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Quantification of p53 protein in tumor cell lines, breast tissue extracts and serum with time-resolved immunofluorometry

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We have developed a highly sensitive time-resolved immunofluorometric procedure for quantifying mutant or wild-type, human or murine, p53 protein. The method uses monoclonal PAb240 or PAb421 antibodies for capture and a polyclonal rabbit antibody for detection. The final immunocomplex is quantified with an alkaline phosphatase substrate which, when hydrolyzed by the enzyme, forms highly fluorescent long-lived complexes with Tb^{3+}-EDTA. The detection limit is approximately 1 pg of protein per assay. The assay was applied for the quantification of p53 protein in lysates from 23 cell lines and overproducers of mutant protein were identified. Eight hundred cancer patients sera tested negative for the presence of p53. We have also applied the quantitative immunofluorometric procedure for measuring mutant p53 protein in breast tumor extracts specifically prepared for steroid hormone receptor analysis. Sixty-four out of the 264 extracts (24%) were positive for p53. Significant negative correlations between levels of p53 and steroid hormone receptors were found. The proposed analytical methodology simplifies the assessment of p53 status in tumor extracts, has many advantages over immunohistochemical techniques and is proposed as a method of choice for routine clinical use and other investigations involving p53.

Introduction

The p53 tumor suppressor gene is localized on chromosome 17 and encodes a nuclear phosphoprotein of 53 kDa with the characteristics of a transcription factor (Fields & Jang, 1990; Raycroft *et al.*, 1990). Sporadic mutations of the p53 gene are the single most common genetic alterations observed in human cancers (Hollstein *et al.*, 1991; Lane & Benchimol, 1991; Levine *et al.*, 1991). Recently, germ-line mutations of the p53 gene have been identified in patients with the Li-Fraumeni syndrome (Malkin *et al.*, 1990; Srivastava *et al.*, 1990) and in patients with other malignant neoplasms (Malkin *et al.*, 1992; Prosser *et al.*, 1992; Toguchida *et al.*, 1992).

The alterations of the p53 gene can be studied by sequencing techniques (Chiba *et al.*, 1990), by single-stranded conformational polymorphism analysis (Murakami *et al.*, 1991; Tamura *et al.*, 1991) or by constant denaturant gel electrophoresis (Borresen *et al.*, 1991). Mutant p53 proteins are examined with antibodies reacting with specific epitopes (Harlow *et al.*, 1981; Wade-Evans & Jenkins, 1985; Greaves, 1988; Gannon *et al.*, 1990; Midgley *et al.*, 1992). Although the p53 gene mutations are scattered throughout the gene, giving rise to many mutant proteins, certain monoclonal antibodies can react with either wild-type and/or many different mutant forms. The mutant p53 proteins are usually much more stable than the wild-type protein, adopt an altered conformation and accumulate within the malignant cells (Bartek *et al.*, 1990; Gannon *et al.*, 1990; Rodrigues *et al.*, 1990). The accumulation of mutant p53 can be detected by immunohistochemical staining. Recently, a polyclonal rabbit antibody and two monoclonal antibodies have been produced which are effective on both frozen and paraffin sections (Bartek *et al.*, 1991).

Although immunohistochemical staining techniques can identify the presence of cells positive for mutant p53 protein (Chang *et al.*, 1991) the information is generally qualitative. Moreover, this technique is unsuitable for the examination of cell-free fluids. Recently, an enzyme-linked immunosorbent assay (ELISA) for p53 protein has been developed and applied for the measurement of p53 in tumor tissue extracts (Bartek *et al.*, 1991). This assay uses a horseradish peroxidase label with colorimetric detection.

