

The contemporary role of antinuclear antibodies in early diagnosis of autoimmune rheumatic diseases

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Antinuclear antibodies (ANA) in blood serum remain the primary diagnostic screening test for systemic connective tissue diseases. This article presents recent literature findings concerning the utilization of ANA in clinical practice. Specifically, it focuses on interpreting analysis positivity, identifying clinically significant types of fluorescence, and categorizing ANA patterns according to specific nosologies. Recommendations for using the name HEp-2-IF instead of ANA and reporting the results of indirect immunofluorescence analysis for antinuclear antibodies on HEp-2 cell substrates are described in a standardized way, presenting immunofluorescence patterns together with the nomenclature of antibodies and informing about the subsequent management of the patient. Changes made to pattern classification to distinguish between competent and expert level patterns and to improve the visual separation between nuclear and cytoplasmic HEp-2 patterns are discussed.

The need for further study of the prevalence and clinical significance of rare ANA patterns, particularly those directed at the mitotic spindle apparatus (NuMA and MSA-2), is emphasized. Prospects for the study and use of autoantibodies against double-stranded DNA not only in diagnosis but also in the treatment of patients with SLE are noted.

It was concluded that there is a need for further clinical research, collection, and arrangement of various models of HEp-2 IF to facilitate the accurate determination of «criterion level» patterns, increase the possibilities of early diagnosis of rheumatological diseases, and improve the management tactics of patients in this category.

Keywords: Antinuclear antibodies, Antibody specificity, Patterns, Systemic connective tissue diseases.

Сучасна роль антинуклеарних антитіл у процесі ранньої діагностики аутоімунних ревматичних захворювань

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На сьогодні рівень антинуклеарних антитіл (ANA) у сироватці крові залишається одним з основних діагностичних скринінгових тестів при виявленні системних захворювань сполучної тканини.

У статті представлено новітні дослідження, наведені у літературі, з використання ANA у клінічній практиці, а саме: інтерпретація позитивності аналізу, виявлення основних клінічно значущих типів світіння, розподіл шаблонів ANA відповідно до конкретної нозології. Наведено рекомендації щодо застосування назви HEp-2-IF замість ANA, результати непрямого імунофлуоресцентного аналізу на антинуклеарні антитіла на субстратах клітин HEp-2 стандартизованим способом з подачею шаблонів імунофлуоресценції разом з номенклатурою антиклітин та інформування щодо подальшого менеджменту пацієнта.

Обговорено зміни, які стосувались класифікації патернів для розрізнення моделей компетентного та експертного рівнів та покращення візуального розподілу між ядерними та цитоплазматичними HEp-2 патернами. Наголошено про необхідність подальшого вивчення поширеності та клінічного значення рідкісних моделей ANA, зокрема спрямованих на веретеноподібний апарат мітозу (NuMA та MSA-2). Зазначено перспективи вивчення та застосування аутоантитіл проти дволанцюгової ДНК не тільки у діагностиці, але й у лікуванні хворих на СЧВ.

Зроблено висновок про необхідність подальшого проведення клінічних досліджень, збору та упорядкуванню різних моделей HEp-2-IF для сприяння точному визначенню патернів «критеріального рівня», збільшення можливостей ранньої діагностики ревматологічних захворювань та покращення тактики ведення пацієнтів даної категорії.

Ключові слова: антинуклеарні антитіла, специфічність антитіл, патерни, системні захворювання сполучної тканини.

The modern approach to managing patients with autoimmune rheumatic diseases requires early and precise diagnosis followed by promptly prescribing the most effective basic therapy. This tactic aims to halt the progression of the pathological process, prevent complications and achieve complete remission [1].

At the stage of diagnostic of the pathological process, providing screening serological tests is mandatory. One of the most crucial diagnostic analyses recommended by the international standardization committee remains the level of antinuclear antibodies (ANA) in blood serum [2, 3].

