Circulating Anti-p53 Antibodies in Esophageal Cancer Patients Are Found Predominantly in Individuals with p53 Core Domain Mutations in Their Tumors

Marie-Charlotte von Brevern, Monica C. Hollstein, Helen M. Cawley, Virna M. G. De Benedetti, William P. Bennett, Linda Liang, An-Guang He, Simin M. Zhu, Thomas Tursz, Nicolas Janin, and Glenwood E. Trivers

ABSTRACT

Serum antibodies reacting with the tumor suppressor protein p53 have been detected previously in cancer patients with a variety of neoplasms. Two initial (although insufficient) prerequisites for a B-cell response to occur have been proposed: p53 protein accumulation in the tumor or a mutant p53 gene, or both.

We have examined 65 esophageal cancer cases (42 from Guangzhou and Shenyang, People's Republic of China, and 23 from Paris, France) to obtain a prevalence estimate of anti-p53 antibodies for this type of cancer and to define the relationship of p53 tumor status to B-cell immune response. Sera were analyzed in a triplicate assay (enzyme-linked immunosassay, immunoprecipitation, and immunoblot) for anti-p53 antibodies. Tumor DNA was screened for mutations in exons 5–8, and tumor tissue was examined by immunohistochemistry for abnormal p53 protein accumulation. p53 mutations were found in 36 (58%) of 62 cases analyzed. Sixteen patients (25%) had circulating antibodies to the tumor suppressor protein. All but two (88%) of the tumors from seropositive cases had a mutation in the DNA binding region of the p53 gene, and with one exception, these tumors also showed nuclear accumulation of the p53 protein. In contrast, tumor mutations were found in just 22 (46%) of the 48 individuals in whom we did not detect anti-p53 antibodies. Among the 22 seronegative cases for which we found no tumor mutations, 11 revealed p53 protein accumulation by immunohistochemical analysis.

Thus, circulating anti-p53 antibodies may be present in one-fourth of esophageal cancer patients, most of whom also would be expected to have a p53 gene mutation in their tumors. Patients without such mutations appear considerably less likely to mount a B-cell response to the p53 tumor suppressor protein than those that do (P < 0.01).

INTRODUCTION

Some cancer patients mount an immunological reaction to the cellular tumor suppressor protein p53, whereas others do not (1–5). Abnormal accumulation and/or structural alteration of p53 in tumors are anticipated to underlie this B-cell response, but experimental demonstration has been difficult to come by. In any case, this tumor component cannot be sufficient in itself to elicit a response, because for all types of solid neoplasms, p53 tumor aberrations are far more common than the prevalence of anti-p53 antibodies in patient sera (6–9). Seropositivity frequency figures vary considerably from study to study, even for a given type of cancer, which may be attributable in part to procedural differences and data interpretation when screening samples by EIA4, the method that has been used most often due to its simplicity.

Few studies have examined serum antibodies directly in conjunction with tumor p53 mutations and protein accumulation in the same set of patients (3, 7, 10–13), and the number of seropositive cases clearly characterized in this way remains small. Breast and lung cancer are two malignancies in which tumors and sera from the same patients have been examined in several studies, but the data still do not provide sufficient basis to clarify whether or how tumor p53 status bears on the likelihood that the patient will be seropositive. It is probable that immune response and the factors that contribute do not stay constant for different types of cancer, and thus must be studied on a cancer-by-cancer basis. A clear example of this has been proposed by Rainov et al. (3), who showed a total absence of seropositive patients among 70 glioma patients despite a high frequency of p53 mutations and protein accumulation in the tumors.

Esophageal cancer, for which there are no full reports yet on serum antibody in relation to tumor p53 status, may be particularly valuable in elucidating associations between immune reaction and specific p53-related molecular defects in the tumor. p53 gene mutations are frequent (usually >50%; Refs. 14–18), missense mutations usually result in unequivocal nuclear accumulation of the p53 protein in the majority of the cancerous cells, and structural alterations of the gene can be already present in lesions that are only mildly dysplastic (19, 20). Several instances have now been described in which an asymptomatic individual who has gone on to develop cancer has been shown to have had circulating anti-p53 antibodies in serum collected months to years before clinical onset of disease, indicating that immune response can be a very early event (21, 22). This would make the serological analysis of p53 a valuable tool for diagnostic screening of individuals at elevated risk for selected types of cancers, in particular for neoplasms in which it has been shown that genetic alterations of the p53 gene occur at initial stages of the disease, presuming that tumor p53 status is indeed relevant to the immune response.

