Antinuclear Antibodies as Potential Markers of Lung Cancer

Félix Fernández-Madrid, Pamela J. VandeVord, Xin Yang, Robert L. Karvonen, Pippa M. Simpson, Michael J. Kraut, José L. Granda, and John E. Tomkiel

Department of Internal Medicine, Divisions of Rheumatology [F. F.-M., P. J. V., X. Y., R. L. K., J. L. G.] and Hematology/Oncology [M. J. K.], Wayne State University School of Medicine; Department of Pharmacology, Children’s Hospital of Michigan [P. M. S.]; and Center for Molecular Medicine and Genetics [J. E. T.], Detroit, Michigan 48201.

ABSTRACT

There are multiple case reports of antinuclear antibodies (ANAs) in patients with malignancies, yet to date there has not been a systematic survey of ANAs in lung cancer. We have previously reported that autoantibodies to collagen antigens resembling those found in the connective tissue diseases are consistently detected in the sera from lung cancer patients. In this work, we looked for the presence of ANAs in the sera from these same patients. Sera from 64 patients with lung cancer and 64 subjects without a history of cancer were retrospectively tested for reactivity on immunoblots of nuclear extracts of HeLa, small cell carcinoma, squamous cell carcinoma, adenocarcinoma, large cell carcinoma of the lung, and of normal lung cells. Associations were sought between the reactivities on immunoblots and lung cancer cell type, diagnosis, and progression-free survival by the method of classification and regression trees (CARTs). Cross-validated CART analyses indicated that reactivities to certain bands in immunoblots are associated with different types of lung cancer. Some of these autoantibodies were associated with a prolonged survival without disease progression. Our data suggest that autoimmunity is often a prominent feature of lung cancer and that molecular characterization of these antigens may lead to the discovery of proteins with diagnostic and prognostic value.

INTRODUCTION

The humoral response in cancer has not received a great deal of attention recently because the quest for tumor-specific antigens has been largely long and fruitless (1–3). However, T-cell antigens have long been recognized to exist on tumor cells (4–6), and a cellular immune response without a concomitant humoral response would be unusual. There are many reasons potentially explaining why tumor-specific antigens have not been found. Most of these relate to the methods used to detect the autoantibodies. Some studies have restricted the search to antigens thought to be present only in tumor cells (2, 3), although it is possible that malignant cell antigens may also be expressed by normal cells. Experience has indicated that even the most restricted tumor antigens generally turn out to be restricted, normal differentiation antigens (7). Other studies have looked for antigens specific for a certain type of malignancy (3, 8–11). It is possible that the many tumor antigens may not be absolutely specific to the type of tumor, but may be expressed in other related malignant cells as well. Another reason has been the focus of previous studies on cell-surface antigens rather than on intracellular antigens (2, 7). A limited number of studies indicate that at least some tumor antigens may be altered forms of cell-cycle regulatory proteins, normally found in the nucleus (12–14). Other studies (15, 16) have focused on antigens known to drive the immune response in the systemic autoimmune diseases (17–19), although there are no a priori reasons suggesting that the same antigens will be associated with cancer. Finally, the possibility that certain antigens may be species- or cell type-specific has been ignored in previous studies using rodent or other unrelated nuclear antigens to detect autoantibodies in cancer sera (15, 20–40).

In view of this background and our previous observations that autoantibodies to collagen antigens are frequently present in the sera of lung cancer patients (41), we decided to reexamine the humoral response in lung cancer. We chose to test for ANAs in cancer sera by using a variety of substrates, posing no constraints on the selection of antigens that may or may not be unique to tumor cells. We used normal as well as specific lung cancer cell types to detect ANAs on immunoblots. Here we report the results obtained by examining associations between ANAs and diagnosis of lung cancer, tumor cell type, and outcome measures.

MATERIALS AND METHODS

Study Population. Sixty-seven consecutive patients who presented to the Detroit Medical Center Hematology/Oncology Clinic at Wayne State University with biopsy-proven, locally advanced or metastatic lung cancer (American Joint Committee on Cancer stages III A, III B, and IV), including both small cell and non-small cell histologies, agreed to participate in this study (41). Only stages III and IV presented in this group. From the original 67 patients, 64 sera obtained before the initiation of therapy were available. Sera were also obtained from 64 con-

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1 This work was supported by the Flora Temple Fund.