We here describe an ultrasensitive procedure for p53 quantification which is based on a newly developed detection system (Christopoulos & Diamandis, 1992). This detection methodology uses alkaline phosphatase as the label and 5-fluorosalicylphosphate as substrate. The liberated 5-fluorosalicylate forms a fluorescent chelate when reacted with a Tb^{3+}-EDTA complex at alkaline pH; this chelate can be quantified with microsecond time-resolved fluorometry (Diamandis, 1988). Using the developed p53 assay, we were able to quantify as little as 1 pg of mutant p53 protein (about 18 attomoles, equivalent to 10^7 molecules). We have verified that this assay can correctly identify mutant p53 in many tumor cell lines bearing different mutations. We have also shown that by varying the capture monoclonal antibody we could also quantify wild-type human p53 protein as well as wild-type or mutant mouse p53. The assay design incorporates assessment of possible non-specific binding effects. We have screened 800 serum samples from cancer patients in an effort to detect circulating p53 protein. All samples were negative, suggesting that mutant p53 is either not circulating in blood, its levels are lower than the detection limit of the method, or it is quickly degraded or

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bound to serum components. We have also analysed mutant p53 levels in 264 breast tumor extracts prepared for steroid hormone analysis and found 24% of tumors to be positive for mutant p53. Quantitative correlation analysis between the levels of mutant p53 and steroid hormone receptors revealed a strong negative correlation.

Results

Assay development and evaluation

Our first objective was to develop a highly sensitive time-resolved immunofluorometric procedure for p53 quantification. For this, we have investigated a number of different assay configurations as follows: (a) monoclonal immoblized capture antibody in combination with monoclonal biotinylated detection antibody (antibodies used were 240 and 421). In this case, both antibodies were first purified by protein A affinity chromatography; (b) monoclonal capture antibody in combination with polyclonal rabbit detection antibody. The latter was further reacted with a biotinylated goat anti-rabbit IgG; (c) as in (b) but the goat anti-rabbit IgG was non-biotinylated, instead, it was directly labeled with alkaline phosphatase (ALP); (d) as in (c) but the solid-phase was coated with a goat anti-mouse Fc-specific antibody as shown in Figure 1. In all cases where biotinylated antibodies were used, they were linked to alkaline phosphatase through a streptavidin-alkaline phosphatase conjugate. The final assay configuration (Figure 1), was chosen because it gave the best sensitivity and for the following additional reasons: solid-phase precoating with affinity purified, goat anti-mouse IgG, which is commercially available, obviates the need to purify the mouse monoclonal antibody (240 or 421) from the tissue culture supernatant and there is about 10-fold economy in consumption of the monoclonals in comparison to direct coating. With this protocol, the assessment of any non-specific binding effects can be accomplished by simply replacing the monoclonal anti-p53 antibody with an irrelevant monoclonal of the same sub-class, during the first assay step. It is also possible to interchange antibodies 240 or 421, using the same solid-phase, in order to assess mutant p53 (240) or wild-type and mutant protein (421). The ALP-labeled goat anti-rabbit IgG was used for detection because the version with biotinylated second antibody gave similar results and had an extra incubation and washing step. The direct labeling of the rabbit polyclonal anti-p53 antibody was not attempted since this antiserum is not purified and expensive. In this assay, the rabbit antibody was used at a dilution of 5000-fold, about 5–20 times less than dilutions used in immunohistochemistry. The ALP substrate and the detection of the reaction products by time-resolved fluorometry have been recently described. This system is capable of quantifying attomoles of analytes in biological fluids (Christopoulos & Diamandis, 1992). The overall assay design obviates the need for either purification or conjugation of any of the reagents used.

