Autoantibodies against the p53 tumor suppressor gene product quantified in cancer patient serum with time-resolved immunofluorometry

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ABSTRACT

Background - Tumors producing mutant p53 protein sometimes immunize the host for the production of autoantibodies which circulate in the serum of cancer patients. The possible clinical usefulness of these serum autoantibodies has not yet been examined in detail, partly because the currently existing methods are difficult to perform and are qualitative procedures.

Methods - We describe here the development of a new method and the modification of a recently developed assay (Clin Biochem 1992;25: 445-9) for measuring anti-p53 antibodies in serum. One method is a ‘competitive’-type immunoassay and the other one is of the ‘non-competitive’ type. These two methods are superior to the methods previously used in terms of speed, cost, quantification and automatability. The availability of two methods based on different principles is useful for cross-checking the results when the assays are used for clinical studies.

Results - Using both methods we were able to detect and quantify serum autoantibodies specific for p53 in patients with breast, colon, ovarian and pancreatic tumors.

Conclusions - These newly developed procedures can be used to study the possible application of p53 antibodies for cancer diagnosis monitoring and prognosis.

Key words: cancer diagnosis, autoantibodies, tumor suppressor genes, p53 protein, time-resolved fluorometry.

INTRODUCTION

Mutations of the p53 tumor suppressor gene are the most frequent genetic alterations in human cancer (1, 2). Currently, more than 350 independent mutations of this gene have been described, occurring in more than 35 different tumor types (3). The mutant p53 protein accumulates in the tumor, presumably due to its increased half-life and can be detected by immunohistochemistry (4) or immunological techniques (5). Recently, it has been found that some mutant proteins, present in tumors, may elicit antibody generation in the host. Such proteins include myb (6), myc (7) and p53 (8-12). These antibodies can be detected in the serum of cancer patients. Although these circulating antibodies are potential tumor markers which could be used for diagnosis, prognosis or monitoring, this approach has not as yet been systematically studied.

In 1982, Crawford et al. (8) detected p53 antibodies in the sera of 9% of patients with breast carcinoma. Caron de Fromentel et al. (9) later found that such antibodies were present in the sera of children with a wide variety of cancers. On average, the frequency was 12% but the frequency in Burkitt’s lymphoma patients was 20%. More recently, Winter et al. reported development of anti-p53 antibodies in 13% of patients with lung cancer (10). Davidoff et al. have also reported incidence of serum anti-p53 antibodies in ~10% of breast cancer patients and found that the antigenic response is dependent upon p53/HSP70 complexes (11). Another recent report described anti-p53 antibodies in the serum of 14% of breast cancer patients and established associations between p53 antibodies and high histological grade and absence of steroid hormone receptors (12). More recently, anti-p53 antibodies have been detected in the serum of patients with colon and ovarian cancers (13) and hepatoma (14).

The techniques for detecting anti-p53 antibodies in patient sera are currently based on radioactive labeling, immunoprecipitation and immunoblotting (8-14). All these methods are qualitative and time consuming and thus not suitable for screening large number of samples. For this reason, the phenomenon of autoantibody generation has been studied for only a few types of tumors and the total number of cancer patient samples tested was limited. Recently, we have devised a quantitative immunological method for measuring p53 antibodies in serum (15). We here describe an improved version of this assay and a newly devised method based on a different principle. We demonstrate that these assays are reliable, quantitative methods which can be used to study the possible clinical applications of the p53 antibody test in cancer patient management.

Nonstandard Abbreviations: HSP70, heat shock protein 70; BSA, bovine serum albumin; FSAP, 5-fluorosalicyl phosphate; GAR Ig, goat anti-rabbit immunoglobulin; GAh Ig, goat anti-human immunoglobulin; GAM Ig, goat anti-mouse immunoglobulin; BCA, bicinchoninic acid.
MATERIALS AND METHODS

Materials - For measuring liquid-phase Tb3+-fluorescence in white microtiter wells we used the Cyberfluor 615 Immunoanalyzer, a time-resolved fluorometer, as described elsewhere (16, 17). The phosphate ester of 5-fluorescein isothiocyanate acid (FSAP) was obtained from CyberFlor Inc., Toronto, Canada. TbCl3. 6H2O was from GPS Chemicals Columbus, OH, USA. All other chemicals were from Sigma Chemical Co., St. Louis, MO, USA unless otherwise stated.