2 To whom requests for reprints should be addressed, at Hutzel Hospital, Center for Rheumatic Diseases, 4707 St. Antoine, Detroit, MI 48201.

3 Present address: CARE, 1120 Marshall, Little Rock, AR 72202.

The abbreviations used are: ANA, antinuclear antibody; CART, classification and regression tree; CREST, calcinosis, Raynaud’s phenomenon, esophageal dysmotility, sclerodactyly, telangiectasia; PFS, progression-free survival; SEREX, serological screening of cDNA expression libraries.
sericutive subjects without a history of cancer. These patients were recruited from the Wayne State University rheumatology clinic and were diagnosed with conditions not known to have autoimmune pathogenesis (either osteoarthritis or fibromyalgia). Demographics and clinical characteristics of these groups are included in Table 1. Patient clinical characteristics including stage and treatment protocols have been reported previously (41). Informed consent was obtained from both patients and control subjects. Serum samples were obtained before the initiation of therapy and stored at −70°C until use.

**Cell Lines.** Human lung cell lines and HeLa cells, obtained from the American Type Culture Collection (Rockville, MD), were cultured according to specifications described for each cell line. Lung cell lines, HTB-119 (small cell carcinoma), HTB-182 (squamous cell carcinoma), HTB-177 (large cell carcinoma), CRL-5800 (adenocarcinoma), and HTB-157 (normal lung) were used. The HTB-157 cell line was developed from the lung of a presumptively normal, Caucasian male fetus in the second trimester. The morphology is fibroblast-like. The number of serial subcultures from the tissue of origin was eight, and in our lab, it was subcultured five times in monolayer to produce enough cells to obtain nuclear extracts for the immunoblot experiments. This is a normal diploid human cell line with 46 XY karyotype and is not a transformed cell line. The human origin was confirmed by isoenzyme analysis, and tests for Mycoplasma, bacteria, and fungi were negative. This cell line is labeled by the American Type Culture Collection as lung, normal fetus, and human.

**Nuclear Extracts and Immunoblotting.** Nuclear extracts were prepared as described previously (42) and separated by SDS-PAGE (43). Gels of different percentages were run to maximize discrimination between antigens of both low and high molecular weights. Proteins were transferred to nitrocellulose as described by Towbin et al. (44). Patient sera were diluted 1:150 in buffer [10 mM NaPO4 (pH 7.5), 0.2% Triton X-100, 0.15 mM NaCl, 1 mM EGTA, and 1 mM NaN3], incubated with membranes at 22°C for 2 h, and then washed 3 times for 3–5 min with 1XGB [50 mM triethanolamine-HCl (pH 7.4), 100 mM NaCl, 2 mM K2-EDTA, 0.5% Triton X-100, and 0.1% SDS]. Sheep antihuman IgG (Amersham, Arlington, IL) and goat antihuman IgM (Sigma, St. Louis, MO) horseradish peroxidase-linked secondary antibodies were used at 1:2500 for 1 h at 22°C, and washes were repeated. Bound antibodies were detected by enhanced chemiluminescence (ECL) reagents (Amersham). Exposure time was 60 s. Developed films were scanned by computer analysis. Intensities and band positions were determined using IPLab Spectrum software (Scanalytics, Fairfax, VA). All of the bands with pixel intensities 20 units more than background pixel intensity were scored as positive. We chose this arbitrary cutoff because under these conditions nuclear reactivities were, on average, absent in more than 97% of normal sera. Although 67% of the cancer sera antibodies showed reactivity even at 1:2000 dilution, only one normal serum showed a titer greater than 1:500 using these criteria. Band detection was consistent when compared by three different operators.

Calibration of immunoblots was performed using molecular mass standards (Life Technologies, Inc., Gaithersburg, MD). A negative serum from a noncancer patient was tested in each assay to control for background. An autoimmune CREST syndrome serum was reacted with each substrate as a control to verify protein integrity, transfer, and activity of detecting reagents. Molecular masses of antigens larger than 40 kDa were estimated to the nearest 5 kDa, and molecular masses smaller than 40 kDa were estimated to the nearest 2 kDa. Consistent estimates of molecular masses were obtained by two or more independent measurements.

