Anti-p53 Antibodies in Sera from Patients with Chronic Obstructive Pulmonary Disease Can Predate a Diagnosis of Cancer

Glenwood E. Trivers, Virna M. G. De Benedetti, Helen L. Cawley, Gail Caron, Anita M. Harrington, William P. Bennett, James R. Jett, Thomas V. Colby, Henry Tazelaar, Peter Pairolero, Roland D. Miller, and Curtis C. Harris


ABSTRACT

Serum anti-p53 antibodies (p53-Abs) may be surrogate markers for both p53 alterations and preclinical cancer. Ancillary to a prospective trial to abate progressive development of clinical stages of chronic obstructive pulmonary disease, we conducted a retrospective, nested case-control study. Twenty-three cases were diagnosed with cancer during the trial. Enzyme immunoassay, immunoblotting, and immunoprecipitation were used to detect p53-Abs in serum, immunohistochemistry (IHC) to detect p53 accumulation, and single-strand conformation polymorphism and DNA sequencing to detect p53 mutations in tumor samples. p53-Abs were detected by three types of assays in five (23%) of the cancer patients, 80% of whom had detectable p53-Abs before diagnosis: 2 lung cancers (7 and 6 months before), 1 prostate cancer (11 months), and 1 breast cancer (5 months). Four Ab-positive patients had IHC-positive tumors. Two of 4 Ab-positive patients and 2 of 14 Ab-negative had p53 missense mutations or base pair deletion and IHC-positive tumors. The 44 noncancer COPD controls, matched with the cancer patients, 80% of whom had detectable p53-Abs before diagnosis: 2 lung cancers (7 and 6 months before), 1 prostate cancer (11 months), and 1 breast cancer (5 months). Four Ab-positive patients had IHC-positive tumors. Two of 4 Ab-positive patients and 2 of 14 Ab-negative had p53 missense mutations or base pair deletion and IHC-positive tumors. The 44 noncancer COPD controls, matched with the cancer cases for age, gender, and smoking habits, were negative for p53-Abs. These results indicate that p53-Abs may facilitate the early diagnosis of cancer in a subset of smokers with chronic obstructive pulmonary disease who are at an increased cancer risk.

INTRODUCTION

Antibodies recognizing p53 protein were first detected in human serum in breast cancer patients (1). Subsequently, serum p53-Abs have been detected in patients with a variety of histologically different tumors, including, as examples, 9 to 25% of breast (1–4), 9 to 13% of lung (5–7), 25 to 33% of liver (8, 9), and 12% of leukemias (10) and childhood lymphoma (11) cases. In addition, several reported studies (2, 5, 7, 9, 10) indicate a positive correlation between p53 missense mutations, and/or p53 accumulation, and p53-Abs. Moreover, most reports indicate that p53 mutations can indicate a poor prognosis of breast (12) and lung (13) cancer cases, and p53 accumulation can indicate a poor prognosis of breast (12, 14, 15), lung (16), and prostate (17) cancer cases. Most previous studies to detect p53-Abs have used sera obtained either at the time of, or after, cancer diagnosis. Under these conditions, p53-Abs also can indicate a poor prognosis in breast (4) and perhaps also in lung (18) cancer.

The utility of measuring p53-Abs as an aid to the early diagnosis of cancer, which requires multiple prediagnostic sera, is currently being evaluated. For example, using multiple serum samples from workers exposed to vinyl chloride in the plastics industry, serum p53-Abs were detected in two of five persons before a diagnosis of liver angiosarcoma and in four of seventy-six heavily exposed workers without a diagnosis for cancer at that time (9). Also, p53-Abs have been reported in 4 of 36 women with a positive family history of breast cancer (3) and in 2 patients with COPD at 5 months and 15 months before a diagnosis of lung cancer (6, 19).

Here we report the results of assays performed on annual serum samples from participants in a 5-year prospective study seeking to abate the clinical progression of COPD (20). During the course of this study, twenty-three subjects developed cancer. In a newly designed retrospective, nested case-control epidemiology study reported here, these individuals were matched by gender, race, and smoking status to 44 COPD subjects who did not develop cancer. Serum p53-Abs were detected before and after cancer diagnosis in smokers with COPD.