The enzyme substrate buffer was a 0.1 mol/l Tris solution, pH 9.1, containing 0.1 mol NaCl and 1 mmol MgCl2 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 phenylmethylsulphonyl fluoride, 2 mg leupeptin and 2 mg aprotonin per liter. The developing solution was a 1 mol/l Tris base solution containing 0.4 mol NaOH, 3 mmol EDTA and 2 mmol TbCl3.6H2O per liter (no pH adjustment). The washing solution was a 5 mmol/l Tris buffer, pH 7.80, containing 0.5 g Tween 20 and 150 mmol NaCl per liter. The coating antibody diluent was a 50 mmol/l Tris buffer, pH 7.80, containing 0.5 g sodium azide per liter. The CM-1 antibody diluent was a 50 mmol/l Tris buffer, pH 7.80, containing 60 g bovine serum albumin (BSA) per liter. The goat anti-rabbit immunoglobulin-alkaline phosphatase conjugate (GAR Ig-ALP) diluent was a 50 mmol/l Tris buffer, pH 7.80, containing 60 g BSA, 0.5 mol KCl and 100 mg albumin per liter.

The cell lines used in this study were colon carcinoma Colo 320 HSR(+) (18), pancreatic carcinoma Mia PaCa-2 (19), breast carcinoma T-47D (20), and human erythroleukemia OCI M2 (21). These cell lines were cultured as described elsewhere; they all have p53 gene mutations and overproduce mutant p53 protein (5). Recombinant wild-type p53 protein, produced as described elsewhere (22), was a gift from Dr. Carol Prives, Columbia University, New York, USA.

Lysates from cell lines producing p53, or recombinant p53, were diluted in a 50 mmol/l Tris buffer, pH 7.80, containing 60 g of BSA per liter for Method A and in 100% goat serum for Method B. The mouse monoclonal anti-p53 capture antibody (PAb240) diluent was a 50 mmol/l Tris buffer, pH 7.80, containing 60 g BSA and 0.5 mol KCl per liter. Serum samples were diluted in a serum diluent which is the same as the PAb240 diluent but supplemented with 10% normal goat serum and 2% normal mouse serum. The goat anti-human immunoglobulin-alkaline phosphatase conjugate (GAH Ig-ALP) diluent was the same as the GAR Ig-ALP diluent.

The mouse anti-p53 monoclonal antibody PAb240 was produced as a tissue culture supernatant from a cell line donated to us by Dr. D.P. Lane, University of Dundee, U.K. Its antibody concentration was approximately 30 μg/ml. The rabbit polyclonal anti-p53 antibody, CM-1, was obtained from Dimension Labs, Mississauga, Ontario, Canada. The goat anti-rabbit and goat anti-human antibodies, conjugated to alkaline phosphatase, and the goat anti-mouse antibody, Fc fragment specific (GAM Ig), all approximately 1 mg/ml, were obtained from Jackson ImmunoResearch, West Grove, Pennsylvania.

Patient Sera - Sera from cancer patients were stored at -70°C until analysis. Sera used were from patients with breast (n=105), ovarian (n=72), colon (n=77) and pancreatic cancer (n=46). For correlation studies we used 38 p53 antibody-positive sera from patients with the above malignancies plus sera from prostate, lymphoma, lung and multiple myeloma patients.

Procedures

Cell lysis - Cells from each cell line were grown until they reached approximately 100 cells/ml or 90% confluency. The cell pellet from a 15 ml culture was lysed in 300 μl lysis buffer, for 30 min, on ice. The cell extract was centrifuged at 12,000 x g for 10 min and the pellet discarded. The lysate was used within two hours. Total protein was measured in the lysates with the bicinchoninic acid (BCA) assay, commercially available by Pierce Chemical Co., Rockford IL. Lysates typically contained 1-3 mg of protein per ml.