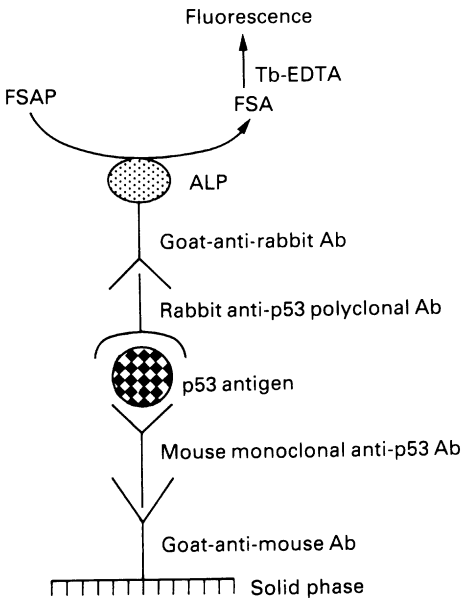

Figure 1 Immunofluorometric assay of wild-type or mutant p53 protein. Solid-phase is polystyrene microtiter well. The mouse monoclonal anti-p53 antibody is either 240 or 421. Ab = antibody; FSAP = 5-fluorosalicylphosphate; FSA = 5-fluorosalicylate; the valency of Tb^{3+} and EDTA is omitted for simplicity. The final fluorescent complex, FSA: Tb^{3+}: EDTA is measured in a time-resolved mode with excitation at 337 nm (nitrogen laser) and emission at 615 nm as previously described (Christopoulos & Diamandis, 1992)

The developed assay was further optimized at each step with respect to antibody dilutions, incubation times and temperature and diluents used. Optimal conditions are given in the assay protocol. The assay can be completed in less than 6 h.

The detection limit of the assay, defined as the concentration of p53 that could be distinguished from zero with 95% confidence was calculated using recombinant human mutant p53 standards (from Oncogene Sciences, Uniondale, NY) and found to be around 0.02 ng ml^{-1}. This detection limit is about three times lower than the detection limit of a commerical assay from Oncogene Sciences and at least 10-fold more sensitive than an assay recently developed (Bartek *et al.*, 1991) using the same antibodies but different design and detection methodology. In many cell lines, p53 could be detected in extracts diluted to contain less than 1 μg ml^{-1} of total protein (see below). The within-run precision (CV, %) assessed at levels of p53 of 0.50, 0.70, 1.00 and 1.70 ng ml^{-1} ($n = 12$) was 5.0, 5.7, 3.7 and 2.0%, respectively. Recovery studies were performed by adding mutant p53 protein to cancer patient sera. Recoveries were between 90–120% with the exception of some samples (approx. 10%) which gave lower values (20–70%). These samples were found to contain anti-p53 antibodies (data not shown) (Crawford *et al.*, 1982).

A typical calibration curve is shown in Figure 2 using lysates from cell line Colo 320 DM (+) (Table 1). Similar curves were obtained with lysates from other overproducing cell lines.

Analysis of p53 in cell lysates

Lysates prepared from the 23 cell lines in Table 1 were analysed by the proposed procedure and the results are shown in Table 2. The following comments apply.

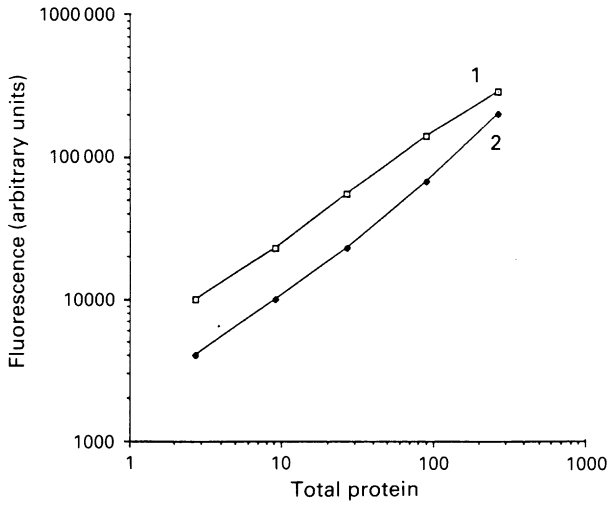

Figure 2 Calibration curve (double logarithmic plot) of the p53 assay using lysates from the Colo 320 DM(+) cell line. Total protein concentration is in ug ml^{-1}. Background signals without lysate were approximately 3500 units and were subtracted from all other readings. Points are means of three replicates with CV's between 1–7%. Coating antibody was 421(1) or 240(2). The p53 concentration at total protein concentration of 200 ug ml^{-1} is approximately 4 ng ml^{-1}