MATERIALS AND METHODS

Serum Donors

Serum samples were obtained from 67 of 518 participants (39% women, 61% men; all white; ages 35 to 59) in a randomized cohort (smokers at risk for clinical COPD) recruited by Mayo Clinic as part of the National Heart, Lung, and Blood Institute 5-year prospective LH (21). The objectives of the
LHS were to test the efficacy of smoking cessation and an inhaled bronchodilator (ipratropium bromide) in slowing the decline in pulmonary function, increases in respiratory morbidity, and mortalities among smokers with early COPD (mild to moderate airflow obstruction). Volunteers were given lung function tests that identified targeted levels of 30% below normal, with exhibited cross-sectional prevalences for coughing, phlegm, day and night wheezing, and shortness of breath. Those accepted into the study were randomly assigned to three study groups: 1, usual care (UC); 2, special intervention with active treatment (SIA; received programming in smoking cessation and inhalation of the bronchodilator three times/day); and 3, special intervention with a placebo replacement (SIP) for the inhalant. Lung function tests and serum cotinine levels were determined annually for 5 years. Annual questionnaires were completed for medical and occupational profiles, job classification, smoking histories, periodic physical examinations, cancer diagnoses and histology (where relevant), and other causes of death. Twenty-three participants (15 men and 8 women) received cancer diagnoses during the study, including 6 women and 12 men ages 50 to 59 years (data not shown). The cancer incidence occurred without any study-related pattern (Table 1): 11 cancers (4 women and 7 men) occurred among participants in the UC group, 7 (1 woman and 6 men) in the SIA group, and 4 (2 women and 2 men) in the SIP group. Both smoking-related and unrelated cancers were detected.

Control Subjects
Sera and medical and smoking histories of the 44 COPD participants who were not diagnosed with cancer (15 women ages 53 ± 5; 29 men ages 59 ± 3; including 11 women and 23 men, ages between 50 and 59 years) were used as the controls for the pS3-Ab detection in those with a cancer diagnosis. For non-LHS controls, sera and medical and smoking histories were obtained from 30 men unrelated to the LHS (ages 41–81, mean ± SD = 64 ± 12; from an ongoing study of lung cancer, smoking, and p53-Ab), 15 of whom had lung cancer, 15 who did not have a history of any other cancer, and all of whom had smoked more than 15 pack-years.

Enzyme Immunoassay
The EIA was described previously (9). Briefly, 2 ng/well of immunofluorescence-purified human p53 (22, 23) and 2 ng/well of BSA fraction 5 (Sigma Chemical Co., St. Louis, MO) as control were dried in 50–μl volumes in triplicate microtiter well columns and stored at −20°C. The plates were washed to remove salt (and following each reaction, at 37°C for 1 h) and blocked with 4% goat serum (GTS; Life Technologies, Inc., Grand Island, NY) in 1× PBS/0.05% Tween-20 (GTS-T20). The sera were diluted 1:100 in 1% GTS-T20 and applied to single, 6-well columns, 10 samples/plate. Rabbit anti-p53 antisera (from Dr. David P. Lane, University of Dundee, Dundee, Scotland) was used as positive control. Detection was by alkaline phosphatase-conjugated, goat antirabbit IgG or goat antihuman IgG anti-
Table 2  Anti-p53-antibody detection in 5 of 23 COPD patients diagnosed with cancer during a 5-year prospective study (MAYO CLINIC COPD COHORT)*