Immuoassay Procedure, Method A - This method is a modification of an assay previously published (15). White, opaque, 12-well microtiter strips (from Dynatech Laboratories, Alexandria, VA) were coated with goat anti-mouse immunoglobulin diluted 500-fold in the coating antibody diluent (100 μl/200 ng/well, overnight incubation at room temperature). We previously found that this indirect coating is superior to direct coating with the PAb240 antibody (15). The wells were then washed six times with an automatic washer and used for the assay as follows: 50 μl of cell lysate (diluted 10-fold in the cell lysate diluent) and 100 μl of mouse monoclonal anti-p53 antibody PAb240 (diluted 20-fold in the PAb240 diluent) were added and incubated for 3 h with shaking at 37°C (airoven). After 6 washes, 100 μl/well of sample serum (diluted 10-fold in the serum sample diluent, in duplicate) and incubate for 1 h with shaking, at room temperature. After six washes, 100 μl/well of an alkaline phosphatase-labeled goat anti-human immunoglobulin G-antibody (diluted 15,000-fold in the GAH Ig-ALP diluent) was added. The wells were incubated for 1 h with shaking at room temperature and washed six times. 100 μl/well of the diluted FSAP substrate solution was added and incubated for 10 min with shaking at room temperature. 100 μl/well of the developing solution was added, mixed for 1 min and the fluorescence was measured on a Cyberfluor 615 TM Immunoanalyzer.

Each assay run was accompanied by a parallel run to assess any nonspecific binding effects. This run was identical to the procedure described above but the cell lysate was related by the lysate diluent. Sera were considered positive for antibodies only if the signal with the lysate exceeded the signal without the lysate by a factor of 1.7 (15).
Imunoassay Procedure, Method B - Microtiter strips were coated as in Method A. Patient serum (200 μl) was then incubated in tubes with 20 μl of a 10-fold diluted cell lysate from Colo 320 HSR (+) cells, for 30 min at room temperature. The p53-supplemented sera (50 μl, in duplicate) were then added to goat anti-mouse IgG-coated wells along with 100 μl/well of mouse monoclonal anti-p53 antibody PAb240, diluted 20-fold as in Method A. The wells were incubated at 370C for 3h with shaking and washed six times. 100 μl/well of the rabbit anti-p53 polyclonal antibody (CM-1) diluted 5,000-fold in the CM-1 antibody diluent was added and incubated for 1h at room temperature with shaking. After washing six times 100 ul/well of the alkaline phosphatase-labeled goat anti-rabbit immunoglobulin (GARlG-ALP) diluted 5,000-fold in the GARlG-ALP antibody diluent was added and incubated for 1h at room temperature with shaking. The wells were washed six times and the procedure continued as in Method A from the point of adding the FSAP substrate solution. Each serum sample was also assayed without the addition of the Colo 320 HSR(+) cell lysate to assess the background signal. Sera were considered positive for antibodies only if the fluorescence signal in the presence of serum was less than 50% of the fluorescence signal obtained with a 6% BSA solution as sample (see also Results section).

Quantification - Due to the lack of a suitable standard solution, we devised an arbitrary system to calibrate Methods A and B. Among the highly p53 antibody-positive sera we selected one and arbitrarily defined its concentration to be 20,480 Units/L (U/L). This serum sample was then used in dilutions to construct calibration curves for assays A and B from which the concentration of the other samples was calculated.

RESULTS AND DISCUSSION

We have recently published an immunological method for measuring anti-p53 antibodies in the serum of cancer patients (15). We have modified this method to improve performance. In comparison to the original report the following changes were made: we supplemented the serum diluent with 10% normal goat serum in order to block human anti-goat antibodies that we found were present in about 3% of the human sera. These human anti-goat antibodies could generate high background signals due to their ability to link the solid-phase goat anti-mouse antibody and the alkaline phosphatase-labeled goat anti-human IgG antibody (Figure 1). An example of such an effect is shown in Table I. This effect was independent of the presence or absence of the PAb240 antibody and of p53 antigen in the assay. The cell lysate diluent in the original report was also modified to 50 mmol/l Tris buffer, containing 60g of bovine serum albumin (BSA) per liter. The PAb240 diluent was changed to a 50 mmol/l Tris buffer, pH 7.80, containing 60g BSA and 0.5 mol KCl per liter and the alkaline phosphatase-conjugated goat anti-human IgG diluent was changed to a 50 mmol/l Tris buffer, pH 7.80, containing 0.5 mol KCl, 60g BSA and 100 ml goat serum per liter. These changes resulted in lower background signals and elimination of non-specific effects due to anti-species antibodies present in some human sera (23).

