ORIGINAL RESEARCH PAPER

Microbiology

Volume - 7 | Issue - 2 | February - 2017 | ISSN - 2249-555X | IF : 3.919 | IC Value : 79.96

PREVALENCE OF ANTINUCLEOLAR ANTIBODY PATTERNS(IFA) AND THEIR DIAGNOSTIC SIGNIFICANCE

KEYWORDS: SLE, SCLERODERMA, CONNECTIVE TISSUE DISEASE., ANTI- NUCLEOLAR ANTIBODIES, IMMUNOFLUORESCENCE, NUCLEOLAR SUBTYPES

Dr.PRAGATHI KOTTAPALLI
LECTURER, DEPARTMENT OF MICROBIOLOGY, NIZAMS INSTITUTE OF MEDICAL SCIENCES, PANJAGUTTA, HYDERABAD -500082, TELANGANA.

DR. TEJA VIJAY
PROFESSOR, DEPARTMENT OF MICROBIOLOGY, NIZAMS INSTITUTE OF MEDICAL SCIENCES, HYDERABAD- 500082, INDIA.

DR. LAKSHMI VEMU
PROFESSOR AND HOD, DEPARTMENT OF MICROBIOLOGY, NIZAMS INSTITUTE OF MEDICAL SCIENCES, HYDERABAD - 500082, INDIA.

Dr.LIZA RAJASEKHAR
PROFESSOR AND HEAD, DEPARTMENT OF RHEUMATOLOGY, NIZAMS INSTITUTE OF MEDICAL SCIENCES, HYDERABAD -500082, INDIA.

SUDHA TALASILA
LAB TECHNICIAN, DEPARTMENT OF MICROBIOLOGY, NIZAMS INSTITUTE OF MEDICAL SCIENCES, HYDERABAD -500082, INDIA.

ABSTRACT

AIMS : To determine the prevalence and clinical significance of anti-nucleolar antibodies in an unselected population.

MATERIALS AND METHODS: Sera positive for anti-nucleolar antibodies (ANoA) were identified during routine autoimmune screening by using indirect immunofluorescence technique. Demographic , clinical and laboratory data of patients were documented .

RESULTS : Prevalence of anti-nucleolar antibodies was 2.16%. The nucleolar subtypes noted were Homogeneous(52%), Speckled (30%),Clumpy(3%),Scl-70 (2%) and Mixed patterns(13%). Nucleolar Homogeneous pattern was the most common among Nucleolar patterns. SLE (12%) and Systemic sclerosis(8%) were the most commonly associated rheumatological conditions in patients with positive ANoA . Presence of ANoA in unselected sera is non-specific for Systemic Sclerosis.

CONCLUSION : Presence of anti-nucleolar antibodies is not uncommon in unselected sera. Neither the presence nor subtype of antinucleolar antibodies is specific for systemic sclerosis.

INTRODUCTION:

Antinucleolar antibodies (ANoA) are a type of antinuclear antibody directed against nucleolar proteins ,identified using indirect immunofluorescence (IIF). [10] Various proteins like nucleolin, fibrillarin, PM-Scl (spliceosome component), RNA-polymerases and annexins (ANX) II and V are present in the nucleoli [14]. These proteins are target molecules for autoantibodies produced in connective tissue diseases (CTD)-especially in scleroderma and overlap syndromes. Other associations include rheumatological and connective tissue diseases, infections and malignancies. Beck in 1961 first described the presence of antinucleolar antibodies (ANoA) in the sera of patients with systemic rheumatic disease .[3]

