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Antibodies to the p53 Tumor Suppressor Gene Product Quantified in Cancer Patient Serum With a Time-Resolved Immunofluorometric Technique

STAVROULA HASSAPOGLIDOU and ELEFTHERIOS P. DIAMANDIS

Department of Clinical Biochemistry, Toronto Western Division, The Toronto Hospital, 399 Bathurst Street, Toronto, Ontario M5T 2S8 and Department of Clinical Biochemistry, University of Toronto, 100 College Street, Toronto, Ontario M5G 1L5, Canada

We have developed new methodology for quantifying antibodies to the p53 tumor suppressor gene product in human serum. The assay involves solid-phase immobilization of a monoclonal anti-p53-specific antibody that is then reacted with a tumor cell line lysate containing mutant p53. The immunopurified p53 antigen acts as an immunosorbent for the serum p53 antibodies that are then detected by reaction with a goat anti-human immunoglobulin G antibody labeled with alkaline phosphatase (ALP). ALP activity is then measured with enzymatically amplified time-resolved fluorometry. The developed assay has many advantages over the radioactively labeled techniques previously used. In a preliminary clinical study involving 790 patient sera, we have identified 16 positive samples (2%). Highest titers were observed in a patient with melanoma and two breast cancer patients. Further studies are needed to improve the sensitivity of this test and to evaluate its possible use for cancer diagnosis, prognosis or monitoring of therapy.

KEY WORDS: tumor suppressor genes; p53; cancer; tumor markers; serum antibodies; time-resolved fluorescence.

Introduction

The p53 protein is a 375-amino acid nuclear phosphoprotein originally discovered in 1979 because of its ability to bind to the simian virus 40 (SV40) large T-antigen (1,2). This binding results in coimmunoprecipitation with anti-T-antibodies from extracts of SV40-transformed cells. It was later observed that other polyomavirus large T-antigens and the adenovirus E1B protein also bind p53 (3). These findings suggested that the transforming proteins of these two distinct DNA tumor viruses are targeting the same host protein as part of their transforming action. A similar interaction between viral transforming proteins and host elements is also seen with the retinoblastoma gene product (Rb) (4). Thus, it has been postulated that modification of the p53 and Rb function by transforming proteins of tumor viruses, through binding, may be essential for viral propagation and expression of their tumorigenic potential.

The p53 protein is present in minute amounts in normal cells. However, relatively high amounts were found in tumor tissue and tumor cell lines (5). It is now known that the accumulation of p53 in tumors and cell lines is due to mutations of the p53 gene and the synthesis of a gene product that has a different conformation and a much longer lifetime than the wild-type protein.

The p53 gene has been cloned and sequenced (6). Mutations can now be studied by PCR amplification of genomic DNA or cDNA and sequencing. Using these techniques, numerous point mutations have been identified (7–9). These mutations do not appear randomly in the gene but cluster within a 600-base pair region that contains codons 110 to 307. This sequence encompasses exons 5–8, where most of the highly conserved amino acids are concentrated. These findings suggest that mutations of the highly conserved regions will likely affect the biological activity of the wild-type protein.

The p53 mutations identified are considered separate somatic mutations because in normal tissue from the same patients, only the wild-type gene was found. However, germline p53 gene mutations have been described in families with the rare Li–Fraumeni cancer syndrome (10).

Mutations of the p53 gene are the most frequent genetic alterations observed in human cancer. It is of interest to know if these mutations change the biological activity of wild-type p53 and in what direction. Recent experiments have shown that wild-type p53 can suppress the growth of transformed cells in culture and the formation of tumors in animals, and the p53 gene was thus designated as a "tumor suppressor gene."

The mutated form, unlike the wild-type protein, cooperates with the *ras* oncogene in transforming primary rat cells in culture (11). Because of its transforming activity, the mutant p53 gene is con-

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Correspondence: Dr. E.P. Diamandis.
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sidered an oncogene. The prolonged half-life of mutant p53 protein allows for its accumulation in relatively vast amounts in tumor tissue.