**Statistical Analyses.** The ability of certain ANAs detected on immunoblots to predict lung cancer cell type and diagnosis was determined by CART analysis as described previously (45, 46) using CART software (Salford Systems, San Diego, CA). CART is particularly useful when there are many variables with a high degree of association among themselves, in this case the ANAs. Additionally, CART does not assume that a function of a linear combination of covariates affects the outcome (45–47). This method of CART analysis was specifically developed to allow cross-validation within the same data set (45–47). Learning and cross-validated trees were grown using 10-fold validation [as recommended by Breiman et al. (45)], which conservatively overestimates the true error rate. The overall correctly predicted percentages of the learning and cross-validated trees were determined as well as the predictive antigens in their descending order of importance (in regard to the particular tree structure) as selected by CART (45–47). For the trees predicting diagnosis, sensitivity is defined as the percentage of patients with disease that were correctly predicted as having lung cancer. Specificity is defined as the percentage of normal subjects that were correctly predicted as not having lung cancer. The ability of the ANAs in predicting PFS was also determined by CART. PFS was analyzed by CART as a categorical variable grouped as follows: 0, up to 6 months; 6, up to 12 months; and >12 months.

**Table 1** Patient and reference group demographics

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Mean age (range)</th>
<th>Sex %</th>
<th>Race %</th>
<th>Histology</th>
<th>Mean pack-years* (range)</th>
<th>% Never smoked*</th>
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</thead>
<tbody>
<tr>
<td>Lung cancer</td>
<td>64</td>
<td>62.2 (31–76)</td>
<td>M 49</td>
<td>W 61</td>
<td>S 13</td>
<td>48.2 (0–120)</td>
<td>8.9</td>
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<td></td>
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<td>F 51</td>
<td>B 39</td>
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<tr>
<td>Reference subject</td>
<td>64</td>
<td>56.1 (22–94)</td>
<td>M 36</td>
<td>W 59</td>
<td>10.0</td>
<td>10.0 (0–75)</td>
<td>55</td>
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<td></td>
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<td>F 64</td>
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* Forty-five of 64 lung cancer patients and 61 of 64 reference subjects have complete smoking history.

W, white; B, black; O, oriental; S, small cell carcinoma; Q, squamous cell carcinoma; A, adenocarcinoma; L, large cell carcinoma.
Kaplan-Meier analysis (48) was used to compare the effect of the presence of combinations of antigens on PFS, and the equality of the distribution was determined by log-rank comparison. Kaplan-Meier and other statistical analyses were performed using SPSS for Windows Version 8.0 (SPSS Inc., Chicago, IL).

We performed CART analysis on nuclear reactivities that were “unique” to each nuclear extract. We defined unique nuclear reactivity as the binding of an antibody of the IgG or IgM class to an antigen band present in only one of the nuclear extracts (HeLa, normal lung, small cell carcinoma, squamous cell carcinoma, adenocarcinoma, or large cell carcinoma).

RESULTS

Autoantibodies to Nuclear Antigens in the Sera of Lung Cancer Patients. We probed immunoblots of nuclear protein isolated from HeLa, normal lung cells, small cell carcinoma, squamous cell carcinoma, adenocarcinoma, or large cell carcinoma with sera from both cancer patients and normal controls. The use of all of the four major lung cancer cell types as well as normal lung cells and a nonlung cancer malignant cell line (HeLa) allowed us to detect autoantigens that were uniquely or preferentially expressed in cancer cells. For purposes of the analyses that follow, we were interested in merely identifying differences in band sizes on immunoblots. We made no assumption about the identities of each antigen because more than one nuclear antigen can exhibit the same electrophoretic mobility.

We compared the antigens recognized by lung cancer sera on nuclear extracts from each cell line and were able to identify autoantigens that were unique to HeLa cells, to normal lung cells, or to cells derived from each of the lung cancer cell types. A representative immunoblot is presented in Fig. 1.