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age/Gender</th>
<th>Pack yrs smoking</th>
<th>Cancer site</th>
<th>Serum prepn. relative to cancer diagnosis</th>
<th>Means of 3 EIA</th>
<th>Blot/PPTN</th>
<th>DNA sequence</th>
<th>IHC</th>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ratio</td>
<td>SD. Diff.</td>
<td>Assay</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>57/M*</td>
<td>35</td>
<td>Colon</td>
<td>4 after</td>
<td>11.3</td>
<td>12.8 (+)</td>
<td>+ /+</td>
<td>x5, c174 (3+)</td>
</tr>
<tr>
<td>12</td>
<td>59/M</td>
<td>68</td>
<td>Lung</td>
<td>7 before</td>
<td>2.78</td>
<td>10.3 (+)</td>
<td>+ /+</td>
<td>NT</td>
</tr>
<tr>
<td>10</td>
<td>55/M</td>
<td>25</td>
<td>Lung</td>
<td>42 before</td>
<td>0.86</td>
<td>NA^</td>
<td>- /-</td>
<td>ND</td>
</tr>
<tr>
<td>56</td>
<td>25</td>
<td></td>
<td>Lung</td>
<td>28½ before</td>
<td>1.55</td>
<td>1.6 (-)</td>
<td>- /-</td>
<td>ND</td>
</tr>
<tr>
<td>57</td>
<td>26</td>
<td></td>
<td>Lung</td>
<td>16 before</td>
<td>1.32</td>
<td>2.7 (-)</td>
<td>- /-</td>
<td>ND</td>
</tr>
<tr>
<td>58</td>
<td>27</td>
<td></td>
<td>Lung</td>
<td>6 before</td>
<td>1.78</td>
<td>5.8 (+)</td>
<td>+ /+</td>
<td>ND</td>
</tr>
<tr>
<td>59</td>
<td>27</td>
<td></td>
<td></td>
<td>6½ after</td>
<td>0.81</td>
<td>NA</td>
<td>- /-</td>
<td>ND</td>
</tr>
<tr>
<td>8</td>
<td>56/F</td>
<td>36</td>
<td></td>
<td>17 before</td>
<td>1.51^</td>
<td>2.5 (-)</td>
<td>- /-</td>
<td>ND</td>
</tr>
<tr>
<td>57</td>
<td>37</td>
<td></td>
<td>Breast</td>
<td>5 before</td>
<td>1.42</td>
<td>2.6 (+)</td>
<td>+ /+</td>
<td>WT</td>
</tr>
<tr>
<td>58</td>
<td>38</td>
<td></td>
<td>Lung</td>
<td>7 after</td>
<td>1.40</td>
<td>2.6 (+)</td>
<td>+ /+</td>
<td>ND</td>
</tr>
<tr>
<td>59</td>
<td>21</td>
<td></td>
<td>Lung</td>
<td>21 after</td>
<td>1.20^</td>
<td>1.8 (-)</td>
<td>- /-</td>
<td>ND</td>
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<tr>
<td>11</td>
<td>58/M</td>
<td>46</td>
<td>Prostate</td>
<td>35 before</td>
<td>1.62^</td>
<td>3.1 (-)</td>
<td>- /-</td>
<td>ND</td>
</tr>
<tr>
<td>59</td>
<td>46</td>
<td></td>
<td>Prostate</td>
<td>18 before</td>
<td>1.38</td>
<td>1.0 (-)</td>
<td>- /-</td>
<td>ND</td>
</tr>
<tr>
<td>60</td>
<td>46</td>
<td></td>
<td>Prostate</td>
<td>11 before</td>
<td>1.39</td>
<td>2.4 (+)</td>
<td>+ /+</td>
<td>WT</td>
</tr>
<tr>
<td>61</td>
<td>46</td>
<td></td>
<td>Prostate</td>
<td>7 after</td>
<td>1.10</td>
<td>1.4 (-)</td>
<td>- /-</td>
<td>ND</td>
</tr>
<tr>
<td>62</td>
<td>46</td>
<td></td>
<td>Prostate</td>
<td>20 after</td>
<td>1.07</td>
<td>1.8 (-)</td>
<td>- /-</td>
<td>ND</td>
</tr>
<tr>
<td>64</td>
<td>57/M</td>
<td>15</td>
<td>Melanoma</td>
<td>24½ before</td>
<td>1.2</td>
<td>1.1 (-)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>58</td>
<td>15</td>
<td></td>
<td>Melanoma</td>
<td>14 before</td>
<td>1.2</td>
<td>1.1 (-)</td>
<td>- /-</td>
<td>WT</td>
</tr>
<tr>
<td>60</td>
<td>16</td>
<td></td>
<td>Melanoma</td>
<td>11½ after</td>
<td>1.1</td>
<td>1.7 (-)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>61</td>
<td>16</td>
<td></td>
<td>Melanoma</td>
<td>21 after</td>
<td>1.2</td>
<td>1.6 (-)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>68</td>
<td>59/M</td>
<td>39</td>
<td>Prostate</td>
<td>47 before</td>
<td>1.1</td>
<td>(-)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>61</td>
<td>40</td>
<td></td>
<td>Prostate</td>
<td>24 before</td>
<td>0.9</td>
<td>NA</td>
<td>- /-</td>
<td>ND</td>
</tr>
<tr>
<td>62</td>
<td>41</td>
<td></td>
<td>Prostate</td>
<td>10 before</td>
<td>1.2</td>
<td>(-)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>63</td>
<td>42</td>
<td></td>
<td>Prostate</td>
<td>0 at diag.</td>
<td>0.9</td>
<td>NA</td>
<td>- /-</td>
<td>ND</td>
</tr>
<tr>
<td>64</td>
<td>43</td>
<td></td>
<td>Prostate</td>
<td>1 after</td>
<td>1.3</td>
<td>(-)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>7</td>
<td>58/M</td>
<td>86</td>
<td>Prostate</td>
<td>12 before</td>
<td>0.82</td>
<td>NA</td>
<td>- /-</td>
<td>ND</td>
</tr>
<tr>
<td>61/</td>
<td>88</td>
<td></td>
<td>Prostate</td>
<td>½ after</td>
<td>1.16</td>
<td>1.5 (-)</td>
<td>- /-</td>
<td>ND</td>
</tr>
<tr>
<td>58</td>
<td>50/F</td>
<td>32</td>
<td>Rectum</td>
<td>8 after</td>
<td>1.0</td>
<td>NA</td>
<td>- /-</td>
<td>ND</td>
</tr>
<tr>
<td>51</td>
<td>33</td>
<td></td>
<td>Rectum</td>
<td>3 after</td>
<td>0.9</td>
<td>NA</td>
<td>- /-</td>
<td>ND</td>
</tr>
<tr>
<td>52</td>
<td>55/M</td>
<td>62</td>
<td>Prostate</td>
<td>13 before</td>
<td>0.9</td>
<td>NA</td>
<td>- /-</td>
<td>ND</td>
</tr>
<tr>
<td>59</td>
<td>59/F</td>
<td>43</td>
<td>Trachea</td>
<td>4½ after</td>
<td>0.8</td>
<td>NA</td>
<td>- /-</td>
<td>ND</td>
</tr>
<tr>
<td>63</td>
<td>51/F</td>
<td>28</td>
<td>Lung Adenoma</td>
<td>6½ after</td>
<td>1.0</td>
<td>NA</td>
<td>- /-</td>
<td>ND</td>
</tr>
<tr>
<td>60</td>
<td>46/M</td>
<td>28</td>
<td>CLL^, myeloma</td>
<td>1 after</td>
<td>0.9</td>
<td>NA</td>
<td>- /-</td>
<td>ND</td>
</tr>
<tr>
<td>56</td>
<td>48/F</td>
<td>52</td>
<td>Breast</td>
<td>1 before</td>
<td>0.9</td>
<td>NA</td>
<td>- /-</td>
<td>ND</td>
</tr>
<tr>
<td>49</td>
<td>52</td>
<td></td>
<td>Breast</td>
<td>11 after</td>
<td>1.0</td>
<td>NA</td>
<td>- /-</td>
<td>ND</td>
</tr>
<tr>
<td>Examples from 44 controls:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>55/M</td>
<td>70</td>
<td>Control^*</td>
<td>NA</td>
<td>1.24</td>
<td>1.7 (-)</td>
<td>- /-</td>
<td>NA</td>
</tr>
<tr>
<td>5</td>
<td>45/F</td>
<td>21</td>
<td>Control</td>
<td>NA</td>
<td>1.14</td>
<td>2.9 (-)</td>
<td>- /-</td>
<td>NA</td>
</tr>
<tr>
<td>6</td>
<td>61/M</td>
<td>65</td>
<td>Control</td>
<td>NA</td>
<td>1.14</td>
<td>0.7 (-)</td>
<td>- /-</td>
<td>NA</td>
</tr>
<tr>
<td>4</td>
<td>63/F</td>
<td>46</td>
<td>Control</td>
<td>NA</td>
<td>1.11</td>
<td>0.9 (-)</td>
<td>- /-</td>
<td>NA</td>
</tr>
<tr>
<td>3</td>
<td>69/F</td>
<td>50</td>
<td>Control</td>
<td>NA</td>
<td>1.08</td>
<td>NA</td>
<td>- /-</td>
<td>NA</td>
</tr>
<tr>
<td>51</td>
<td>59/M</td>
<td>58</td>
<td>Control</td>
<td>(Zero time, 10 later)</td>
<td>1.06</td>
<td>NA</td>
<td>- /-</td>
<td>ND</td>
</tr>
<tr>
<td>61</td>
<td>58</td>
<td></td>
<td></td>
<td></td>
<td>0.87</td>
<td>(-)</td>
<td>- /-</td>
<td>NA</td>
</tr>
</tbody>
</table>