The mode of action of p53 is still unclear. Strong evidence exists that p53 is a regulator of the cell cycle. The finding that wild-type p53, but not mutant p53, binds to the SV40 large T-antigen, a necessary promoter of viral DNA replication, prompted some to speculate that wild-type p53 binds to a cellular homolog of large T-antigen. The complex formed then prevents the cell from entering the S-phase, likely by arresting formation of the DNA replication–initiation complex in the G_1 phase. An alternative theory speculates that p53 is a regulator of transcription. Evidence for this comes from the finding that p53 exhibits DNA-binding ability that changes with mutations. Whether p53 regulates specific sets of genes necessary for passage from late G_1 to S is still unknown (8).

Mutant p53 protein is conformationally different from the wild-type protein and accumulates in tumor tissue (12). This led some to speculate that mutant p53 may act as an immunogen that could trigger an immune response for the production of anti-p53 antibodies. These antibodies, if produced early during tumor initiation and growth, could be used as tumor markers for diagnosis, monitoring, or prognosis.

To date, only three groups published data on the production of anti-p53 antibodies in cancer patients (13–15). Crawford *et al.* (13) detected antibodies in the serum of about 9% of patients with breast cancer. DeFromentel *et al.* (14) detected antibodies to p53 in 21% of children with B-cell lymphoma and in a smaller proportion of children with other malignancies. Recently, Davidoff *et al.* (15) studied anti-p53 antibody production in patients with breast carcinoma and found that antibody-negative tumors have mutations exclusively in exons 7 and 8 whereas antibody-positive tumors have mutations primarily in exons 5 and 6. Interestingly, they also found that all antibody-eliciting tumors contained complexes between p53 and a 70 kDa heat-shock protein (HSP70) suggesting that the HSP70 is involved in the antigenic presentation of p53.

All reported studies related to p53 antibody production detect antibodies to p53 by using the following principles: radioactively labeled p53 (metabolic labeling) from cell extracts is immunoprecipitated with the patient's serum, run on a gel under denaturing conditions, and detected by autoradiography (13,15). Alternatively, monoclonal antibody-immunoprecipitated p53 from tumor cell extracts is run on a gel and the patient's serum is used in a Western blot configuration as a possible source of anti-p53 antibodies (14). These methodologies suffer from a number of limitations: (a) they are labour intensive, time-consuming, and they are not suitable for screening large numbers of samples; (b) they are based on radioactivity; (c) they are mainly qualitative procedures.

In view of the interest in studying p53 antibody production in cancer patients and its possible use as a tumor marker, we have developed a highly sensitive non-isotopic procedure that does not involve metabolic labeling, electrophoresis, or autoradiography. This method is simple, quantitative, can be completed in one day, and is amenable to automation. The procedure was preliminarily applied for the detection p53 antibody production in a group of 790 patients with diverse cancers. Sixteen (2%) of patients were tested positive. The highest titers were associated with a melanoma patient. Detailed clinical studies are needed to establish if the developed procedure is useful for diagnosis, prognosis, or treatment of cancer patients.

Materials and methods

Instrumentation

For measuring liquid-phase Tb^{3+} fluorescence in white microtiter wells we used the CyberFluor 615 Immunoanalyzer, a time-resolved fluorometer, as described elsewhere (16).

Materials

The phosphate ester of 5-fluorosalicylic acid (FSAP) was obtained from CyberFluor Inc., Toronto, Canada. $TbCl_3 \cdot 6H_2O$ was from GFS Chemicals, Columbus, OH, USA. All other chemicals were from Sigma Chemical Co., St. Louis, MO, USA unless otherwise stated.