Table 2 p53 levels in cell lysates

Cell line	*p53, ng mg^{-1} Protein* 240*	421
OCI M2	38	24
Colo 320 HSR (+)	62	39
Colo 320 DM (+)	100	42
MIA PaCa-2	108	47
BT 474	2.8	9.8
T47 D	49	16
BT20	9.7	21
MDA-MB-468	36	35
HOS	30	29
SK-LU-1	6.5	4.2
C1	2.3	3.9
Ito-II	2.3	3.5
W2	1.3	1.9
ENB	1.3	0.8
Borton	1.2	1.4
K562	ND†	ND
Jurkat	ND	ND
U937	ND	ND
MLA	ND	ND
DP20-2	1.1	1.4
DP16-1	ND	ND
SK-N-MC	ND	0.91
SK-N-SH	ND	0.19

* Captured antibody was either 240 or 421
† ND not detectable

Table 1 Cell lines used

Cell line	*Description*	*p53 gene status*	*Supplier*	*Culture conditions**	*Reference*
1. OCI M2	Human erythroleukemia	P.M. 274/W.T. lost†	Dr S. Benchimol	C	Singerland *et al.* (1991)
2. Colo 320 HSR (+)	Colon Ca‡	P.M. 248/W.T. lost	ATTC**	A	Murakami *et al.* (1991)
3. Colo 320 DM (+)	Colon Ca	P.M. 248/W.T. lost	ATCC	A	Murakami *et al.* (1991)
4. MIA PaCa-2	Pancreatic Ca	P.M. 248/W.T. lost	ATCC	E	Barton *et al.* (1991)
5. BT 474	Breast Ca	P.M. 285/W.T. lost or silent	ATCC	G	Bartek *et al.* (1990)
6. T47 D	Breast Ca	P.M. 194/W.T. lost or silent	ATCC	H	Bartek *et al.* (1990)
7. BT20	Breast Ca	P.M. 132/W.T. lost or silent	ATCC	D	Bartek *et al.* (1990)
8. MDA-MB-468	Breast Ca	P.M. 273/W.T. lost or silent	ATCC	F	Bartek *et al.* (1990)
9. HOS	Osteosarcoma	P.M. 156/W.T. lost	ATCC	D	Romano *et al.* (1989)
10. SK-LU-1	Lung Ca	P.M. 193/W.T. lost	ATCC	B	Lehman *et al.* (1991)
11. C1	Colon Ca	No mutations by SSCP††	Dr Y. Murakami	A	Murakami *et al.* (1991)
12. Ito-II	Testicular Ca	No mutations by SSCP	Dr M. Sekiguchi	A	Murakami *et al.* (1991)
13. W2	Wilm's tumor	No mutations by SSCP	Dr M. Sekiguchi	A	Murakami *et al.* (1991)
14. ENB	EBV-transformed leukocytes‡‡	Unknown	Dr E. Nisbet-Brown	A	–
15. Borton	EBV-transformed B-cells	Unknown	Dr E. Nisbet-Brown	A	–
16. K562	CML***	Deletion of p53 gene	Dr S. Benchimol	A	Lozzio & Lozzio (1975)
17. Jurkat	T-cell lymphoma	P.M. 196,256,259,260/W.T. present	Dr E. Nisbet-Brown	A	Cheng *et al.* (1990)
18. U937	Histiocytic lymphoma	Unknown	Dr E. Nisbet-Brown	A	–
19. MLA	Gibbon lymphoma	Unknown	Dr E. Nisbet-Brown	A	–
20. DP20-2	Murine erythroleukemia	P.M. 236/W.T. lost	Dr S. Benchimol	B	Chow *et al.* (1987)
21. DP16-1	Murine erythroleukemia	Deletion of p53 gene	Dr S. Benchimol	B	Mowat *et al.* (1987)
22. SK-N-MC	Neuroepithelioma	No mutations in exons 5–8/200–300 bp deletion	ATCC	D	Davidoff *et al.* (1992)
23. SK-N-SH	Neuroblastoma	No mutations in exons 5–8/increased p53 half-life	ATCC	D	Davidoff *et al.* (1992)