Nuclear Antigen Reactivity as a Potential Predictor of Lung Cancer Cell Type. We tested whether nuclear reactivities might be useful predictors of lung cancer cell type. Cross-validated CART analysis was used to determine the ability of different sets of unique nuclear antigens to predict cancer cell types (Table 2). The cross-validated CART analysis using all of the six sets of antigens selected nine antigens, designated hg90, sm70, hg110, lg160, hg65, qg85, ag180, ng55, and sg30, as the most useful predictors with a 50% overall correctly predicted cancer cell type (classification tree presented in Fig. 2). The presence of three of the antigens (hg110, lg160, and hg65) was associated with small cell carcinoma, one (qg85) with squamous cell carcinoma, three (ag180, ng55, and sg30) with adenocarcinoma, and two (hg90 and sm70) with large cell carcinoma. Fig. 2 shows that antibodies binding a limited number of selected antigens predict cancer cell type with a 50% accuracy, well beyond the 25% attributable to random chance (Table 2). Examining the patient numbers in the nodes of the tree for Fig. 2 shows that predictions about our patient population can be made with great accuracy using the selected antibodies. These data clearly indicate that groups of nuclear antigens recognized by autoantibodies have the potential to discriminate among different cell types within a group of lung cancer patients.

Nuclear Antigen Reactivity as a Potential Predictor of Lung Cancer Diagnosis. We also tested whether nuclear reactivities might be useful predictors of lung cancer diagnosis. The cross-validated CART analysis using all of the 6 sets of
Three of the antigens associated with the diagnosis of lung cancer were also associated with small cell carcinoma (lg160) and adenocarcinoma (ag180 and ng55), respectively, as shown in Table 2. Once again, examining the subject numbers in the nodes of the tree for Fig. 3 shows that predictions about our subject population can be made with great accuracy using the selected antibodies.

**Nuclear Antigen Reactivity as a Potential Predictor of PFS.** Cross-validated CART analysis was used to determine the ability of different sets of unique nuclear antigens to predict PFS (Table 2). The cross-validated CART analysis using all of the six sets of antigens selected nine antigens, designated lm160, am45, ag150, lg180, ng30, qg160, hg65, am70, and sm220, as the most useful predictors with a 52% overall correctly predicted PFS range (antigen designations in Table 2). Four of the antigens (lm160, ng30, qg160, and sm220) were associated with a longer survival without progression (>9 months); four of the antigens (am45, lg180, hg65, and am70) were associated with an intermediate length of survival without progression (4–9 months); and one antigen (ag150) was associated with a shorter survival without progression (<4 months). Kaplan-Meier analysis of PFS revealed that subjects whose sera had antibodies recognizing one or more of antigens lm160, ng30, qg160, or sm220 (median PFS of 403 days), had a higher probability ($P = 0.01$) of surviving without progression than those lacking these antigens with a median PFS of 111 days (Fig. 4), which suggests a potential prognostic value. Although there was a significant difference between these curves, it should be noted that, for the entire observation window, they terminated at similar times (921 and 958 days, respectively), which suggests that patients possessing these antibodies had short-term benefits that eventually dissipated. The ability of these antibodies to detect differences between these two groups of patients is striking in view of the dismally short survival of the whole study group.

The disease stage at the time of diagnosis and treatment obviously may affect PFS. The stage 3 patients in our study were treated with radiation and chemotherapy, and stage 4 patients were treated with chemotheraphy alone (41). We found no association between groups of antigens with disease stage or treatment. However, larger patient numbers that can be further stratified will be needed to study this question in more depth.
Nuclear Antigen Reactivity as a Potential Predictor of the Diagnosis of Particular Cell Types of Lung Cancer. To demonstrate the occurrence of nuclear antigen reactivities specific and/or common to different cancers, we assumed for our next analysis that the four different cell types of lung cancer are independent and unrelated forms of cancer. Separate CART analyses were performed on the set of control subjects and each of the patient groups with the four cell types to identify variables that can predict diagnosis of each cell type. Variables identified by CART analysis as important in predicting each lung cancer cell type as a specific independent cancer and their overlapping relationships are represented in Fig. 5. Some nuclear antigen reactivities were identified as predictors specific to one cancer type; some were identified as predictors common to two cancer types; and some were identified as predictors common to three cancer types (Fig. 5). These results demonstrate the specific and common nuclear antigen reactivities that can apparently occur between different types of cancers. Some of these nuclear antigen reactivities (as annotated in Fig. 5) were previously identified (Table 2) as predictors of lung cancer diagnosis for the total group of lung cancer patients, which further suggests their potential importance. Similarly, some of these nuclear antigen reactivities were previously identified (Table 2) for the total group of lung cancer patients as predictors of cancer cell type and PFS.