Solutions

The enzyme substrate buffer was a 0.1 mol/L Tris solution, pH 9.0, containing 0.1 mol of NaCl and 1 mmol of $MgCl_2$ per liter. The stock FSAP substrate solution was a 10^{-2} mol/L solution in 0.1 mol/L NaOH. Fresh FSAP substrate working solutions were prepared just before use by dilution (10-fold) of the stock in the enzyme substrate buffer. The cell lysis buffer was a 20 mmol/L Tris solution, pH 8.1, containing 150 mmol NaCl, 10 g Nonidet P-40, 0.5 mmol phenylmethylsulfonyl fluoride, 1 mg leupeptin, and 50 mg aprotinin per liter. The detection antibody diluent is a 50 mmol/L Tris solution, pH 7.80, containing 60 g bovine serum albumin (BSA) per liter. The developing solution is a 1 mol/L Tris base solution containing 0.4 mol NaOH, 3 mmol EDTA and 2 mmol $TbCl_3 \cdot 6H_2O$ per liter (no pH adjustment). The washing solution is a 5 mmol/L Tris buffer, pH 7.80, containing 0.5 g Tween 20 and 150 mmol NaCl per liter. The coating antibody solution is a 50 mmol/L Tris buffer, pH 7.80, containing 0.5 g sodium azide per liter. The serum sample diluent is a 50 mmol/L Tris buffer, pH 7.80, containing 60 g of BSA, 150 mmol NaCl, 200 mg mouse immunoglobulin, and 500 mmol KCl per liter. The cell lysate diluent is a 0.1 mol/L phosphate buffer, pH 7.40, containing 10 g of BSA per liter.

Cell lysates positive or negative for mutant p53 protein

The human cell lines COLO HSR (colon carcinoma) and K562 (chronic myeloid leukemia) were obtained from the American Type Culture Collection, Rockville, MD, USA. The cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum. The COLO HSR cell line was found to produce relatively large amounts of mutant p53 protein as measured by an immunofluorometric procedure (17). The p53 gene in this cell line is mutated at codon 248 (CGG to TGG) resulting in the replacement of arginine by tryptophan (18). The K562 cell line is known not to produce any p53 protein and was used for the assessment of nonspecific binding effects (19).

The cells were grown until they reached at least 10^6 cells/mL. The cell pellets from a 10 mL culture were lysed in 300 μL of lysis buffer for 30 min on ice. The cell extract was centrifuged at 12,000 *g* for 10 min and the pellet was discarded. The supernatant was either used immediately or stored in aliquots at −70 °C until use.

Antibodies

The mouse anti-p53 monoclonal antibodies PAb421 and PAb240 were kindly provided by Dr. S. Benchimol, Princess Margaret Hospital, Toronto. These are tissue culture supernatants containing about 30 μg/mL of antibody. Antibody PAb421 reacts with an epitope of wild-type p53 between amino acids 370 and 378 (20,21) but also recognises mutant human and wild-type or mutant p53 from other species. Antibody PAb240 recognises an epitope specifically exposed when p53 is present in an altered, mutated form (22). Goat anti-mouse immunoglobulin and alkaline phosphatase-labeled goat anti-human immunoglobulin G (both approximately 1 mg/mL and affinity purified) were obtained from Jackson Immunoresearch, West Grove, PA, USA.

Patient samples

Seven hundred and ninety serum samples from patients with various malignancies were obtained from the Princess Margaret Hospital, Toronto. The samples were stored at 4 °C for up to 2 weeks.

Assay procedure

White, opaque 12-well microtiter strips (from Dynatech Laboratories, Alexandria, VA, USA) were coated with goat anti-mouse immunoglobulin diluted 500-fold in the coating antibody solution (100 μL/well, overnight incubation at room temperature).

The wells were then washed ×4 with an automatic washer and used for the assay as follows: pipet 100 μL of mouse monoclonal anti-p53 antibody (421 or 240, diluted 20-fold in the coating buffer) and 50 μL of COLO HSR cell lysate (diluted 10-fold in the cell lysate diluent) and incubate for 3 h with shaking at 37 °C (air oven). Wash ×4, add 100 μL/well of serum sample diluted 10- to 40-fold in the serum sample diluent (in triplicate) and incubate for 1 h with shaking, at room temperature. Wash ×4 and add 100 μL/well of an alkaline phosphatase-labeled goat anti-human immunoglobulin G antibody diluted 15,000-fold in the detection antibody diluent. Incubate for 1 h with shaking at room temperature and wash ×4. Add 100 μL/well of the diluted FSAP substrate solution and incubate for 10 min with shaking at room temperature. Add 100 μL/well of the developing solution, mix for 1 min and measure the fluorescence on the CyberFluor 615 immunoanalyzer.