Antinuclear antibodies (ANA) constitute a heterogeneous group of autoantibodies that bind to cellular components of the nucleus, including proteins, DNA, RNA, and the nucleic acid-protein complex. Initially described in 1948, the identification of ANA served as the cornerstone for diagnosing autoimmune connective tissue diseases, particularly systemic lupus erythematosus (SLE), as more than 95% of patients with this condition exhibit positive ANA [4]. Positivity for ANA is also characteristic in certain infectious and endocrine diseases such as tuberculosis, HIV infection, diabetes mellitus, chronic active hepatitis [5]. Additionally, this

test can be useful for patients with venous thrombosis and allergic reactions [6, 7]. However, significantly high titers of ANA are specific to individuals with systemic connective tissue diseases [8–13].

Based on this, the European League Against Rheumatism (EULAR) and the American College of Rheumatology (ACR) proposed new classification criteria for SLE in 2019, where the initial criterion is the presence of ANA at a titer of 1:80 or higher on HEp-2 cells or an equivalent positive test [8].

Over 80 years ago, it was discovered that antinuclear antibodies not only play a role in the pathogenesis of diseases but also hold a significant place in the diagnosis and treatment of systemic connective tissue diseases. Throughout this time, the primary goal has been to enhance the specificity and sensitivity of diagnostic methods.

As of today, over 100 different types of antinuclear antibodies (ANA) have been described. ANAs are commonly categorized into two groups: antibodies against DNA and histones, and antibodies against nuclear material. Specifically, antibodies against DNA and histones encompass antibodies to double-stranded (native) DNA and antibodies directed against histones directly. The second group includes additional targeted nuclear antigens, the first of which was identified as anti-Sm, followed by others such as anti-SSA/Ro, anti-SSB/La, anti-U3-RNP, anticentromere, Scl-70, and Jo-1 [9, 14].

Currently, the spectrum of diagnostic methods includes the indirect immunofluorescence test (IF-ANA), indirect immunofluorescence method (IIF) with simultaneous examination of patterns and fluorescence intensity, measurement of antinuclear antibody titers, and enzyme-linked immunosorbent assay (EIA/ELISA). Consequently, today we can not only confirm the presence of antinuclear antibodies but also classify them into subtypes, determine their quantity, and specify their antigen specificity [10, 15].

It is also important to emphasize the complexity of interpreting a positive result in the ANA analysis in clinical practice, as ANAs are evidently present in systemic autoimmune rheumatic diseases (SARDs), but they can also be detected in many other conditions such as infectious diseases, inflammatory processes, and their detection tends to increase with age.

The indirect immunofluorescence test (IIF) used to detect ANA is a screening method. Therefore, whenever there is a positive or negative result accompanied by symptoms suggestive of SARDs, a solid-phase assay (SPA) should be performed to determine the specificity of autoantibodies [9].

In recent years, various solid-phase assays (SPAs) including ELISA, fluorescence enzyme immunoassay (FEIA), and chemiluminescent immunoassay (CIA) have been increasingly introduced into clinical laboratories for screening ANA-associated SARDs. They are also utilized to confirm the specificity of autoantibodies in cases of positive screening results. Furthermore, consideration should be given to both line and dot blots, although they are typically not used for screening ANA-associated SARDs. Instead, they are commonly employed for identifying specific autoantibodies [9, 16].

During the investigation using IIF, it was found that the serum of patients with autoimmune diseases exhibits distinct nuclear staining and selective fluorescence of certain structures. Each variety of antinuclear antibodies targets specific cellular sites, thereby reflecting the interaction of patient serum antinuclear antibodies with antigen-

containing structures within the cell. The use of cell lines such as HEp-2 allows for the detection of various patterns of nuclear and cytoplasmic staining [17].