Esophageal cancer mainly affects certain high-risk groups in the world and has an extremely poor prognosis (23, 24). By the time diagnosis and treatment occur, usually the cancer is metastatic, and treatment is a palliative measure (25).

Thus, there is both clinical and scientific purpose to investigating the prevalence of serum antibodies and the tumor-component determinants of anti-p53 antibody response in esophageal cancer patients. We examined 65 individuals with this cancer (predominantly squamous cell carcinomas) for most of whom we also had matched sets of fixed tumor tissue and tumor DNA. Patient anti-p53 seropositivity measured by three separate techniques was determined, tumor p53 protein nuclear accumulation was examined, and tumors were examined for p53 gene mutations, which included prescreening by DGGE,
a method sensitive enough to detect a mutant allele present at 10% of a sample with predominantly wild-type sequences.

**MATERIALS AND METHODS**

**Sample Collection and Tissue Extraction.** All specimen donors were provided with cancer research information and given the opportunity to refuse participation in projects approved by local boards reviewing human research risks. Serum samples were collected from 65 patients (smokers, 64%; non-smokers, 34%) with esophageal cancer at the time of initial diagnosis or surgery and stored at ~70°C for 2-5 years before analysis. Twenty-three patients were from Paris, France, and the rest were from Guangzhou (28 cases) and Shenyang (14 cases), People's Republic of China. Although individual records on smoking and drinking were not available to us for the European cohort, the typical profile of esophageal cancer patients from this region includes heavy consumption of tobacco and alcoholic beverages, and generally poor nutritional status. Case-by-case dietary information for Guangzhou patients was available and indicated patterns typical for rural areas with a high esophageal cancer incidence, such as consumption of pickled vegetables (a known risk factor), consumption of hot foods, and long-term history of dysphagia or other symptoms of esophagitis. Biopsies or ethanol-fixed tumor tissues from each patient were collected for DNA extraction and for paraaffin embedding in preparation for immunohistochemistry. Tissue blocks were sectioned serially at 5 or 50 µm and mounted with minimal heating onto poly-L-lysine-coated slides. Additionally, fresh-frozen tumor biopsies were collected from 23 cases from Paris, France, from which pieces were paraffin embedded and also sectioned for further immunohistochemistry. DNA was extracted from ethanol-fixed or fresh-frozen tumor tissues according to standard procedures.

**Immunohistochemistry.** Immunohistochemical analysis was performed essentially as described previously (26). Briefly, after dewaxing, inactivating endogenous peroxidase activity, and blocking with normal goat serum, the sections were incubated overnight at 4°C with either monoclonal antibody against p53 (DAKO, Glostrup, Denmark). Localization of the primary antibody was achieved by subsequent application of a biotinylated horse antireabbit antibody, avidin-biotin peroxidase complex (Vectorstain Elite Kit, Vector Laboratories, Burlingame, CA), and diaminobenzidine, together with nickel chloride. H&E-stained sections were reviewed to confirm the presence of malignant tissue.

**PCR and Mutational Analysis.** DNA fragments were amplified in a DNA thermocycler (Perkin-Elmer Cetus) in 50-µl reaction mixtures containing 200 ng of genomic DNA, 20 pmol of each primer, 160 µM of each dNTP, and 1 unit of Taq polymerase (Boehringer Mannheim) in a buffer containing 10 mM Tris-HCl (pH 8.3), 50 mM potassium chloride, 0.5 mM magnesium chloride, 2.5% formamide, and 0.02% BSA. The DNA fragments were screened for mutations by DGGE as described by Hamelin et al. (27) and Renault et al. (28). For DGGE analysis, the sequences of the used primers for exons 5, 7, and 8 and of DGGE analysis, the sequences of the used primers for exons 5, 7, and 8 were scored clearly positive if results in two or more of the three methods used to examine each sample were positive and marginal if one test was positive. Twelve of 15 were positive showing an abnormal migration pattern on the DGGE gels. Primers and sequencing conditions were as described previously (26), except that deoxy sequencing was performed on biotinylated PCR product affixed to streptavidin magnetic bead support (Dynal, Oslo, Norway). Ten of the tumors from Shenyang have been examined previously for mutations (26). DNA stocks and PCR reagents were kept physically separated from areas where PCR products were handled, and setup was performed in an Oncor Template Tames hood (Oncor, Inc., Gaithersburg, MD) equipped with UV light.