Each assay run must be accompanied by a parallel run that assesses any nonspecific binding effects. This is identical to the run described above but instead of using the cell lysate from COLO HSR cells, we use the cell lysate from the K562 cells. Sera were considered positive for antibodies, only if the signal with the COLO HSR lysate exceeded the signal with the K562 lysate by a factor of 1.7.

Results and discussion

The final immunocomplex formed on the solid phase is shown in Figure 1. Conventionally, human antigen-specific antibodies are detected by using immobilised antigen as described elsewhere (23). However, pure p53 is not available in quantities sufficient for immobilisation. We have developed an al-

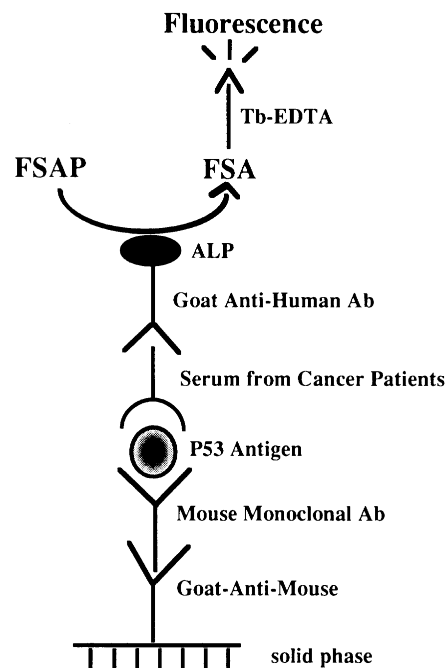

Figure 1 — Assay configuration for the detection of anti-p53 antibodies in human serum. More details are given in the text. The detection technique is described in detail in (16). Ab, antibody; ALP, alkaline phosphatase; FSAP, fluorosalicyl phosphate; FSA, fluorosalicylate.

ternative strategy that involves coating with a monoclonal anti-p53 specific antibody. This antibody binds p53 present in tumor cell lysates and simultaneously immunopurifies it from other proteins present. The monoclonal antibody-captured p53 acts as an immunosorbent for antibodies present in human serum. The detection system that we have used is based on enzymatically amplified time-resolved fluorescence with terbium chelates and is described in detail elsewhere (16). Briefly, alkaline phosphatase hydrolyses FSAP and the released 5-fluorosalicylate is reacted at alkaline pH with a Tb^{3+}-EDTA complex. The ternary complex formed, when excited at 337 nm, emits long-lived Tb^{3+}-specific fluorescence quantified by time-resolved fluorometry (24). This technique allows detection of attomole quantities of analytes.

This assay configuration would allow for the quantification of antibodies to p53 irrespective of the position of the mutation of the immunizing antigen since it has already been shown that the serum antibodies are polyclonal in nature and recognize a wide variety of mutant p53 proteins and in addition, wild-type murine p53 (15).

The quantitative nature of the assay is shown in Figure 2. Varying dilutions of a positive specimen were run. Near-linear response is obtained at serum dilutions between 8- to 128-fold. At lower serum dilutions there is a saturation effect, as expected. Assay imprecision was assessed at two levels of antibody titers by diluting a positive serum 4-fold or 40-fold. The obtained coefficients of variation were 5.7 and 6.2%, respectively ($n = 12$). This imprecision is typical for this type of immunological assay.

To ensure that the assay described is free of any nonspecific effects, we have incorporated a measurement with a cell lysate that does not contain any p53. This measurement is necessary because different sera exhibit variable background signals that may be misleadingly interpreted as specific signals. We have arbitrarily defined the positive samples as these that exhibit a signal-to-background fluorescence ratio of at least 1.7.

