ($p=0.016$). When ANCA patterns were compared to age groups, no significant difference was found ($p>0.05$).

Of the patients in the present study, 12 patients (24.5%) were diagnosed as vasculitis, 11 patients (22.4%) as chronic renal failure (CRF), 10 patients (22.4%) as granulomatosis with polyangiitis (Wegener's), 10 patients (22.4%) as pulmonary disease, 9 patients (18.4%) as autoimmune disease, six patients (12.2%) as diabetes mellitus (DM), six patients (12.2%) as cholelithiasis, 5 patients (10.2%) as chronic liver disease and 4 patients (8.2%) as malignancy.

In addition, ulcerative colitis, nasal polyp, urolithiasis and gout disease were seen in individually in one patient. These patients were classified in a group called "others". Half of patients had multiple diagnosis. Vasculitis group was involving small vessel vasculitis other than granulomatosis with polyangiitis (Wegener's). Chronic liver diseases were caused by hepatitis B virus (HBV) and hepatitis C virus (HCV) in 3 and one cases, respectively. Etiologic origin of liver disease was unknown in one case. Among autoimmune diseases, there were 4 autoimmune thyroiditis, 2 collagen tissue diseases, one primary sclerosing cholangitis, one Good-Pasture syndrome and one autoimmune hemolytic anemia. Malignancies were including acute lymphoblastic leukemia, non-hodgkin lymphoma, pleural mesothelioma and myelodysplastic syndrome.

There was concomitant diseases in six patients with CRF, 5 patients with granulomatosis with polyangiitis (Wegener's), 5 patients with pulmonary disease and six patients with autoimmune diseases. All patients in DM, cholelithiasis, chronic liver disease, malignancy and others groups had various co-morbid diseases.

Anti-PR3 antibody was detected in 8 of patients in vasculitis group, whereas anti-MPO anti body in 3 patients; both anti-PR3 and anti-MPO antibodies were detected in one patient in vasculitis group. No significant difference was found regarding ANCA patterns ($p>0.05$). Among ten patients with granulomatosis with polyangiitis (Wegener's), anti-MPO antibody was detected in one patient and anti-PR3 antibody in 8 patients, while both two antibodies were detected in one patient.

When only MPO-ANCA and PR3-ANCA patterns were compared, a significant difference was found in favor of PR3-ANCA ($p=0.025$). However, no significant difference was found in terms of all 3 ANCA groups ($p>0.50$). When all vasculitis cases were assessed together by including patients with granulomatosis with polyangiitis (Wegener's), it was found that there was anti-MPO antibody in 4 patients and anti-PR3 antibody in 16 patients, while both two antibodies in two patients. There was a significant difference in favor of PR3-ANCA regarding ANCA patterns ($p=0.01$). Anti-MPO antibody was detected in six patients and anti-PR3 antibody in 5 patients among eleven patients with CRF. No significant difference was found regarding ANCA patterns ($p>0.05$). In pulmonary disease group, anti-MPO and anti-PR3 antibodies were detected in 7 and 3 patients, respectively. When all 3 ANCA groups were considered together, a significant difference was found in favor of MPO-ANCA ($p=0.047$). However, when we only compared MPO-ANCA and PR3-ANCA patterns, we failed to found a significant difference ($p>0.05$). In autoimmune diseases group, there was anti-MPO antibody in six and anti-PR3 antibody in two cases, whereas both two antibodies in one patient. No significant difference was found between ANCA groups ($p>0.05$). There was anti-MPO antibody in 4 of patients with cholelithiasis, while anti-PR3 antibody in two of them. No significant difference was observed between ANCA groups ($p>0.05$). For chronic liver diseases, anti-PR3 antibody was found in 3 patients with HBV origin, while anti-MPO antibody in one patient with HCV. Both two antibodies were detected in one patient with unknown origin. When ANCA patterns were assessed, no significant difference was found ($p>0.05$).