Bernstein et al described three distinct nucleolar immunofluorescent patterns ie, speckled, homogeneous, and clumpy [3]. Among these, homogeneous staining pattern is the most frequently found nucleolar pattern and can be found in a variety of autoimmune diseases and in apparently normal individuals. Homogeneous staining of the nucleoli associated with weak homogeneous or speckled staining of the nucleoplasm is seen in this pattern. It is characteristic of anti-PM-Scl 70 (scleroderma polymyositis overlap) and anti-Th/To- RNP (limited cutaneous SSc) antibodies [7,11,12]. Nucleolar clumpy pattern has high specificity for systemic sclerosis found approximately in 5% of patients. Fibrillarin or U3-snoRNP are the associated antigens in the Nucleolar clumpy pattern. Clustered large granules are seen in the nucleoli of interphase cells which tend towards homogeinity.There is no staining of nucleoplasm and in mitosis the condensed chromatin is stained [1,6,13].The speckled nucleolar staining pattern represents antibodies directed against anti-RNA polymerase I/III and has high specificity for Systemic sclerosis (4-20%). It is strongly associated with diffuse cutaneous systemic sclerosis.[5]

Nucleolus is the site for the biogenesis of ribosomes. Three of the four ribosomal RNAs (18S, 5·8S and 25/28S) are synthesized as one large precursor. Methylation of the precursor rRNA at specific position is mediated by one class of snoRNPs. Fibrillarin is also a protein subunit of the coiled body and is involved in ribosomal RNA processing. The box H/ACA snoRNPs (hGar1, Nap57/dyskerin, hNHP2 and hNOP10)have been implicated in the conversion of uridine residues in pre-rRNA to pseudouridines. The RNase MRP and RNase P complexes contain the Th/To autoantigen and these ribonucleoprotein particles function as endoribonucleases and help in the cleavage of pre-rRNA to mature rRNA and the processing of pre-tRNA, respectively .[11] In addition to antibodies against small nucleolar ribonucleoprotein complexes, antibodies to the PM/Scl100 autoantigen, are also known to result in nucleolar staining in immunofluorescence.

The purpose of our study was to determine the diagnostic significance of antinucleolar antibodies in unselected patients. In our study, we examined the demographic ,clinical and immunological features of all the patients found to be ANoA positive during routine anti-nuclear testing. This is important as clinicians use the ANA test indiscriminately and hence ANoA are found in patients thought not to have Scleroderma. However,in patients with strong suspicion of connective tissue disease classification of nucleolar subtypes can be done by the indirect immuno fluorescence technique and it may be a useful tool for confirming diagnosis and predicting prognosis of certain autoimmune diseases.

MATERIALS AND METHODS :

This was a retrospective study carried out between May 2014 and May 2015 (12 months). Sera from 2,776 consecutive patients with suspected or known rheumatic disease sent to the Microbiology laboratory at NIMS Hospital during a one year period were screened for the presence of the antinuclear antibodies by a standard indirect immunofluorescence technique. Demographic, clinical and

Volume - 7 | Issue - 2 | February - 2017 | ISSN - 2249-555X | IF : 3.919 | IC Value : 79.96

laboratory data of patients were documented . 5 ml of blood was drawn and sera were separated from the clotted blood samples of by centrifugation.Each of the serum samples was tested for ANA by Indirect immunofluorescence on HEp-2 cell line and ELISA for specific antibodies as requested by the clinician.

INDIRECT IMMUNOFLUORESCENCE USING HEp-2 CELLS :

Sera sent to the Microbiology laboratory for routine auto-immune screening were tested for the presence of anti-nucleolar antibodies (ANoA) by standard immunofluorescence technique using HEp-2 cells (EUROIMMUN, Lubeck,Deutschland) as substrate. Serum diluted in phosphate buffered saline, pH 7.4 at 1:100 dilution , was overlaid onto fixed HEp-2 cell and incubated for 30 minutes at room temperature. Slides were washed twice for five minutes each with PBS, overlaid with fluorescence labeled conjugate, which is antihuman IgG heavy and light chain specific and incubated for an additional 30 minutes. After washing twice, a coverslip was placed over the slide, and the slides were read using a fluorescence microscope at 40 power. The intensity of fluorescent nuclear staining was graded from 0-4 + using Ayrus Epi fluorescence microscope. Sera giving 1+ or greater nuclear pattern were considered positive. Interpretation of IIF patterns was performed independently by two different observers .