* A, RPMI-1640 medium supplemented with 10% fetal calf serum (FCS); B, MEM medium supplemented with 10% FCS; C, DMEM supplemented with 10% FCS; D, MEM with non-essential amino acids and Earle's balanced salt solution, supplemented with 10% FCS; E, DMEM supplemented with 10% FCS and 2.5% horse serum; F, L-15 supplemented with 10% FCS; G, RPMI-1640 medium suplemented with 10% FCS, 10 $\mu g\ ml^{-1}$ insulin and 300 $\mu g\ l^{-1}$ L-glutamine; H, RPMI-1640 medium supplemented with 10% FCS and 10 $\mu g\ ml^{-1}$ insulin
† P.M. = point mutation at codon indicated; W.T. = wild-type allele
‡ Ca = Carcinoma
** ATCC, American Type Culture Collection
†† SSCP = single-stranded conformational polymorphism
‡‡ EBV = Epstein-Barr virus
*** CML = Choronic myelogenous leukaemia

Three cell lines that produce the highest levels of mutant p53 (Colo 320 HSR(+), Colo 320 DM(+), MIA PaCa-2) have a mutation at codon 248 which leads to aminoacid substitution from Arg to Trp (CGG--TGG). Another two overproducing cell lines (OCI M2 and MDA-MB-468) have mutations at adjacent codons, 274 and 273, respectively. The remaining overproducing cell lines (BT 474, T47 D, BT20, HOS and SK-LU-1) have mutations scattered throughout the p53 gene (codons 285, 194, 132, 156 and 193, respectively). These data can be interpreted as follows: (a) In comparision to cell lines without mutations, the levels of p53 protein detected in all the above cell lines are much higher, as expected from previous reports; (b) The response of the assay with various lysates, using the mutant-specific 240 antibody, varies considerably in comparison to results with the wild-type and mutant-specific 421 antibody. In two cell lines (MDA-MB-468 and HOS) the response is similar but in others (OCI M2, Colo, 320 HSR(+), Colo 320 DM(+), MIA PaCa-2, T47D and SK-LU-1) the response with the 240 antibody is higher. In the remaining cell lines, BT474 and BT20, the response with the 421 antibody is higher. We postulate that these differences are due to different affinities of the two monoclonal antibodies and the rabbit polyclonal antibody used, for the various mutant proteins. These differences have also been observed by others (Bartek *et al.*, 1991) and suggest that the quantitative nature of the assay results must be viewed with caution. The final contributors to the signal are both affinity and concentration factors of each mutant. Unfortunately, no individual mutant p53 standards are available to standardize such assays.

The remaining cell lines tested fall into the following categories. (a) Human cell lines C1, Ito-II, W2, ENB and Borton which bear a wild-type p53 gene or have unknown p53-gene status, tested positive for the presence of p53 protein by both 240 and 421 antibodies. However, the levels are much lower in comparison to the cell lines producing mutant p53. These consistently reproduced data suggest that we are likely detecting wild-type p53 protein which has partially denatured during lysis or assay and thus being reactive with both antibodies 240 and 421. (b) The human cell line K562 and the murine cell line DP16-1 are known to have p53 gene deletions and do not produce p53 protein, in accordance with undetectable levels found in this study. (c) Another two human cell lines, Jurkat and U937 and a Gibbon lymphoma cell line, MLA, showed undetectable levels of p53 protein. Although Jurkat was shown to have p53 gene mutations, the wild-type allele was also there. While the reason for undetectable levels of p53 protein in this line is obscure, one possibility may involve heteropolymers between wild-type and mutant p53 (Vogelstein & Kinzler, 1992).

The cell lines SK-N-MC and SK-N-SH have been previously studied (Davidoff *et al.*, 1992). The p53 gene in the SK-N-SH cell line is wild-type and this protein is detected only by the 421 antibody, as expected. The SK-N-MC cell line contains no mutations in the p53 gene but there is a 200–300 bp deletion affecting the aminoterminal part of p53. Although no p53 was detected by immunoprecipitation, the shortened transcript, is produced at high levels (Davidoff *et al.*, 1992). With our assay, we detected p53 with antibody 421 but not with 240. This finding suggests that the truncated protein is detected by our assay, a finding that should be expected since 421 antibody binds to the carboxy-terminal end of p53, which is not affected by the deletions.