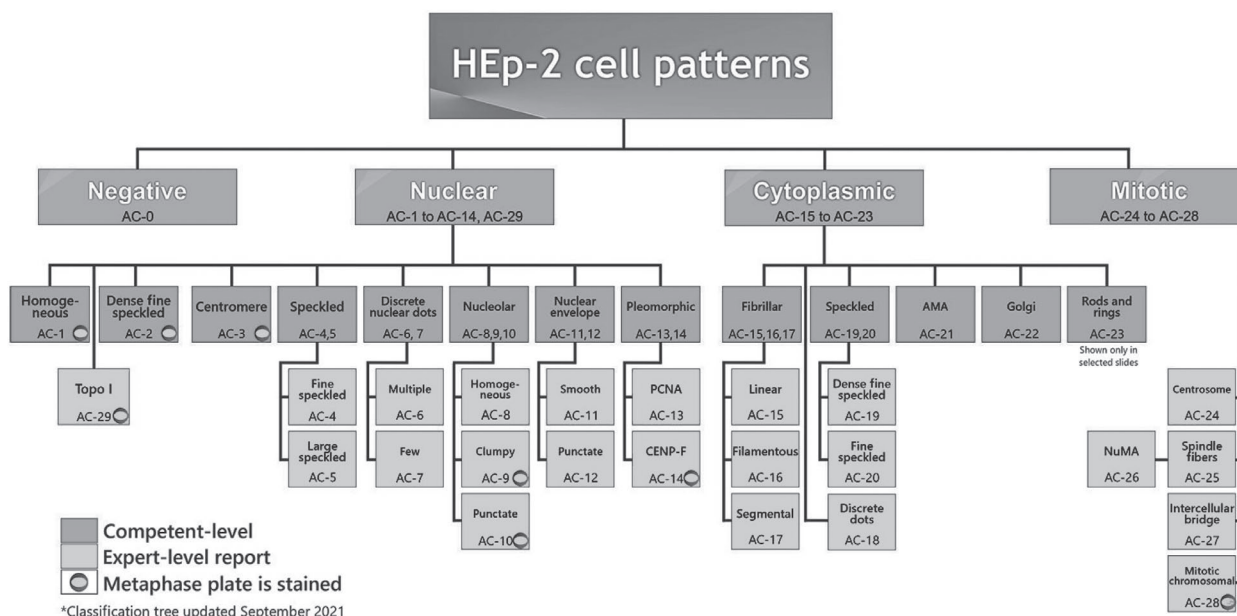
Currently, there are three main groups of antigens that define different fluorescence patterns: DNA-associated, RNA-associated, and membrane-associated. The nuclear fluorescence patterns, in turn, provide more information. For a long time, ANA fluorescence patterns were divided into 6 types: homogeneous, speckled, nucleolar, centromere, and cytoplasmic. Each type has one or several subtypes, for example: peripheral subtype for homogeneous, fine and large speckled subtype for speckled, ribosomal/synthetase, mitochondrial, and filamentous for cytoplasmic. Occasionally, dot patterns on the nucleus, proliferating cell nuclear antigen (PCNA) and non-specific centrioles may also be encountered [8].

For certain conditions, specificity in ANA fluorescence types and subtypes already exists. For instance, in systemic sclerosis, the homogeneous, nucleolar, and centromere subtypes are considered specific, while for SLE, the homogeneous and ribosomal subtypes are noteworthy. The nucleolar type and ribosomal subtype of cytoplasmic fluorescence may also suggest polymyositis. Autoimmune liver diseases are detectable using the peripheral homogeneous and filamentous subtypes of cytoplasmic fluorescence [18]. The mitochondrial subtype of cytoplasmic fluorescence is highly specific and indicative of autoimmune liver disease [19]. Fine speckled fluorescence has very low specificity and may indicate the presence of systemic rheumatic diseases, atopic dermatitis, psoriasis, or an oncological process [20].

Thus, identifying HEp-2 IIF morphological patterns in patient serum testing is not a definitive confirmation of systemic autoimmune rheumatic diseases (SARDs) and can be used for diagnosing various internal illnesses. In recent years, indirect immunofluorescent analysis using HEp-2 has extended beyond rheumatological conditions. For instance, detecting ANA in lymphoma patients, while not diagnostically valuable on its own, aids in the identification and prognosis of this condition, yet research continues [21]. Currently, the primary goal remains the unified and accessible classification of various ANA fluorescence patterns, contributing to the refinement of diagnostic algorithms.

In 2009, the American College of Rheumatology, in its position statement by the ANA Working Group, recommended the indirect immunofluorescence assay (IIF) using HEp-2 cells (human laryngeal epidermoid carcinoma cell line type 2) panel as the 'gold standard' for primary ANA detection. There is a continuous need to standardize methods for identifying autoantibodies and reporting, both in research settings aimed and in clinical laboratories. Some laboratories use quantitative immunological techniques employing a solid phase immunological assays for ANA detection, either as a reflex test to supplement the screening HEp-2 IIF test or even as a replacement for HEp-2 IIF testing. However, considering the high sensitivity and specificity of the HEp-2 IIF method for detecting ANA, it remains a crucial diagnostic method [22].