Table I: Elimination of background problems in Method A due to human anti-goat antibodies present in a patient serum1,2

<table>
<thead>
<tr>
<th>Serum diluent</th>
<th>GAHlg-ALP diluent</th>
<th>Background signal (fluorescence, arbitrary units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6% BSA/KCl</td>
<td>6% BSA</td>
<td>391,270</td>
</tr>
<tr>
<td>6% BSA/KCl</td>
<td>6% BSA/KCl</td>
<td>265,000</td>
</tr>
<tr>
<td>6% BSA/KCl/10% G.S.</td>
<td>6% BSA/KCl</td>
<td>13,880</td>
</tr>
<tr>
<td>6% BSA/KCl</td>
<td>6% BSA/KCl/10% G.S.</td>
<td>38,880</td>
</tr>
<tr>
<td>6% BSA/KCl/10% G.S.</td>
<td>6% BSA/KCl/10% G.S.</td>
<td>8,880</td>
</tr>
</tbody>
</table>

1:6% BSA = 6%(w/v) bovine serum albumin. KCl was used at a concentration of 0.5 mol/L. 10% G.S. = 10%(v/v) of normal goat serum. GAHlg-ALP = goat anti-human IgG, conjugated to alkaline phosphatase.
2: In another representative example, by diluting the p53 antigen in goat serum in Method B, instead of a 6% BSA solution, we were able to reduce the background signal from 70,000 to 3,742 arbitrary fluorescence units.
3: Background signal in Method A was measured as described in "Methods".

![Figure 1](https://example.com/figure1.png)

Figure 1. Mechanisms of interference of human anti-goat antibodies present in some patient sera. (A). Cross-linking of coating and detection goat antibodies without the need for p53 antigen or anti-p53 antibodies, operating in both Methods A and B. (B). This mechanism, which operates in Method A only, involves attachment of human anti-goat antibodies to the solid-phase goat antibody. These human antibodies are then detected by the goat anti-human immunoglobulin conjugated to alkaline phosphatase (ALP). See Figure 2 for more details.

The optimal Colo 320 HSR(+) cell lysate dilution for Method A, prepared as described in Methods section was 10-fold, as proposed in the original assay. For screening purposes, the optimal serum dilution for Method A is 10-fold. At this dilution, samples with relatively low anti-p53 antibody titers could be effectively identified. With highly positive samples, dilutions ranging from 20 to 5,000-fold were used.

In the original Method A assay configuration (15) the source of p53 antigen was a lysate from the cell line Colo 320 HSR(+) cells. This cell line bears a mutant p53 gene with a point mutation at codon 248 (CGG→TGG) which substitutes arg by trp. In order to examine whether other
mutants or wild-type p53 are recognized by the human anti-p53 antibodies in the serum, we have used lysates prepared from other cell lines i.e. the MIA PaCa-2 pancreatic carcinoma cell line (point mutation at codon 248, CGG→TGG, arg to trp) the T-47D breast carcinoma cell line (point mutation at codon 194, CTT→TTT, leu to phe) and the OCI M2 human erythroleukemia cell line (point mutation at codon 274, GTT→GAT, val to asp). We have also used a purified wild-type recombinant p53 protein produced as described by Wang et al (22). Some data are presented in Table II. Human anti-p53 antibodies bind to various mutants as well as to wild-type recombinant protein, in accordance with previous reports (8-14). However, in some cases where the antibody titers are relatively low the sera tested were negative (ratio <1.7) with lysates from T-47D or OCI M2 cells, or with the recombinant p53 protein, suggesting different affinities of certain anti-p53 antibodies against various mutants or wild-type p53. For subsequent studies, we used only the lysate from Colo 320 HSR(+) cell lines to screen all the clinical samples for anti-p53 antibodies. This lysate and the one from MIA PaCa-2 cells gave superior results in comparison with other lysates or with the recombinant p53 protein, but the Colo 320 HSR(+) cells are easier to culture. Method A is able to detect anti-p53 antibodies of the IgG class but by substituting the alkaline phosphatase-labeled goat anti-human IgG antibody with anti-IgA or anti-IgM antibodies it is also possible to detect IgA or IgM anti-p53 antibody classes in serum (see below).