DISCUSSION

Lung cancer is the most frequently diagnosed major cancer in the world (49). Although there are many reports of positive ANA tests in patients with malignancies (23–40, 50, 51), including lung cancer (29), this is the first report on the consistent presence of ANAs in the sera of patients with lung cancer. In this work, we found that most sera from patients with lung cancer presented significant nuclear reactivity at a 1:500 dilution. It is likely that our data reported on unique nuclear reactivity are conservative because our nuclear antigens were derived from single cell lines. Certain antigens may not be detectable in the particular cell lines that we used but, nevertheless, may be present in a patient’s own tumor cells. It is also possible that the same autoantigens may be processed differently in normal and cancer cell lines, for example, by specific protein degradation or by posttranslational modifications, and these differences may alter mobility in SDS-PAGE. These variations would result in recording those antigens as different, thus diluting the strengths of the associations.

Fig. 3 Diagram of the CART tree of lung cancer diagnosis between 64 lung cancer sera and 64 reference sera using all of the six antigen sets as shown in Table 2. Antigen variables selected by CART analyses are presented and labeled as follows: first letter (h, s, q, a, l, n, as in Table 2), the antigen set; second letter (g or m), the recognizing isotype IgG or IgM; and the following number, the antigen size in kDa. The number of subjects with correctly predicted diagnosis over the total number is included for each terminal node.

Fig. 4 Kaplan-Meier estimate of the survival distribution function, a measure of the probability that an individual survives past a given time without progression, plotted against the PFS in days. The black curve (■) represents subjects with serum antibodies that bind one or more of the nuclear antigens lm160, ng30, qg160, or sm220. The presence of these antibodies predicted a survival without progression >9 months by CART analysis in Table 2. The gray curve (○) represents those with serum antibodies that did not bind the first four antigens but did bind one or more of the nuclear antigens am45, lg180, hg65, or am70. The presence of these antibodies predicted a survival without progression from 4 to 9 months. Those without serum antibodies binding any of these eight antigens (□) had a predicted survival without progression of <4 months. Testing for equality by log rank indicated a significant difference in survival distribution between the curves (P = 0.006). Antigen nomenclature is presented in Table 2.

Antigen nomenclature is presented in Table 2.
We attribute our success in demonstrating such reactivity in the sera of patients with lung cancer to our use of both normal and malignant cell nuclear extracts as substrates and to the use of the more sensitive immunoblotting method. This approach allowed us to analyze the data statistically using CART.

We considered alternative explanations that could influence our results, including patient age and tobacco use. The lung cancer patients and reference group are well matched by gender and race (Table 1). There is a small difference in mean age between the groups, but this difference is unlikely to have affected our results. Although autoantibodies are more prevalent in the elderly population (52), they are usually of low titer, unlike those reported here. The large difference in smoking habits between our groups could potentially be more important because cigarette smoking is a major determinant of lung cancer (53). Although there are several reports of DNA damage caused by tobacco-specific pulmonary carcinogens (53–55), autoimmune phenomena potentially caused by cigarette smoking has received little attention. Recently, Pulera et al. (56) reported on the presence of serum anti-benzo(a)pyrene diol epoxide-DNA adduct antibodies in smokers. It is conceivable that ANA development in heavy smokers may be an early indication of DNA damage in susceptible subjects. However, even if smoking is able to induce autoimmunity, this does not fully explain our results. The ability of ANAs to discriminate among the four major types of lung cancer as well as the association of ANAs with PFS are both independent of the reference sera used in our study. The relationship of smoking to autoimmunity remains an important issue that deserves investigation in a prospective study.

ANAs found in malignancy have been regarded as an expression of autoimmunity that may occur sporadically, but the mechanism by which it develops is not understood (15, 20, 52). Although ANAs in systemic autoimmune diseases have shown considerable disease specificity, ANAs in cancer are thought to be characterized by reactivity to generally distributed nuclear antigens (18, 52). However, our data demonstrate that a large proportion of these patients have autoantibodies that seem to react preferentially with normal lung, HeLa, and different lung cancer cell nuclear antigens.