The nucleolar staining patterns were further classified according to a system described by Bernstein *el a/* [11] on the Antibodies Inc. HEp-2 slides. Titres greater than or equal to 1 in 100 were considered positive and classified into homogeneous, clumpy and speckled antinucleolar subtypes.

The serum specimens were tested for nucleolar staining morphology without the tester having any knowledge of the clinical situation. Subsequently, the clinical diagnoses and disease manifestations were compared with the different nucleolar staining patterns.

ANTIBODY DETECTION BY ELISA :

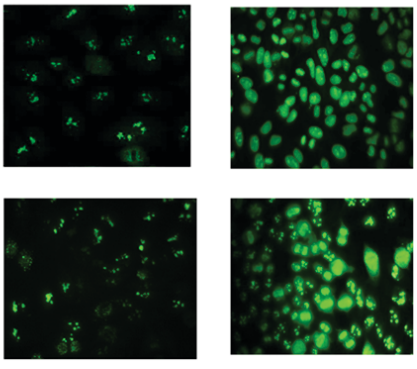
All sera positive for Anti-nucleolar antibodies were tested by ELISA (EUROIMMUN, Lubeck,Deutschland) for various antibodies.Tests were performed as per manufacturers instructions.

RESULTS:

ANoA were demonstrated in sera of 60/ 2,776 patients when HEp-2 cells were used as substrate.Prevalence of Nucleolar staining among the tested sera was 2.16%. Among the 60 patients with anti-nucleolar antibodies ,17 were males and 43 were females.Mean age of onset of the disease was 35 yrs.

Fig 1 shows nucleolar immunofluorescence patterns using Hep-2 cells. The nucleolar subtypes noted were Homogeneous(52%), Speckled (30%),Clumpy(3%),Scl-70 (2%) and Mixed patterns(13%).Subsequent testing for antibodies showed 5 patients with anti-dsDNA,1 patient with anti-Scl-70 and 1 patient with anti-jo antibodies. These patients had clinical diagnosis of SLE, Scleroderma and Dermatomyositis respectively.

FIG 1 NUCLEOLAR IMMUNOFLUORESCENCE PATTERNS USING Hep-2 CELLS A SUBSTRATE.



ANoA were more commonly found in patients with SLE (12%) and Systemic sclerosis(8%).Other connective tissue diseases with ANoA patterns were Myositis/Myopathies (8.3%) and Sjogrens syndrome (1.67 %).

Systemic sclerosis was evident in only 5 ANoA positive patients. Musculoskeletal and mucocutaneous features were more common among these patients. Out of 5 , 4 had limited cutaneous sclerosis(3-Nuclear Homogeneous,1- Nucleolar speckled) and 1 had Diffuse systemic sclerosis (Scl-70 pattern). None of these patients had any association with any specific ANoA pattern. Table 1 shows various non-specific conditions associated with antinucleolar antibodies.

Table 1 showing non-specific conditions associated with ANoA patterns.

S.NO	Clinical Disease	Homogeneous ANoA. (n=19)	Clumpy ANoA (n= 1)	Speckled ANoA (n=15)	Mixed Patterns (n=7)
1.	JUVENILE IDIOPATHIC ARTHRITIS	1	NR	1	NR
2.	VIRAL INFECTIONS	1	NR	2	NR
3.	STROKE	NR	NR	1	NR
4.	MALIGNANCY	2	1	NR	NR
5.	RENAL DISEASE	4	NR	4	2(N+S)
6.	PARKINSONS DISEASE	NR	NR	NR	1(N+S)
7.	KOCHS DISEASE	2	NR	2	1(N+S+CYTO)
8.	MYASTHENIA GRAVIS	1	NR	NR	NR
9.	CHRONIC LIVER DISEASE.	NR	NR	3	NR
10.	GUILLAIN BARRE SYNDROME.	1	NR	1	NR
11.	SENSORY NEUROPATHY	NR	NR	1	NR
12.	PERIPHERAL VASCULAR DISEASE.	1	NR	NR	1(H+N)
13.	PARA-INFECTIOUS ACUTE DEMYELINATING ENCEPHALOMYELITIS.	1	NR	NR	2(N+CYTO)
14.	INCONCLUSIVE.	5	NR	NR	NR

NR- Not reported N- Nucleolar pattern.
H- Homogeneous pattern. S- Speckled pattern.
Cyto- Cytoplasmic pattern.