**Enzyme Immunoassay for p53 Serum Antibodies.** The assay was performed as described previously (21). Briefly, purified human p53 protein, BSA as control (Sigma Chemical Co., St. Louis, MO), and all other reagents were added to wells of microtiter plates in 50 µl volumes. The plates were washed five times after each reaction (37°C for 1 h). Two ng/well of p53 and of BSA were dried in triplicate wells and stored at ~20°C. Plates were washed, then blocked with 4% goat serum (Life Technologies, Inc., Gaithersburg, MD) in PBS containing 0.05% Tween 20 (GTS-T20). The serum samples were diluted 1:100 in 1% GTS-T20 and applied simultaneously to p53 and BSA in single, triplicate-well columns. Rabbit anti-p53 antiserum (1:40,000 dilution, CM-1 from Dr. David Lane, University of Dundee, Scotland, UK) in 1% GTS-T20 was added in a single column as positive control. Detection of bound antibody involved use of alkaline phosphatase-conjugated, goat anti-rabbit IgG or phosphatase-conjugated, goat anti-human IgG antiserum (Jackson Immunoresearch Laboratories, West Grove, PA). p-Nitrophenyl phosphate (0.1%; Sigma Chemical Co.) in 10 mM magnesium chloride and 10 mM dithiothreitol (pH 9.6) was added to the wells, and the color reaction was measured at 405 nm after 15 min, up to 6 h, and after storage overnight at 4°C.

**Immunoblotting.** Procedures used were essentially as described previously (21). p53 and BSA fraction as control antigen were fractionated in 12% polyacrylamide gels containing 10% SDS. The proteins were transferred by electrophoresis to nitrocellulose membranes (Bio-Rad Laboratories, Richmond, CA) in buffer (×1 PBS, 0.1% Tween 20, 0.02% NaNO3, and 3% BSA) for 1 h, then incubated for 2 h with 1:100 human sera and 1:10,000 CM-1 as positive control. Antibodies were detected by incubation with 1:1000 anti-rabbit IgG and 1:1000 antihuman IgG (Jackson Immunoresearch Laboratories, West Grove, PA). p-Nitrophenyl phosphate (0.1%; Sigma Chemical Co.) in 10 mM magnesium chloride and 10 mM dithiothreitol (pH 9.6) was added to the wells, and the color reaction was measured at 405 nm after 45 min, up to 15 min, and to 6 h, and after storage overnight at 4°C.

**Immunoprecipitation.** In vitro translation of 35S-labeled p53 was carried out according to previous descriptions (21). For the immunoprecipitation procedures, 10 µl of serum were diluted in 90 µl of 1% goat serum in GTS-T20. Twenty µl of this reaction were added to 80 µl of the reaction solution, containing 4–16 ng of 35S (DuPont NEN, Boston, MA)-labeled p53 protein. The positive control was 1:10 monoclonal antibody DO-1 (Oncogene Science, Uniondale, NY). Protein A- and G-conjugated agarose beads (Oncogene Science) were added, and the precipitated mixture was fractionated in a polyacrylamide gel and exposed to film. The negative controls consisted of sera from cancer-free donors.

<table>
<thead>
<tr>
<th>Patient origin</th>
<th>No. of cases</th>
<th>Histology</th>
<th>p53 protein (IHC +)</th>
<th>Gene mutation (DGGE +)</th>
<th>Immune response Serum Ab+</th>
</tr>
</thead>
<tbody>
<tr>
<td>China</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Guangzhou</td>
<td>28</td>
<td>SCC/C5 ADC</td>
<td>81%</td>
<td>71%</td>
<td>32%</td>
</tr>
<tr>
<td>Shenyang</td>
<td>14</td>
<td>SCC/C3 ADC</td>
<td>71%</td>
<td>50%</td>
<td>14%</td>
</tr>
<tr>
<td>Europe</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>France</td>
<td>23</td>
<td>SCC/C ADC</td>
<td>47%</td>
<td>45%</td>
<td>22%</td>
</tr>
<tr>
<td>All patients</td>
<td>65</td>
<td></td>
<td>69% (40/58)</td>
<td>58% (36/62)</td>
<td>25% (16/65)</td>
</tr>
</tbody>
</table>

Table 1: Anti-p53 serum antibodies, tumor p53 gene mutations, and p53 protein accumulation in 65 esophageal cancer patients.