* Results by patient, order of serum collection, and descending EIA.
^ prepn., preparation. Data shown in months.
^ SD. Diff., SD difference.
^ Immunoblot and immunoprecipitation.
^ Male or female.
^ p53 mutation: x = exon; c = codon.
^ NT, no tissue available.
^ NA, not applicable.
^ ND, not done.
^ One of three assays that were positive.
^ WT, wild type.
^ NEG, negative.
^ CL, chronic lymphocytic leukemia.
^ Non-cancer COPD participants shown as examples of the results from 44 matched controls. Tissue from 19 of the 23 cancer patients was studied by IHC for overexpression of p53, SSCP, and DNA sequencing for p53 alterations, both of which have been shown to associate with the presence of p53-Abs in some patients.
Fig. 2 Three-assay detection of serum p53-Abs in chronic obstructive pulmonary disease patients nos. 13, 12, and 10 with colon cancer, small cell lung cancer, and a lung adenocarcinoma, respectively. Marker: molecular weight indicator; Positive Control, rabbit anti-p53 specific serum (CM-1); Non-Cancer Sera, normal human serum as negative antibody control; M, months; Dx, diagnosis; p53, the M, 53,000 marker indicated by the band produced by the positive control. WESTERN, immunoblotting at dilutions of 1:200, 1:100, and 1:50 for nos. 13, 12, and 10, respectively. Results show that all p53 bands for all sera except that taken 16 months before diagnosis in no. 10. IMMUNOPRECIPITATION, all sera were assayed at a 1:50 dilution. All sera produced a p53 band at M, 53,000, including the first sample of no. 10. In the EIA, all sera were assayed at 1:100 in triplicate-well assays two or more times.
Fig. 3 Three-assay detection of serum p53-Abs in COPD patient no. 11, who had prostate cancer. Marker, molecular weight indicator; Positive Control, rabbit anti-p53 specific serum (CM-1); Non-Cancer Sera, normal human serum as negative antibody control; M, months; Dx, diagnosis; p53, the Mr 53,000 molecular weight marker indicated by the band produced by the positive control. WESTERN, immunoblotting; IMMUNOPRECIPITATION, PPTN. The figure contains the results obtained in the three-antibody detection methods used for p53-Abs in three of five annual sera from this patient, including 35, 18, and 11 months before diagnosis. The 35-month (35M) sample was erratic and EIA(-) at 1:100, negative in PPTN at 1:50, and positive in the Western at 1:100. The 18-month (18M) sample was EIA(-), positive in PPTN at 1:50, and negative in the Western at 1:200. The 11-month sample was only technically EIA(-) and positive in both PPTN and immunoblotting at 1:50.