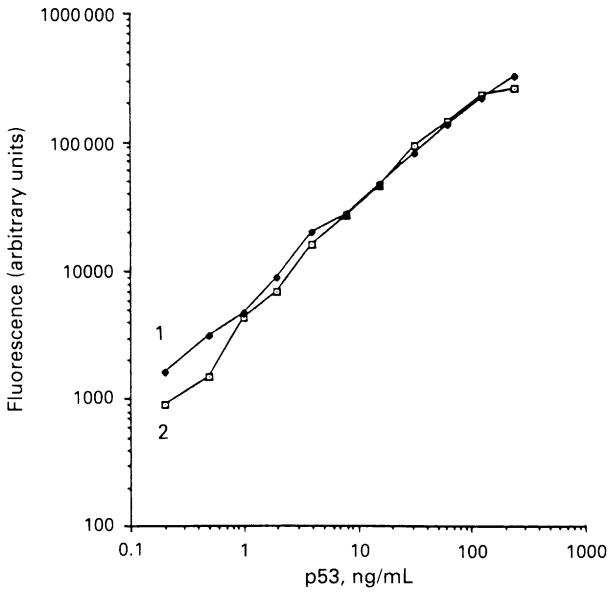

Figure 3 Assay response using recombinant wild-type p53 protein produced in insect cells with the baculovirus system. The capture antibody was PAb421 (1) or PAb 240 (2)

Our assay can also be used to detect murine wild-type or mutant p53 proteins. This is shown by the data of cell line DP20-2 which has a point mutation at codon 326 (asn--asp). Low, but easily detectable levels of mutant p53 protein were found by using both 240 and 421 antibodies. Additionally, easily measurable levels of wild-type murine p53 were found in an embryonic stem cell line, D3, which is known to overproduce the protein (data not shown) (S. Benchimol, personal communication).

Analysis of recombinant p53 protein

The assay was applied for the determination of human wild-type p53 protein, produced in insect cells with the baculovirus system as previously described (Wang *et al.*, 1989). The data are shown in Figure 3. The assay response is similar with both antibodies, 240 and 421. This finding suggests that the recombinant protein is either not conformationally equivalent to authentic wild-type protein or that the recombinant protein loses its conformation when diluted in the sample diluent or during assay.

Analysis of p53 in human serum

Eight hundred cancer patient sera, representing variety of malignancies and disease stages, were screened by the developed procedure in an effort to identify the mutant protein in serum. All samples tested negative, in accordance with other recent reports (Winter *et al.*, 1992).

Analysis of mutant p53 in breast tumor extracts

There is no universal agreement as to what level of estrogen or progesterone receptor in tumor tissue is considered positive or negative. In this study, the cut-off values for ER and PR were set at 10 fmol mg^{-1} of cytosolic protein as also suggested by others (Henderson, 1991; Thor *et al.*, 1992). The analytical data of our study are summarized in Table 3 and graphically presented in Figures 4 and 5. From all tumor extracts, 24% were positive for p53. It is clearly shown that from the tumors that do not produce p53, the majority (75–83%) are estrogen and/or progesterone receptor-positive. The percentage of steroid hormone receptor-positive tumors decreases proportionally in tumors producing mutant p53, as clearly shown in Figure 5 (lines 1, 3, 5 and 9) and Table 3. Additionally, the percentage of steroid hormone receptor-negative tumors increases proportionally in tumors producing mutant p53 (Figure 5, lines 2, 4, 6 and 10 and Table 3). Interestingly, in the small proportion of cases where there is discordance between ER and PR levels [ER(+)/PR(−) or ER(+)/PR(−)], no significant changes were observed in the groups of patients who are either positive or negative for p53 (Figure 5, lines 7, 8 and Table 3). In these cases, there were uniform proportions of patients detected for each level of p53.