In our study, musculoskeletal and mucocutaneous features were more common among patients with SLE and Scleroderma. Whereas general features like fever, eight loss,loss of appetite etc were more common among patients with non-specific conditions.

DISCUSSION:

The patterns of nuclear immunofluorescence using Hep -2 cells are not usually diagnostic but may help in identifying specific autoantibodies responsible for the pattern demonstrated by a particular serum.

The present study on the analyses of ANoA by IIF using Hep-2cells in an unselected patient population showed that presence of ANoA is not uncommon on routine auto-immune screening. Similar results were observed in a study where the prevalence of ANoA was 3.8% .[8] Other studies by Satoh M et al, Robert F. Ritchie, M.D and Suchela Janwityanuchit et al showed higher prevalence of 6.1%, 8.3 % and 4.9 % respectively. Probably this was because most of the sera in these studies were obtained from rheumatology department.

Previous studies showed that antinucleolar antibodies are widely

Volume - 7 | Issue - 2 | February - 2017 | ISSN - 2249-555X | IF : 3.919 | IC Value : 79.96

associated with Systemic sclerosis.[3,13]Systemic sclerosis is a connective tissue disease characterized by inflammation, fibrosis, and degenerative changes in the blood vessels, skin, synovium, skeletal muscle, and multiple internal organs. Several autoantibodies are associated with Scleroderma. Three antinucleolar antibodies have been identified- anti–U3 RNP, anti–PM-Scl, and anti-Th/To.[9]

Earlier studies showed the close clinical association of antigenic specificities of anti-nucleolar antibodies analysed by immunoprecipitation method, and indicated the usefulness of detecting these anti-nucleolar antibodies in subgrouping of patients with SSc.[5a,b] In the present study , ANoA were found to be associated with various non-specific conditions like Juvenile idiopathic arthritis, Poncets disease ,Rapid progressive renal failure with IgA nephropathy,Myasthenia gravis, pauci-immune glomerulonephritis, viral infections,Malignancy, Liver disease,Para-infectious Acute Demyelinating encephalomyelitis and Stroke.The results are consistent with previous studies .[10,11]The nucleolar pattern detected by IF could not be correlated with any specific antibody as sera were not tested by immunoprecipitation techniques.

Antinuclear antibodies (ANoAs) are not exclusive to Scleroderma patients as they can occur in other autoimmune diseases, such as systemic lupus erythematosus (SLE),sjogrens syndrome and Polymyositis/Dermatomyositis. Our results are consistent with one study on unselected sera where ANoA reactivity was more frequent among patients with SLE (3.8%) compared to Scleroderma (2%).[10] Other studies showed higher prevalence rates of 36% and 54 % among patients with Scleroderma [3] compared to SLE (35% and 26%). [3,4] According to some studies the significance of nucleolar staining and antinucleolar antibodies (ANoA) in SLE is still unknown.[2]

There are 3 distinct nucleolar staining patterns which may be associated with different rheumatic diseases as previously described by Bernstein et al.Nucleolar Homogeneous pattern was the most common nucleolar subtype identified in our study. Other investigators have reported previously that speckled nucleolar staining was the most common nucleolar subtype and was associated with systemic sclerosis. Clumpy nucleolar pattern was found in patients with non-rheumatic diseases such as Hashimoto's thyroiditis and interstitial pulmonary fibrosis. Homogeneous nucleolar pattern was found to be associated with polymyositis and polymyositis overlap syndromes. [3,10] But our study showed that presence of antinucleolar antibody and specific subtype has low specificity for Systemic sclerosis. Our results are concordant with study by S.Khan et al.