Table II: Reactivity of human anti-p53 antibodies against various p53 mutant proteins and against wild-type recombinant p53 protein

<table>
<thead>
<tr>
<th>source of p53 antigen</th>
<th>recombinant p53 protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HSR(+)</td>
</tr>
<tr>
<td>breast</td>
<td>1.8(5)</td>
</tr>
<tr>
<td>breast Ca</td>
<td>4.2</td>
</tr>
<tr>
<td>breast Ca</td>
<td>4.0</td>
</tr>
<tr>
<td>lung Ca</td>
<td>2.0</td>
</tr>
<tr>
<td>lung Ca</td>
<td>2.8</td>
</tr>
<tr>
<td>cell line/mutations</td>
<td>248</td>
</tr>
<tr>
<td>coton</td>
<td>(CGG-TGG)</td>
</tr>
<tr>
<td>amino acid substitution</td>
<td>Arg-Trp</td>
</tr>
<tr>
<td>malignancy</td>
<td>colon</td>
</tr>
</tbody>
</table>

In order to confirm and extend the data obtained by Method A, we devised another method for anti-p53 serum antibody detection based on a different principle. The new method (Method B) is able to detect any p53 antigen-binder in serum, including non-immunoglobulin molecules. Recovery experiments performed by adding p53 antigen in serum (from Colo 320 HSR(+) cells) to serum and quantifying it with a p53 antigen assay described by our group (5) revealed that the p53 antigen added could be quantitatively recovered. Recovery was dramatically reduced when the p53 antigen-spiked sera were positive for anti-p53 antibodies. Based on this observation, we devised Method B for measuring anti-p53 antibodies as follows: Spiking a human serum with p53 antigen and incubating for 30 min. During this period, any p53 antigen-binder present in serum will react with the p53 antigen. Subsequent analysis for p53 antigen by the method previously described (5) will result in low recovery due to the inability of the mouse monoclonal anti-p53 capture antibody and/or the rabbit
CM-1 antibody to react with the diagrammatically already bound. In Figure 2 we present both Methods A and B p53 antigen. Method B was carefully optimized because the amount of p53 antigen added affects the sensitivity of the method. The amount of antigen added should not be very high, so that even relatively low amounts of antibodies or other binders can neutralize it. However, the amount of antigen added should not be too low because in this case the difference in fluorescence between the zero reading (no antigen added) and the reading with p53 added, will not be significant. With the Colo 320 HSR(+) lysate, prepared as described in Methods, a 100-fold final dilution was found to be optimal. For maximum sensitivity, the serum should be used undiluted with this method. The lysate was diluted in goat serum in order to neutralize any anti-goat antibodies present in some patient sera after the spiking. We used the same arbitrary serum standard to construct calibration curves for both Methods A and B (Figure 3). When we compared 38 positive sera by the two methods we obtained the correlation shown in Figure 4.

The sensitivity of Methods A and B was compared by using the standard p53 antibody-containing serum at various dilutions. Method A was found to be approximately 5-fold more sensitive than Method B. The detection limit for Method A was ~2 U/L (or 20 U/L in the original serum since the sera are diluted (x 10 in this method) and ~100 U/L for Method B. Precision studies have shown that antibody titers could be quantified with either method with coefficients of variations (CVs) of about 10% or less.

For three hundred cancer patient serum samples (105 breast; 72 ovarian; 77 colon and 46 pancreatic) we performed the analysis by both methods in an effort to identify any positivity-rate differences between Methods A and B (Table III). The results show that of the p53 antibody-positive sera, only two were positive by Method B and negative by Method A while five sera were positive by Method A and negative by Method B. Another 26 samples were positive by both methods. We have attributed the differences in the five sera to the higher sensitivity of Method A over Method B because these sera contained relatively low concentrations of p53 antibodies.