The Committee on ANA Serology (Committee on Standardization of Autoantibodies in Rheumatic and Related Diseases) was established in the early 1980s to standardize human autoimmune serum, crucial for the operations of



ICAP. Nomenclature and classification tree (ANApatterns.org)

scientific and clinical laboratories. Today, the organization operates under the abbreviated name – the Committee on Standardization of Autoantibodies (ASC). The primary goal of ASC is to enhance the accuracy of autoantibody testing and ensure the highest standards of patient care. The official website of ASC www.AutoAb.org serves as a free resource providing general information about the committee’s activities and grants access to a list of 23 available reference reagents (www.AutoAb.org under the «Reference Materials» section), which are distributed free of charge among scientific and diagnostic laboratories, as well as commercial enterprises involved in the development of autoantibody diagnostic kits. These reagents are also distributed by the Plasma Services Group (www.plasmaservicesgroup.com) on a non-commercial basis [16, 23].

One of ASC’s achievements is the establishment of the International Consensus on ANA Patterns (ICAP) initiative during the 12th International Workshop on Autoantibodies and Autoimmunity held in São Paulo, Brazil, in 2014. There was proposed, described, and categorized HEp-2 morphological patterns into three major groups: nuclear, cytoplasmic, and mitotic, classifying a total of 29 patterns (Figure) [18, 20].

The goal of ICAP is to facilitate harmonization and comprehension of the nomenclature of HEp-2 IIF morphological patterns and optimize their usage by providing recommendations for interpreting research findings. Apart from fluorescence intensity or titers, the test also offers a fluorescence pattern, encompassing the nucleus and cytoplasm of interphase cells, along with structures associated with mitotic cells [24]. The panel of autoantibodies detected in the HEp-2 IIF test can be more accurately described as antibodies targeting cellular antigens or, as recently recommended by ICAP, anti-cellular antibodies [22, 25].

The initiative continued its work until September 4, 2019, during the XXXVI Brazilian Congress of Rheumatology held in Fortaleza (CE, Brazil). The latest ICAP seminar took

place on September 6, 2021, in Dresden, where updated classification tables with several enhancements were presented. The ICAP consensus recommended replacing the outdated term ANA with HEp-2-IIF, and the results of indirect immunofluorescence analysis for anti-nuclear antibodies on HEp-2 cell substrates should be reported to clinicians in a standardized manner. This involves providing immunofluorescence patterns along with the nomenclature of anti-cellular antibodies and informing about further patient monitoring or reevaluation [26].

Changes also pertained to the classification of patterns. The previous pattern distribution model was revised to provide better visual distinction between nuclear and cytoplasmic HEp-2 patterns, as well as clear differentiation between competent and expert level patterns [22].

It is worth noting that competent-level patterns are those intended to be easily recognizable. The distinction between expert and competent-level patterns is based on their clinical significance and ease of identification [23, 25]. Hence, to achieve a clear differentiation between different pattern levels, several changes have been implemented. Firstly, general nuclear membrane (AC-11,12) and pleomorphic (AC-13,14) patterns, easily recognizable and clinically relevant, have been classified under the competent level, while identifying their specific subtypes still remains an expertise-level task. Secondly, the nuclear dense fine speckled (AC-2) and Topo I-like (AC-29) patterns on the scheme are placed closer to the homogeneous nuclear pattern (AC-1) to emphasize their similarity in staining both interphase nuclei and mitotic chromatin condensation. These alterations have led to the highlighting of a distinct nuclear dense fine speckled pattern (AC-2) at the competent level and a separate pattern – topoisomerase 1 (Topo I-like / AC-29) – at the expertise level [27, 28].

Fine speckled (AC-4) and large speckled (AC-5) patterns remain subtypes of nuclear speckled fluorescence at the expert level. Such categorization is more practical as it

aligns with the understanding that many laboratories do not differentiate between AC-4 and AC-5 but do differentiate between AC-2 and AC-29. Thirdly, the cytoplasmic discrete dots (AC-18) pattern is separated from the cytoplasmic dense fine speckled (AC-19) and cytoplasmic fine speckled (AC-20) into a distinct type of cytoplasmic fluorescence based on the visual difference between AC-18 and the more similar AC-19 and AC-20. The AC-18 pattern remains at the expert level [22, 29].