EIA results were converted to specific activities and compared in SAS (PROC NPAR1WAY; SAS Institute, Inc., Cary, NC 27513), using the Kruskal-Wallis $\chi^2$ approximation, and the Wilcoxon Two-Sample test for normal approximation. All $P$s are two sided.

Immunoblotting

Procedures were modifications (9) of methods described previously (24). Ten ng/lane of p53 and 10 ng/lane of BSA fraction 5 (Sigma), as control antigen, were fractionated in 12% SDS-polyacrylamide gels and transferred by electrophoresis to nitrocellulose membranes (Bio-Rad, Richmond, CA). Nonspecific binding was blocked in buffer containing BSA (1 X PBS, 0.1% Tween 20, 0.02% NaN$_3$, and 3% BSA), and then membranes were incubated in 1:100 human sera and 1:10,000 rabbit anti-p53 (CM-1) as positive control. Antibodies were detected with anti-human IgG (Jackson Laboratories and antirabbit IgG (Jackson Laboratories) as control, followed by incubation in ECL Western detection reagents (Amersham Corp., Arlington Heights, IL). Positive sera were repeated at 1:100 or higher dilutions; negative sera were reassayed at 1:100 or lower dilutions (1:50 and 1:25), making interpretation occasionally difficult, but frequently allowing confirmation of an EIA result.

Preparation of Purified Human p53

Purified p53 was prepared by modification of procedures described previously (22, 23). Recombinant baculoviruses transfected with the wild-type human p53 gene (25) were used to infect SF9 insect cells (Invitrogen, San Diego, CA) grown in culture. The cells were lysed, and the p53 protein was immunoaffinity purified on p53-specific monoclonal antibody (PAB421, Oncogene Science, Uniondale, NY) columns (A-Sepharose; Oncogene Science). The choice of a control antigen was determined by experimentation, as described previously (9). Immunoaffinity purified, mock protein from SF9 cells, serum albumin from several species (BSA; Sigma), and proteins from human tumor cells transfected with p53 mut143ala (26) were
tested with p53 Ab(+) and Ab(−) serum. No control antigen was better than BSA, fraction 5.

**Immunoprecipitation**

**In Vitro Translation of 35S-labeled p53.** The procedures were as reported previously (9). We used Promega kit TnT lysate systems, a p-Select wild type p53 DNA plasmid prepared in our laboratory (26), 20 μCi of 35S-labeled cysteine (Du Pont NEN, Boston, ME), and kit reagents incubated at room temperature for 90 min. Two μg of plasmid DNA per reaction produced 200–300 ng of p53 (by Coomassie Blue staining) in 50 μl of the TnT system.