A recent review suggests that it is unnecessary to perform testing for antibodies against nuclear antigens by ELISA and immunoblot for routine detection of Scl-70 antibodies on ANoA+ samples. Reflex testing for various antibodies is not required. Subsequent identication of anti-nucleolar antibodies by ELISA is difficult as commercial kits are not available for most of the -nucleolar antigens. Western blotting and immunoprecipitation techniques are expensive and can only be performed in research institutes.One study on detection of small nucleolar ribonucleoproteins by immunoprecipitation showed that 40% of antinucleolar reactivity remains uncharacterized. Hence diagnostic specificity of these antibodies is low as evident from our study.[11]

As per our literature search there are very few studies on isolated antinucleolar antibodies and our study is the first of its kind on Indian population.

CONCLUSION:

Our data indicate that presence of anti-nucleolar antibodies is not uncommon in unselected sera Identification of antinucleolar antibodies by IIF is non-specific for systemic sclerosis in unselected sera. However,identification of specific antibodies provides valuable diagnostic and prognostic information in systemic sclerosis patients with evidence of organ involvement.

Reporting of nucleolar subtype is not required during routine screening for antinuclear antibodies by immunofluorescence technique.

REFERENCES:

1. Bernstein RM et al (1982), Association of antinuclear and antinucleolar antibodies in progressive systemic sclerosis. Clin Exp Immunol ; 48: 43-51.
2. C.M.Cabrera et al (2016),Serological profile and clinical features of nucleolar antinuclear pattern in patients with systemic lupus erythematosus from southwestern Spain. Lupus 0961203316629557, first published on February 3, 2016.
3. Janwityanuchit et al (1994) Antinucleolar antibodies and their disease association; Asian Pac J Allergy Immunol :43-9.
4. Jolly M et al(2003) , Are isolated antinucleolar antibodies a marker of scleroderma? J Clin Rheumatol. Oct;9(5):291-5.
5. a.Kuwana M et al (1993) ,Autoantibody reactive with three classes of RNApolymerases in sera from patients with systemic sclerosis. J Clin Invest ; 91: 1399-1404.
   b. Kuwana M1, Mimori T, Hama N, Kaburaki J, Okano T, Tojo T, Clinical significance of anti-nucleolar antibodies detected by immunoprecipitation method in patients with systemic sclerosis. Article in Japanese.
6. Ochs RL et al (1985) , Fibrillarin: a new protein of the nucleolus identified by autoimmune sera. Biol Cell ; 54: 123-133.
7. Reddy R et al (1983) ,Detection of a nucleolar 7-2 ribonucleoprotein and a cytoplasmic 8-2 ribonucleoprotein with autoantibodies from patients with scleroderma. J Biol Chem ; 258: 1383-1386.
8. Reimer G et al (1987) , Autoantibody to RNA polymerase I in scleroderma sera.J Clin Invest ; 79: 65-72.
9. Rohit Aggarwal et al (2009),Anti–U3 RNP Autoantibodies in Systemic Sclerosis: ARTHRITIS & RHEUMATISM Vol. 60, No. 4, pp 1112–1118.
10. S.Khan et al (2008) The clinical significance of antinucleolar antibodies, J Clin Pathol; 61:688.
11. Van Eenennaam H et al(2002) ,Autoantibodies against small nucleolar ribonucleoprotein complexes and their clinical associations. Clin Exp Immunol; 130: 532-540.
12. Yamane K (2001),Antibodies to Th/To ribonucleoprotein in patients with localised scleroderma. Rheumatology ; 40: 683-686.
13. Yang JM et al (2003), Human scleroderma sera contain autoantibodies to protein components specific to the U3 small nucleolar RNP complex. Arthritis and Rheumatism ; 48: 210-217.
14. Zabek J et al (2008) Krzewska I,Antinucleolar antibodies in diagnostics of antiphospholipid syndrome, Pol Arch Med Wewn. ;118 Suppl:25-30.
15. Robert F. Ritchie, M.D, Antinucleolar Antibodies — Their Frequency and Diagnostic Association. N Engl J Med 1970; 282:1174-1178May 21, 1970DOI: 10.1056/NEJM197005212822104.