In patients with malign diseases, there was anti-PR3 antibody in two and anti-MPO antibody in two of these patients, whereas both anti-MPO and anti-PR3 antibodies in one patient. There was a significant difference in favor of MPO and PR3-ANCA between ANCA patterns ($p=0.005$). However, when only MPO-ANCA and PR3-ANCA patterns were included in comparison, no significant difference was found ($p>0.05$).

Among the patients in "other" group, anti-MPO antibody was found in patients with urolithiasis and gout, whereas anti-PR3 antibody in patients with ulcerative colitis and nasal polyp. Anti-PR3 positivity was found in single patient without a diagnosis. Specific antigens and ANCA patterns of ANCA positive patients according to diagnosis were shown in Table 1.

Table 1. Specific antigens in relation to diagnosis
(Tablo 1. Tanı ile ilşkili spesifik antijenler)

Diagnosis (number of patients)	MPO** (n)	PR3*** (n)	MPO and PR3 (n)
Vasculitis (12)	3	8	1
Chronic renal failure (11)	6	5	-
Granulomatosis with polyangiitis (Wegener's) (10)	1	8	1
Pulmonary diseases (10)	7	3	-
Autoimmune diseases (9)	6	2	1
Diabetes mellitus (6)	3	3	-
Cholelithiasis (6)	4	2	-
Chronic liver diseases (5)	1	3	1
Malignancy (4)	-	2	2
*Other (4)	2	2	-
Undiagnosed (1)	-	1	-

*Other; ulcerative colitis, nasal polyp, urolithiasis and gout disease.
**Myeloperoxidase
***Proteinase-3

When ANCA positive patients were assessed, MPO-ANCA pattern was identified in 21 patients (42.9%) and PR3-ANCA pattern in 24 patients (49%). MPO and PR3-ANCA pattern was identified in 4 patients (8.1%).

5. DISCUSSION (TARTIŞMA)

In ANCA evaluations, most commonly used tests are IIF and direct and capture ELISA [8]. IIF neutrophil preparations are more sensitive, but not specific, than ELISA techniques for ANCA determination. In small vessel vasculitis, IIF techniques should be confirmed by antigen-specific ELISA technique in order to enhance specificity [8 and 18]. Direct ELISA-ANCA tests show which target antigen is present as well as confirming IIF positive serum samples [8]. Savige et al reported that many laboratories screen samples by IIF first, then evaluate positive serum samples by direct ELISA for PR3 and MPO-ANCA in small vasculitis. Target antigens of ANCA is related to recurrence and clinical characteristics of disease. Therefore, knowledge about target antigens is also important in this regard [8]. In present study, all samples referred for ANCA evaluation were studied by IIF first and p-ANCA, c-ANCA or p and c-ANCA patterns were detected in 320 of samples. Two hundred fifty two of these samples were considered as either anti-nuclear antibody (ANA) positivity or undefined antigen positivity other than our ELISA-ANCA panel. PR3 and MPO or both were found in 68 of samples (4.1%) by antigen-specific ELISA-ANCA tests. Also in the our study, overall ANCA positivity was found as 4.1%, MPO-ANCA positivity as 1.7% and PR3-ANCA positivity as 1.9%. This condition, it may be depend on high rate of men in granulomatosis with polyangiitis (Wegener's) in our study.

Defendenti et al implied that 40 of 50 patient with positive ANCA were female [19] . In a study on 190 patients diagnosed as ANCA-related vasculitis, Chen et al reported that majority of patients were female [20]. In our study, it was seen that number of females were higher than males among patients with positive ANCA. Furthermore, when MPO-ANCA and PR3-ANCA patterns (2 groups; MPO or PR3) were compared in terms of gender, a significant difference was found in favor of PR3-ANCA in men (p=0.016).