Also of importance is that a positive result of HEp-2 IIF with the AC-2 pattern (or some other rare patterns) may not necessarily reflect a «positive ANA test». Similar reasoning likely applies to other patterns such as centromere (AC-3), discrete nuclear dots (AC-6 and AC-7), nucleolar (AC-8, AC-9, and AC-10), CENP-F-like (AC-14), as well as certain patterns of cytoplasmic and mitotic apparatus. For instance, anti-centromere antibodies are detected in less than 2% and anti-nucleolar antibodies are found in less than 10% of sera with SLE, but when detected, they are usually associated with overlapping syndromes (i.e., presence of antibodies to PM/Scl and myositis, Reynaud's phenomenon).

Or the NuMA-like pattern (AC-26), which has been identified in some patients with SLE, yet more commonly observed in patients with Sjögren's syndrome. HEp-2 IIF patterns represent a wide spectrum of autoantibodies and their specific molecular targets, but only a minority of them are associated with SLE. Thus, generalizing any HEp-2 IIF pattern used as a criterion for classifying a patient as having SLE may be misleading since patterns associated with SLE (for example, AC-1 homogeneous nuclear or AC-5 nuclear large speckled) could be equally assessed as patterns unrelated to SLE (for example, nuclear dense fine speckled). A specific study aimed at determining the frequency of HEp-2 IIF patterns in a large cohort of SLE patients and appropriate controls should contribute to identifying which patterns should be considered a «benchmark» for SLE classification [30].

During the analysis of a large cohort of ANA tests, rare patterns were identified. The prevalence and clinical significance of these unusual patterns targeting the spindle mitotic apparatus (MSA) are currently insufficiently studied. According to retrospective studies, the most frequent anti-MSA patterns were nuclear mitotic apparatus (NuMA) and MSA-2. The NuMA pattern was associated with the highest ANA levels and was most commonly encountered in Sjögren's syndrome, rheumatoid arthritis

(RA) and SLE. Undifferentiated connective tissue disease (UCTD) was linked to centrosome patterns ($P < 0.001$), NuMA ($P < 0.02$), and MSA-2 ($P < 0.45$) [30, 31]. Such rare ANA subpatterns as mitotic spindle, cytoplasmic anti-mitochondrial antibodies, and discrete nuclear dots were more prevalent in women than in men and were most significantly associated with SLE and RA among the most common autoimmune diseases [32–34].

Despite the low detection rate of rare HEp-2 IIF patterns in the studied cohort, their prevalence in the search for unusual ANA patterns turned out to be higher than expected. Additionally, there are various autoantibodies of unknown significance that require further investigation [21, 35, 36]. In our opinion, it is worthwhile to conduct further studies to explore these patterns along with their corresponding antibodies to elucidate their roles and potential applications in clinical practice.

Studying antibodies against double-stranded DNA (dsDNA) in patients with SLE is interesting and promising. Their levels fluctuate depending on the activity of the pathological process and contribute to various internal organ damages, particularly in lupus nephritis. The discovery that antibodies against dsDNA have the capability to trigger the complement cascade, modulate gene expression, and induce fibrotic processes' activation is significant. Their involvement in the hydrolysis of DNA and peptides within cells is crucial for understanding the pathogenesis of SLE. Scientists believe that blocking these antibodies might prevent the progression of the pathological process, prompting research into antibodies against dsDNA as a therapeutic target in treating SLE [37–40].

Thus, in summary, achieving consensus on the nomenclature and definitions of HEp-2 IIF patterns to date enables the standardization of pattern descriptions across various laboratories. The integration of computer-assisted immunofluorescence microscopy (CAIFM) further enhances consistency in pattern designation [24, 41, 42]. Continuing the collection, refinement, and dissemination of information regarding the clinical significance of HEp-2 IIF patterns could significantly assist clinicians in expediting diagnostic searches, early detection, and treatment of patients with systemic connective tissue diseases, as well as in monitoring patients with atypical clinical presentations. Integration of HEp-2 IIF pattern information may also help identify novel prognostic factors for rheumatological diseases and refine diagnostic and treatment algorithms for patients of this category.

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