**Immunoprecipitation (PPTN).** Twenty μl of a 1:10 dilution of serum in GTS-T20 was further diluted to 1:50 in a reaction solution containing 4 μg to 16 μg (estimated by immunoblotting) of 35S (Du Pont NEN)-labeled p53 protein. Monoclonal antibody DO-1 (1:10; Oncogene Science) was used as positive control. Protein A- and G-conjugated agarose beads (Oncogene Science) were added, and the precipitated mixture was fractionated in a gel along with Rainbow 14C-methylated, molecular weight markers (Amersham) and exposed to film. Noncancer COPD donor sera were used as negative controls. Assays of matched sera from the same individual were assayed blindly and not necessarily on the same day with the same lot of 14C-labeled p53 translation product.

**p53 IHC**

Immunohistochemical analysis was performed as described previously (27) using dewaxed paraffin sections, monoclonal p53-Ab, DO-7 (Oncogene Science), biotinylated horse antimouse IgG, and avidin-biotin-conjugated horseradish peroxidase (Vectorstain Elite kit; Vector Laboratories, Burlingame, CA) for detection and diamobenzidine as chromogen. Positive and negative controls were used in each experiment. Staining was recorded numerically according to: (a) intensity, 0 to 3 (for none, equivocal, moderate, and intense); (b) distribution: 0 to 4 (for the percentage of cells stained: none, <10%, 10–50%, 51–90%, and >90%); and (c) the sum of intensity distribution reported on a four-point scale as negative, (0), positive, 1+ (1–3), 2+ (4–7), or 3+ (8–10).

**DNA Analysis**

Tumor DNA from 19 of the 23 tumors were examined by the SSCP assay and DNA sequencing. Samples were microdissected from paraffin sections of the tumors. Exons 5–8, including splice sites, of the p53 gene were amplified by two sequential rounds of PCR using nested, intronic primers as described previously (9).

**SSCP.** PCR samples were assayed using the modifications of methods described previously (28). Briefly, after purification on 3% NuSieve:1% agarose (FMC) gel, isolated bands were heat denatured and cooled before applying 1 μl into an 8-well comb of the PhastSystem 20% precast polyacrylamide gels (Pharmacia Biotech, Inc., Piscataway, NJ). Wild-type p53 DNA, from B-cell lymphoma cell lines, was used as negative control. Gels (20%) were run at 4°C and at 20°C. Mutations were recorded for locations in wild-type exons corresponding to reproducible band shifts in the test exons.

**DNA Sequence Analysis.** Tumor DNA from 19 tumors were examined by sequencing the same purified PCR products used for SSCP. Exons 5–8 were sequenced as described previously (9). Each exon was sequenced in both directions; complete coding sequences, including splice sites, were examined and compared to the normal sequence for that exon. Positive samples were confirmed by sequencing from a second independent PCR product from genomic DNA.

**RESULTS**

**Characteristics of Serum Donors**

Following the completion of the 5-year prospective intervention study, it was determined that some participants in the Mayo Clinic cohort had received cancer diagnoses in the period. We obtained and tested 93 coded sera for p53-Abs (see "Materials and Methods") after 15 to 85 pack-years of smoking cigarettes and a diagnosis of COPD. Sera from 23 smokers with cancer were studied, and five had p53-Abs. The antibodies in four (80%) of the p53-Ab(+) cancer patients (nos. 12, 10, 8, and 11) were detectable before the diagnosis. Forty-four sera from 44 smokers without a cancer diagnosis were negative; 15 sera from 15 age-matched noncancer smokers were significantly higher when compared to smokers without a cancer diagnosis (P = 0.01), and p53-Ab(+) noncancer smokers unrelated to the study population were negative also. EIA results in smokers with cancer were significantly higher when compared to smokers without a cancer diagnosis (P = 0.0025), smokers unrelated to the COPD study (P = 0.01), and p53-Ab(+) normal volunteers (3 of 736) compiled from the published studies cited previously (Ref. 9; i.e., P = <0.0001).