Defendenti et al clinically and serologically evaluated 50 patients with positive ANCA and found anti-MPO antibody in 9 and anti-PR3 antibody in other 9 patients. Moreover, they reported presence of atypical ANCA pattern in 14 patients [19]. In a study, Chen et al reported p-ANCA positivity in 90% and c-ANCA positivity in 10% of patients [20]. In our patients, anti-MPO antibody was detected in 42.9% of 49 patients with positive ANCA; anti-PR3 was found in 49% of patients. Both anti-PR3 and anti-MPO were found in 8.1% of patients. Different rates between studies could be resulted from distinct patient groups.

In previous studies, it was reported that PR3 and MPO-ANCA ELISA tests were extremely sensitive and specific for diagnosing active small vessel vasculitis, but ability of detecting ANCA in IBD was variable [8 and 18]. It was also reported that c-ANCA pattern was predominantly seen in patient with granulomatosis with polyangiitis (Wegener's) [8 and 10]. Trevisin et al found c-ANCA pattern in 90% and p-ANCA pattern in 5% of patients with active granulomatosis with polyangiitis (Wegener's). They also reported that they found c-ANCA positivity in 71% and p-ANCA positivity in 8% of patients with treated granulomatosis with polyangiitis (Wegener's). In the same study, it was reported that p-ANCA pattern was seen in 85% patients with MPA, whereas c-ANCA pattern in 12%. Presence of p-ANCA was reported in 96% of patients with treated MPA. Moreover, c-ANCA was reported in 44% of patients with active necrotizing vasculitis, whereas p-ANCA in 56%. In addition, 18% positivity rate was found in healthy blood donors by IIF test. Of these, 6% was considered as c-ANCA, 9% as p-ANCA and 3% as ANA pattern [18]. In the our study, when only PR3-ANCA and MPO-ANCA patterns were compared in patients with granulomatosis with polyangiitis (Wegener's), it was found that there was a difference in favor of PR3-ANCA (p=0.025). When all vasculitis patients were considered together, a significant difference was found in favor of PR3-ANCA (p=0.01).

Besides vasculitic diseases, p-ANCA pattern was seen predominantly in autoimmune disease [6, 8 and 18]. In the our study, no significant difference was found between MPO-ANCA and PR3-ANCA in autoimmune disease group, cholelithiasis cases, diabetic patients and patients with chronic liver diseases. When all three ANCA patterns were compared, it was found that there was a significant difference in favor of MPO and PR3-ANCA in malignancies and in favor of MPO-ANCA in pulmonary diseases. When only MPO-ANCA and PR3-ANCA were compared, no significant difference was found between ANCA patterns in both groups. In their study, Chen et al found that renal and pulmonary involvement were similar in both ANCA groups. They emphasized that ANCA type, laboratory tests and clinical manifestations are helpful in diagnosing ANCA-related vasculitis [20]. Consistent with findings of Chen et al it was found that similar renal and pulmonary involvement in both ANCA groups, when those were compared only in MPO-ANCA and PR3-ANCA groups in our study (p>0.05). When all three ANCA pattern were compared, it was found that there was a significant difference in favor of MPO-ANCA

in pulmonary involvement (p=0.047). ANCA is positive in about 70% of patients with ulcerative colitis and, generally, p-ANCA pattern is seen [9 and 11]. Trevisin et al reported that they found c-ANCA in 35%, p-ANCA in 61% and atypical ANCA pattern in 4% of patients with IBD [18]. In our study, there was only one patient with ulcerative colitis and PR3-ANCA pattern was found in this patient. It is difficult to make a conclusion due to small number of patients.

6. CONCLUSIONS (SONUÇLAR)

ANCA has an important role in diagnosis of diseases which cause vasculitis. As ANCA could be positive in diseases which cause vasculitis, however, it should become positive in several diseases such as CRF, pulmonary diseases, autoimmune disorders, DM, cholelithiasis, chronic liver disease, malignancy, ulcerative colitis, nasal polyp, urolithiasis and gout diseases. Thus, considering solely as positive is inadequate in determination of ANCA. In ANCA positive cases, determining antibodies directed against specific antigens will enhance sensitivity.

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