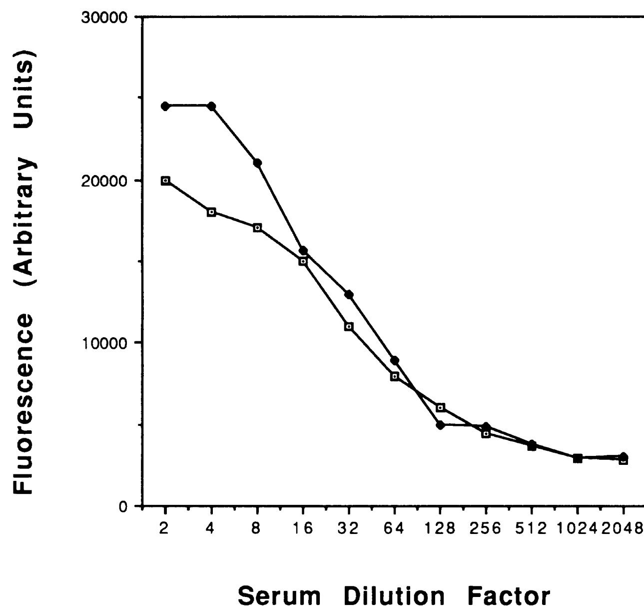

Figure 2 — Plot of fluorescence versus serum dilution factor for a serum positive for p53 antibodies. Coating antibody was PAB421 (♦) or PAB240 (⊡). A near-linear response is obtained at dilutions between 8- to 128-fold.

The type of assay that we have developed has numerous advantages over the previously described techniques. Our assay does not use metabolic labeling, radioactivity, immunoprecipitation, autoradiography, or Western blot techniques. It is thus much faster, it can be used to analyze many hundreds of samples in one working day and it can be automated. Additionally, it gives quantitative information that allows for accurate monitoring of antibody titers with time or as related to surgical or pharmacological treatment.

It is not accurately known if anti-p53 antibodies are produced early during the initiation of malignancy or are relatively late events. Nor has it been reported if the antibody titers are correlating with the cancer stage or any other clinical parameter. Anti-p53 antibody titers have only been studied in breast carcinoma and some childhood malignancies. Studies involving other tumor types are lacking. This simplified methodology should be a useful tool to address the above questions. It is conceivable that anti-p53 antibody titers may find usefulness for the diagnosis, monitoring, and treatment of cancer patients. Although the prevalence of anti-p53 antibodies already reported in some cancer patient populations is relatively low, this may be partially due to the fact that not all patients with malignancy bear tumors that produce p53. The p53-negative tumors do not induce anti-p53 antibody production (15).

In a preliminary screen of 790 cancer patient sera, we have identified 16 patients (2%) who have anti-p53 antibodies in their serum (Table 1). Antibody titers, arbitrarily defined as the ratio between the specific and nonspecific fluorescence, ranged from about 2.0–22. Highest antibody titers were obtained in a patient with melanoma followed by two breast cancer patients. The presence of anti-p53 antibodies in the sera from patients with melanoma, brain, stomach, bladder, lung, prostate, or ovarian tumors, reticulosarcoma, leukemia, or Hodgkin's lymphoma has not been previously reported. Sera from 300 noncancer patients tested negative for anti-p53 antibodies.

Further clinical investigations are needed to accurately define the prevalence of anti-p53 antibodies in various groups of cancer patients. Methodological improvements of the proposed technique may also be necessary in order to improve the sensitivity of the test. More specifically, direct antigen coating, when p53 becomes available in high quantities, or capture of various mutated forms of p53 produced by tumor cell lines may yield higher detectabilities. These analytical improvements and detailed clinical studies are currently under investigation.

TABLE 1
Diagnosis of Patients Tested Positive for Anti-p53 Antibodies

Sample	Tumor/Malignancy	Antibody Titer	
		PAb240	PAb421
1[a]	Brain	6.5	5.1
2	Stomach	4.7	4.9
3	Breast	7.0	6.8
4	Breast	14	10
5	Melanoma	22	11
6	Bladder	2.7	2.1
7	Hodgkin's lymphoma	6.0	4.0
8	Leukemia	8.8	3.2
9	Reticulosarcoma	4.8	4.2
10	Lung, kidney	3.2	2.8
11	Prostate	3.7	3.3
12	Hodgkin's lymphoma	1.7	2.2
13	Leukemia	2.8	3.5
14	Lung	2.2	2.5
15	Ovary	2.0	2.0
16	Breast	2.0	1.7

Antibody titer is the ratio of fluorescence in the presence of cell lysate positive or negative for mutant p53. Coating antibody was either PAb240 or PAb421.
[a]This patient was identified positive for p53 antibodies four times during blinded testing, over 4 weeks. Titers with Ab240 were 6.5, 7.3, 5.0, and 6.0.

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