![Fig. 4 EIA results shown as specific activities (mean p53 A_{505} = mean BSA A_{505}) of individual sera in groups of patients screened for p53-Abs (see "Materials and Methods") after 15 to 85 pack-years of smoking cigarettes and a diagnosis of COPD. Sera from 23 smokers with cancer were studied, and five had p53-Abs. The antibodies in four (80%) of the p53-Ab(+) cancer patients (nos. 12, 10, 8, and 11) were detectable before the diagnosis. Fourty-four sera from 44 smokers without a cancer diagnosis were negative; 15 sera from 15 age-matched noncancer smokers were significantly higher when compared to smokers without a cancer diagnosis (P = 0.01), and p53-Ab(+) noncancer smokers unrelated to the study population were negative also. EIA results in smokers with cancer were significantly higher when compared to smokers without a cancer diagnosis (P = 0.0025), smokers unrelated to the COPD study (P = 0.01), and p53-Ab(+) normal volunteers (3 of 736) compiled from the published studies cited previously (Ref. 9; i.e., P = <0.0001).](image-url)
of cases. Fourteen (61%) of the cancer patients were ages 55–59 years (4 women and 10 men), and 18 (80%) were 50–59 years (6 women and 12 men).

Detection of Anti-p53 Antibodies

Table 2 shows the antibody detection results for 14 of the 19 cancer patients who had tumor tissue available for analysis, including the 5 with p53-Abs. They are arranged in descending EIA p53:BSA ratios found in patient serum sets.

**EIA.** A total of 93 sera were tested (triplicate well) a minimum of two times in the EIA. The results were: three EIA(+) sera (the colon, no. 13; one small cell lung, no. 12; and a lung adenocarcinoma patient, no. 10); seven EIA(−) sera that were marginally (±) positive (two breast patients and one prostate patient); and five sera with 1 positive assay in three assays (Table 2). Mean p53:BSA ratios ranged from 1.8 to 11.1 for positive sera, 1.4 to 1.6 for (±), less than 1.0 to 1.3 for a negative sera, and an occasional 1.6 for sera with 1 positive of three assays. These sera and a group of decidedly EIA(−) sera were tested in the immunoblot and the PPTN assays. Two of the 15 lung cancer patients unrelated to the COPD study were marginally (±) positive in EIA. Fig. 4 shows a scatter plot of the specific activity obtained in the EIA. The antibody activities obtained from cancer sera were significantly higher when compared to sera from the 44 noncancer, COPD controls ($P < 0.0025$) or the 15 noncancer, heavy smokers unrelated to the LHS ($P = < 0.01$).

**Immunoblotting.** Sixteen sera were tested a minimum of two times in the immunoblot assay. The results were seven positive sera, including the three EIA(+), the three EIA(±), and one of the five sera that were EIA(−) (Table 2; Figs. 1–3).

**PPTN.** Twenty-two sera were tested a minimum of two times in the PPTN assay. Seven sera were (+), including the three EIA(+), three EIA(±), and two of the 5 sera that were EIA(−) with one positive of three assays (Table 2; Figs. 1–3).

Three Assay p53 Antibody Detection

Table 1 and Figs. 1–3 show that the 11 sera identified by EIA results for testing in the other two assays belonged to five patients; there were two EIA(+) sera from each of two patients (nos. 8 and 11), who provided multiple sera. Six sera (nos. 13, 12, 10d, 8b, 8c, and 11c) were determined to be triple-assay positive by the minimum requirement of a positive result in at least two of the three different detection assays. All six of the sera (nos. 13, 12, 10d, 8b, 8c, and 11c) with EIA ratios of 1.39 and above were positive by both immunoblot and PPTN. The three remaining high ratio EIA(−) sera gave inconsistent results in PPTN as they had in EIA. However, PPTN was the only assay to give positive results in the earliest serum of patient no. 8 [Ab(+) breast cancer], and immunoblotting was the only positive result in the earliest serum of patient no. 11 [Ab(+) prostate cancer]. Both sera were marginally positive in PPTN but negative in EIA. Fig. 4 shows a scatter plot of the specific activity obtained in the EIA. The antibody activities obtained from cancer sera were significantly higher when compared to sera from the 44 noncancer, COPD controls ($P = < 0.0025$) or the 15 noncancer, heavy smokers unrelated to the LHS ($P = < 0.01$).

Immunohistochemistry. A total of 7 (37%) of the 19 tumors assayed for nuclear accumulation of p53 were IHC(+) (Table 2); 5 were strongly (3+) positive, and two were moderately (2+) positive. All four of the p53-Ab(+) patients with available tumor tissue had IHC(+) tumors; three were strongly positive (nos. 13, 10, and 8). The prostate patient (no. 11) was moderately positive. Three (21%) of the 14 Ab(−) patients with available tissue had IHC(+) tumors. Two (no. 56, breast; no. 59, tracheal cancer) stained strongly, and one (no. 58, rectal cancer) stained moderately.
p53 Mutations: SSCP and Sequencing. Two of four Ab(+) cases and two (14%) of 14 Ab(−) cases had SSCP banding patterns indicating p53 alterations. Sequencing confirmed (Fig. 5) p53 mutations in exons 5 of Ab(+) patients no. 13 (colon) and no. 10 (lung) and of Ab(−) patient no. 59 (trachea). Patient no. 13 had a CTC→TTC transition (Ser→Phe); no. 10, a GCC→CG 1 base frameshift deletion, and no. 59 had a GTG→GAG transversion (Val→Glu). All were strong IHC(+) Ab(−) patient no. 52, with an IHC(−) prostate cancer, had a GCC→GAC transversion (Arg→Pro) in exon 8. No alterations in the p53 gene were detected in the IHC(+) and Ab(−) patients nos. 56 and 58.