Two cancer patient sera were positive by Method B and negative by Method A (Table III) and six other sera exhibited titers which were 2-4 times higher with Method B. We speculated that these sera might contain antibodies of the IgA or IgM subclass or other non-immunoglobulin p53 binders. To examine this possibility, we reanalyzed twelve
samples with Method A but substituted the goat anti-human immunoglobulin G antibodies with goat anti-human immunoglobulin A or M. From the six samples which showed differences in p53 antibody concentrations between Methods A and B, we found five that were positive for IgA and two that were positive for IgM. However, in all six samples, the fluorescence signals with goat anti-human immunoglobulin G were much higher. These data suggest that although IgA and IgM antibodies against p53 exist in some patient sera, their concentrations or affinities are much lower in comparison with the co-existing IgG antibodies. From the six samples with good agreement in p53 antibody concentrations between Methods A and B, two samples were positive for IgA and one sample was positive for IgM. High performance liquid chromatography with a molecular sieve column of one sample of which gave about 4-fold higher p53 antibody concentration with Method B was performed and fractions analyzed by both Methods A and B. This experiment failed to identify any p53 antigen-binders which could be detected only by Method B. Taken together, these data suggest that the differences between titers observed between Methods A and B in some cases are probably due to lower affinity anti-p53 IgG, IgA or IgM antibodies which are detected only by Method B. This notion is suggested, because in Method B, low affinity antibodies could effectively block p53 antigen capture by the coating antibody (Figure 2). In Method A, low affinity antibodies could escape from the p53 antigen and pass undetected during the two washing steps of the assay.

The prevalence of p53 antibodies in cancer patient sera has only been occasionally studied, the cancer types examined were few and the results reported have been qualitative (8-14). We here describe two new, quantitative methodologies which are based on different principles. Both methods employ a detection methodology using alkaline phosphatase as label and time-resolved fluorometry with terbium chelates. This detection method is among the most sensitive reported and is suitable for measuring analytes at atomol levels (14, 15).

Our methods are free from interference from heterophilic antibodies which occur with a frequency of about 3% in the general population. The methods are also fast, economical, quantitative and amenable to automation.

The clinical usefulness of p53 antibodies in cancer patient sera needs to be further examined. Currently, there are suggestions that the test may be used as a diagnostic aid in patients with hepatoma who have normal alpha-fetoprotein levels (14). The diagnostic usefulness of the test in other cancers, e.g. ovarian, lung and colon cancers, is currently under investigation by our group. The application of the test for patient monitoring has not yet been published. Other reports suggest that the p53 antibody-positive patients are a subgroup with poor prognosis (12). Clearly, more studies are needed to further establish the possible use of the p53 antibody test for patient diagnosis, prognosis and monitoring. The quantitative procedures described in this paper are well suited to studying these questions in detail.

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REFERENCES

Quantification des autoantigènes dirigés contre le gène suppresseur de tumeur p53, dans le sérum des malades atteints de cancer, à l'aide d'une épreuve d'immunofluorométrie

RESUME

Contexte - Les tumeurs productrices de protéines p53 mutantes immunisent quelques fois l'hôte pour la production d'autoantigènes qui circulent dans le sérum des cancéreux. L'utilisation clinique de ces autoantigènes sériques n'a pas encore été examinée en détail, en partie car les méthodes courantes existantes sont difficiles à pratiquer et parce qu'il s'agit de procédés qualitatifs.

Méthodes - Nous décrivons ici le développement d'une nouvelle méthode et les modifications d'un essai mis au point récemment (Clin Biochem 1992;25: 445-9) pour mesurer les anticorps anti p53 dans le sérum. Une des méthodes est du type immuno-essai compétitif et l'autre du type non compétitif. Ces deux méthodes sont supérieures aux méthodes utilisées, avant tout en termes de rapidité, de coûts, de quantification et d'automatisation. La disponibilité des deux méthodes basées sur des principes différents, est utile pour croiser les résultats quand les essais sont pratiqués lors d'études cliniques.

Résultats - En utilisant les deux méthodes nous sommes susceptibles de détecter et de quantifier l'autoantigène sérique spécifique du p53 chez les malades atteints des tumeurs des seins, du colon, des ovaires et du pancréas.

Conclusions - Ces nouvelles procédures développées peuvent être utilisées pour étudier l'application possible des anticorps p53 pour le diagnostique le suivi et les pronostics du cancer.