Criteria for positivity: IHC, nuclear staining in 20% or more of tumor cells; DGGE, abnormal migration pattern of PCR amplified p53 gene segment compared to wild type; sera were scored clearly positive if results in two or more of the three methods used to examine each sample were positive and marginal if one test was positive. Twelve of 15 were positive in all three assays. EIA results were positive when the ratio (see "Materials and Methods") was >1.5, marginal at 1.2 < x = 1.5, and negative at a ratio <1.2.

Mutant by DGGE analysis and DNA sequencing. Constitutive polymorphisms and silent mutations are considered negative. NS, undifferentiated, anaplastic, or no material available for histology.

Tumor tissue was not available for seven cases, and tumor DNA was lacking for two patients.

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RESULTS

p53 serum antibodies, tumor DNA mutations, and tumor tissue protein accumulation were examined in esophageal cancer patients from three geographical areas: Guangzhou and Shenyang, China, and Paris, France (Table 1). Of the 65 sera tested in our three antibody detection assays (EIA, IB, and IP), 16 were positive (25%) for p53 antibodies in at least one of the three assays we used to examine each sample, and 12 of these were positive in all three tests (Table 2). Tumor DNA was available from 62 of the patients for analyzing exons 5-8 of the p53 gene for mutations. In more than half (36 cases, or 59%), sera showed a tumor-specific abnormal DGGE profile, indicating structural aberration in the midregion of the p5.3 gene (Table 2). The correlation between tumor-specific p53 gene mutation and anti-p53 seropositivity was clearly significant (P < 0.01, χ² statistic, one-sided; P < 0.017, Fisher’s Exact test, two-sided), whereas the association between p53 protein accumulation in the tumor and the presence of serum p53 antibodies was not, despite the clear relationship between p53 missense mutation and elevated protein levels in tumor cell nuclei. p53 protein accumulation in tumors with wild-type p53 genes may be attributable to stabilization of p53 protein by other mechanisms, such as binding to cellular or viral proteins (32, 33). Approximately one-fourth of the tumors that were positive in our p53 immunohistochemistry assay had normal DGGE patterns, suggesting that p53 stabilization was not due to missense mutation in the core
ANTI-p53 ANTIBODIES IN ESOPHAGEAL CANCER PATIENTS

DISCUSSION

Of the 65 sera assayed from esophageal cancer patients, 16 (25%) were positive for anti-p53 antibodies. This prevalence falls at the high end of the 5–25% range generated from studies in most other major cancer types (4–6, 10, 34). It is important to note that, as reported previously, we and other investigators have obtained essentially negative results (~0.4%) when assaying noncancer control sera for anti-p53 antibodies (21). However, because sensitivity and background in serum assays can be problematic in screening sera, here we have examined all blood samples for anti-p53 antibodies by three separate methods: EIA, IP, and IB. Twelve of the 16 serum samples were positive in all three assays, so that even if marginal cases are excluded, the positive cases would still constitute 19% of all sera tested.

Most of the mutations were DNA base changes leading to amino acid substitutions in the conserved region of the gene that encodes the specific DNA binding domain of the p53 protein. Amino acid substitutions in this domain disrupt interaction at the p53 protein-DNA target sequence interface.

A significant association between tumor mutation and seropositivity in previously published reports may be due to: (a) insufficient numbers of seropositive cases per study to establish a correlation; (b) the cancer type examined, if anatomical site has a bearing on immune competence in the local milieu of the tumor (3, 35); or (c) the methods used to determine tumor mutations. Some core-domain mutations are not detected by single strand conformational polymorphism, or by DGGE when primer sets are used that do not identify mutations in the sample located at proximal or distal ends of midregion exons (10, 28). Direct sequencing of tumor DNA without a more sensitive prior prescreening step is also likely to allow some mutations to go unnoticed due to wild-type sequences from contaminating normal cells that block out mutant signals.