DISCUSSION

We have studied p53-Ab(s) in sera from extensive smokers in a LHS intervention of early COPD (21). Twenty-three of the smokers developed cancer during the 5 years of the study. The objective of the ancillary study was to determine the prevalence and the timing of the appearance of p53-Ab(s) in this population as compared to noncancer COPD controls. Five (22%) of the 23 COPD patients with cancer had serum p53-Ab(s), and 4 (80%) of the p53-Ab(+) patients had detectable antibodies before their cancer diagnosis, including 2 lung cancers, 1 breast cancer, and 1 prostate cancer. p53-Ab(s) were not detected in 44 COPD matched controls who did not develop cancer, nor in 15 geographically unrelated smokers.

A review of the published reports (1–6, 8, 9, 11) using methods to detect p53-Ab(s) similar to those we used reveals an incidence of approximately 14% (9 to 33%) Ab(+) donors among cancer patients, compared to 0.4% Ab(+) donors among noncancer control donors, including breast (3), lung (6), and liver (8) patients with organ specific, noncancer clinical symptoms. Thus, similarly exposed and treated noncancer controls at twice the number of cancer patients appear sufficient to study the prevalence of serum p53-Ab(s) in cancer patients in the COPD intervention trial.

These results from COPD patients are similar to those reported from our previous study of vinyl chloride-induced, angiosarcoma of the liver (9) in which 5 (33%) of 15 workers had angiosarcoma of the liver and 2 (5%) of 42 workers without cancer had p53-Ab(s). Two of the patients with angiosarcoma of the liver had p53-Ab(s) prior to diagnosis, and two of five Ab(+) patients with angiosarcoma of the liver had tumors with p53 mutations. Therefore, this COPD study provides a second indication of a possible role for p53-Ab(s) in the early detection of cancer.

In our study, all p53-Ab(+) cancer patients with available tumor tissue (four of five) had IHC(+) tumors. Two p53-Ab(+) cancer patients and one that was Ab(−) had IHC(+) tumors and p53 gene alterations in exon 5; one patient (no. 52) was p53-Ab(−) but had an IHC(+) tumor that had p53 gene alterations in exon 8. Similar results were reported previously in studies of breast (2, 29) and lung (5, 6, 7, 30) cancer. In two studies, either all (2) or the majority (7) of p53-Ab(+) patients had IHC(+) tumors and p53 missense mutations in exons 5 and 6, and some Ab(−) patients had IHC+ tumors with mutations. Explanations for the failure to demonstrate mutations in some IHC+ tumors have been suggested as the result of either: (a) the cloning heterogeneity in tumors, and errors in sampling certain tumors (31); or (b) false-positive IHC tests (12). These conditions could apply to p53-Ab(+) patients who have IHC+ tumors and no mutations (7, 30) or to those who were Ab(+) without any demonstrated p53 abnormalities (7, 29, 30). To the contrary, failure to find p53-Ab(s) in patients with mutations and or IHC(+) tumors in this study and others (2, 5, 7, 29, 30) may represent clues to the mechanism(s) of the differential immunogenicity or host responses observed with some p53 mutations. Certainly, it indicates that all p53-mutated proteins are not immunogenic (2, 5), and that p53 immunogenicity relates to key exons in some cancers (2), i.e., exons 5 and 6, identified in this study and in others (2, 7).

Our results are consistent with the finding that p53 mutation and/or nuclear protein accumulation can occur in preinvasive lesions during carcinogenesis in human esophagus (32–34), lung (35–37), and skin (38) cancers. The presence of p53-Ab(s) prior to a diagnosis for breast, lung, and prostate cancer indicates that prospective studies to test the utility of p53-Ab(s) in the early detection of cancer are warranted. In addition, changes in the titer of p53-Ab(s) may be of value in following the clinical course of patients after cancer therapy.

REFERENCES


Anti-p53 antibodies in sera from patients with chronic obstructive pulmonary disease can predate a diagnosis of cancer.


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