Statistical analysis of our total lung cancer group using cross-validated CART to predict cancer cell type suggests that our nuclear antigen sets have the potential to reveal some degree of tumor specificity (Table 2). Our additional analyses, using CART to predict the diagnosis of lung cancer cell types assumed as independent unrelated cancers, suggests the possibility that our nuclear antigen sets may reveal some degree of specificity even between different types of cancer and other reactivities common to all cancers (Fig. 5). The suggestion of an association between these autoantibodies and lung cancer diagnosis as well as with cancer cell type should be experimentally pursued because most reported markers of lung cancer are presently of limited clinical use (57). Additionally, we predicted the diagno-
sis of almost 50% of lung cancers correctly with as few as three antigens (nm60, am115, and ag180). This suggests that cloning these antigens will provide useful diagnostic tools.

Research on autoantibodies in the systemic autoimmune diseases may shed light on the significance of our observations of ANAs in lung cancer. By far, the most common autoantibodies found in systemic autoimmune diseases are those directed against nuclear antigens (17). There are clear indications that ANAs in the systemic autoimmune diseases are immunological footprints of previous biological events (19). ANAs are used as serological markers in systemic lupus erythematosus and other systemic autoimmune diseases. For example, antibodies to dsDNA and to Sm antigen in systemic lupus erythematosus, antibodies to Scl-70 and nucleolar antigen RNA polymerase I and fibrillarin in scleroderma, antibodies to centromere/kinetochore in CREST syndrome, and antibodies to tRNA synthetase called JO-1, PL-7, and PL-12 have considerable disease specificity (17, 18). It is likely that the failure to recognize an association between nuclear reactivities and malignancy in the past may be related to the use of nuclear antigens commonly recognized by serum antibodies from patients with systemic autoimmune diseases (15, 16). This failure may simply reflect molecular differences between the autoantigens involved in cancer and those involved in the systemic autoimmune diseases. Our suggestion—that the nuclear antigen sets recognized by autoantibodies in the sera of patients with lung cancer may have some degree of tumor specificity—may seem unorthodox because the question of the existence of cancer-specific antigens has long been debated (1–3). However, T-cell-recognized epitopes have long been known to occur on human tumor cells (4–6). Because the cellular and humoral immune responses work in concert, it is likely that both a cellular and a humoral immune response may occur in cancer. Recently SEREX methodology has awakened the old hope of finding antibody-based screening tests for general use in the diagnosis of cancer (58–62). Similar to SEREX, our approach offers the potential of identifying tumor-specific antigens. Some degree of tumor specificity is also suggested by the reports of seropositivity for type 1 antineuronal nuclear autoantibodies (ANNA-1, also know as anti-Hu), which is considered a valuable marker of small cell lung carcinoma (8–11).

Our data showing a relationship of autoantibodies to lung cancer cell type and patient outcome also suggest that the autoimmune response in cancer may reflect biological events related to the process of carcinogenesis. In agreement with this interpretation, Imai et al. (50) found that some patients developing hepatocellular carcinoma from preexisting chronic liver disease sero-converted from ANA-negative to ANA-positive status or showed changes in ANA specificity in close temporal relationship with clinical changes. Imai et al. (50, 51) suggested that changes in ANA responses may reflect early carcinogenic events and that, in hepatocellular carcinoma, such changes in ANA may reflect an autoimmune response to nuclear antigens that are perturbed in cellular transformation. Similarly, our observation on the relation between some autoantibodies and PFS may be related to the expression of particular antigens at different stages of tumor progression (Fig. 4).

Previous studies on humoral immunity and cancer found antibody reactivities to be of relatively low titer and not useful as molecular probes (61). In contrast, in preliminary work, we have identified a large subset of sera with reactivities at high titers, which suggests that these antibodies may be potentially useful as immunoprobes for the identification of nuclear antigens.

Our work suggests that the molecular characterization of these antigens may lead to the discovery of proteins with diagnostic and prognostic value. Also, it is possible that the sequential finding of certain ANAs—uniquely associated with certain types of lung cancer developing over time—may provide clues on the critical genetic events essential for malignant transformation.

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REFERENCES