In a study of breast cancer patients, Davidoff et al. (11) found that tumor p53 mutations, predominantly in exons 5 and 6, were immunogenic, and they linked this to selective binding of heat shock protein (HSP70) to p53 protein with amino acid substitutions arising from mutations in the proximal exons of the core domain. In our study, tumor mutations in seropositive individuals were primarily in exons 7 and 8, and included one that was equivalent to an exon 8 mutant in a breast cancer that did not show HSP70 binding activity (11).

Unexpectedly, p53 antibodies found in human sera are directed against immunogenic epitopes that are present in both wild-type and tumor-mutant p53 proteins (36, 6), i.e., in the N- and C-terminal ends, which are generally devoid of tumor-specific mutations. This being the case, it is not immediately apparent how core domain mutations elicit an autoimmune response, except insofar as the mutation causes protein accumulation (see below). An explanation for this paradox has been discussed by Milner (37), who suggested that certain conformational changes leading to variant proteolytic cleavage of mutant p53 might yield novel peptides for MHC presentation that would not necessarily map to the site of mutation.

It remains to be clarified to what extent (normal) p53 protein accumulation in the tumor cells is an independent determinant of immune response to tumor p53 protein in esophageal cancer. Our results indicate that it is structural mutation rather than the p53 protein abundance per se that is more likely to cause production of p53 antibodies in esophageal cancer patients, because only 1 of 12 patients with p53 accumulation, but no mutation in exons 5–8, was seropositive. An undetected tumor mutation, or one lying outside the gene segment we screened, may be present in this case (Table 2).

Accumulation of (mutant) protein could nevertheless contribute to further the immune response. If mutation is the primary (although clearly insufficient) prerequisite for an immune response in this type of malignancy, then the two seropositive cases with no tumor mutation in exons 5–8 would also require explanation. One possibility is the presence of a second undiagnosed primary cancer with mutant p53, which has caused the B-cell response. Metachronous second primary cancers are not uncommon in esophageal cancer patients.

Table 3 Tumor status of patients tested for serum anti-p53 antibodies in three-assay analysis: seronegative cases

<table>
<thead>
<tr>
<th>IHC</th>
<th>DGGE</th>
<th>Missense</th>
<th>Frameshift/nonsense/splice</th>
<th>Silent</th>
<th>RFLP</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>+</td>
<td>+</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>11</td>
<td>+</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>+</td>
<td>1</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>11</td>
<td>+</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>5</td>
<td>NA</td>
<td>2+/3NM</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* RFLP, restriction fragment length polymorphism; NA, not applicable; NM, no material available.

**Fig. 2.** A, p53 sequence analysis in esophageal cancer. Tumor DNA was isolated from patients HE91.07, HE-91.10, and wild-type control, amplified by PCR, and sequenced directly. Both cases showed a nucleotide substitution in the second base of codon 175, as indicated by the arrow. Wild-type (CGC) and mutant (CAC) alleles are present in each tumor sample. B, serological analysis of patients with the same missense mutation. IHC, IB, IP, and EIA ratio results from sera of patients HE91.07 (high titer) and HE91.10 (marginal) are shown. Patient HE91.01 (not shown), with the identical tumor mutation, was negative for p53 antibody in all three assays. The IB result for patient HE91.10 was an unreadable high background (HB, not shown).
from the high-risk groups to which these patients belong. A potentially immunogenic structural mutation in the tumor that lies outside the core domain we analyzed is also plausible. Alternatively, in some cases, cellular or viral protein complexes to normal p53 protein (33), with or without consequent stabilization and accumulation, may elicit an immune response due to altered antigen processing (38).

Although our results do not provide evidence to suggest that accumulation of structurally unaltered p53 protein is immunogenic, this certainly cannot be excluded. More patients with tumors that have wild-type p53 sequences, yet abnormally high p53 protein levels in tumor cells, would be required to pursue this further.

It is becoming evident that individual host response is a critical factor in p53 antibody synthesis, and one cannot presume that a given specific mutation will be uniformly immunogenic. In the present study, we found two cases from Guangzhou, in which a missense mutation at codon 175 resulted in p53 antibody production, whereas in two other patients with the identical tumor mutation, we found no circulating p53 antibody. Two similar instances were also described in a recent study on lung cancer patients (10). The importance of individual genetic background would be in keeping with recent work indicating that certain p53 mutations, in combination with specific human MHC molecules, may determine whether a B-cell response will occur or not (34, 39). Future studies should investigate whether the presence of p53 serum antibodies and mutations can be linked to patient HLA haplotypes.

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