The results of our study were separated into distributions of samples which were either p53-negative or p53-positive, with a cut-off value of p53 of 0.02 ng per mg of total protein in the extract (Table 3, columns 2 and 3). Likewise, we separated the two groups into four categories each, based on their ER and PR values (in fmol mg^{-1} total protein) as follows, respectively: <10, <10; <10, >10; >10, <10; >10, >10. When we compared the distributions with the chi-square test (χ^2) with three degrees of freedom, we found that the results were highly statistically significant to chi-square equal to 20.6, $P<0.001$.

Discussion

The p53 tumor suppressor gene and its product has attracted considerable interest because of its possible relation to cancer initiation and development and to possible implications which relate to cancer diagnosis, prognosis and therapy. Studies of the gene are usually carried out with well established cloning and sequencing techniques. The protein product is best studied with a panel of monoclonal and polyclonal antibodies which show specificity for either mutant and/or wild-type proteins. With such antibodies, the protein is studied by immunoprecipitation, immunoblotting or immunohistochemistry. Very recently, an enzyme-linked immunosorbent assay for p53 has been developed (Bartek *et al.*, 1991) and is also commercially available. We have used different assay designs and a highly sensitive detection methodology, based on time-resolved fluorometry, to develop assays for wild-type and mutant p53 protein. This new method is capable of detecting about 1 pg of mutant protein per assay. This new tool has a number of notable advantages: It is fast (6 h), precise, simple to perform and objective, obviating the need for interpretation by trained personnel as in immunohistochemistry. This method was tested for its ability to detect mutant and wild-type protein in a large number of cell lysates (Table 2). Although the quantitative nature of this procedure must be interpreted with caution since the final signal may relate to p53 concentration and affinity for the antibodies used, it is clearly demonstrated that all overproducers are, as expected, the cell lines bearing p53 gene mutations. In some cell lines with normal p53 genes, low but detectable levels were found while in others, the protein was not detected. We also show that this tool is useful for murine wild-type and mutant p53.

The assay was applied to measure wild-type recombinant p53 protein produced in insect cells using a baculovirus system. The equivalent signal obtained with both 421 and 240 antibodies prompted us to speculate that the recombinant protein may not be conformationally equivalent to authentic p53.

A large number of cancer patients sera were screened by the proposed method in an effort to identify the mutant protein in serum. All 800 samples were negative. These data can be interpreted as follows: In a proportion of cases, no mutant protein is produced by the tumor and p53 should not be expected in serum. In tumors that produce p53, it may be possible that p53 is not secreted into the circulation or when it does, the levels are lower than the detection limit of our method. Alternatively, p53 in serum may be unstable or is bound to serum components. These possibilities are currently under investigation. Other authors have also reported absence of p53 in the serum of patients with lung carcinoma (Winter *et al.*, 1992).

One of the current priorities of cancer research is the

Table 3 Estrogen or progesterone receptor and p53 status in 264 breast tumor extracts

	p53 negative			*p53 positive*		
Receptor status	<0.02*	($n=200$)†	$\geqslant 0.02$ ($n=64$)	$\geqslant 0.1$	($n=40$)	$\geqslant 0.5$ ($n=20$)
ER (+)	150	(75%)	32 (50%)	15	(38%)	4 (20%)
ER (−)	50	(25%)	32 (50%)	25	(62%)	16 (80%)
PR (+)	150	(75%)	31 (48%)	15	(38%)	4 (20%)
PR (−)	50	(25%)	33 (52%)	25	(62%)	16 (80%)
ER or PR (+)	166	(83%)	36 (56%)	17	(42%)	5 (25%)
ER or PR (−)	34	(17%)	28 (44%)	23	(58%)	15 (75%)
ER (+), PR (−)	15	(7.5%)	6 (9.4%)	3	(7.5%)	2 (10%)
ER (−), PR (+)	15	(7.5%)	4 (6.2%)	2	(5.0%)	1 (5.0%)
ER (+) & PR (+)	135	(68%)	27 (42%)	13	(32%)	3 (14%)
ER (−) & PR (−)	20	(10%)	23 (36%)	18	(45%)	12 (57%)

* Values of p53 are expressed as ng of p53 protein per mg of total protein in the breast tumor extract

† n = number of samples

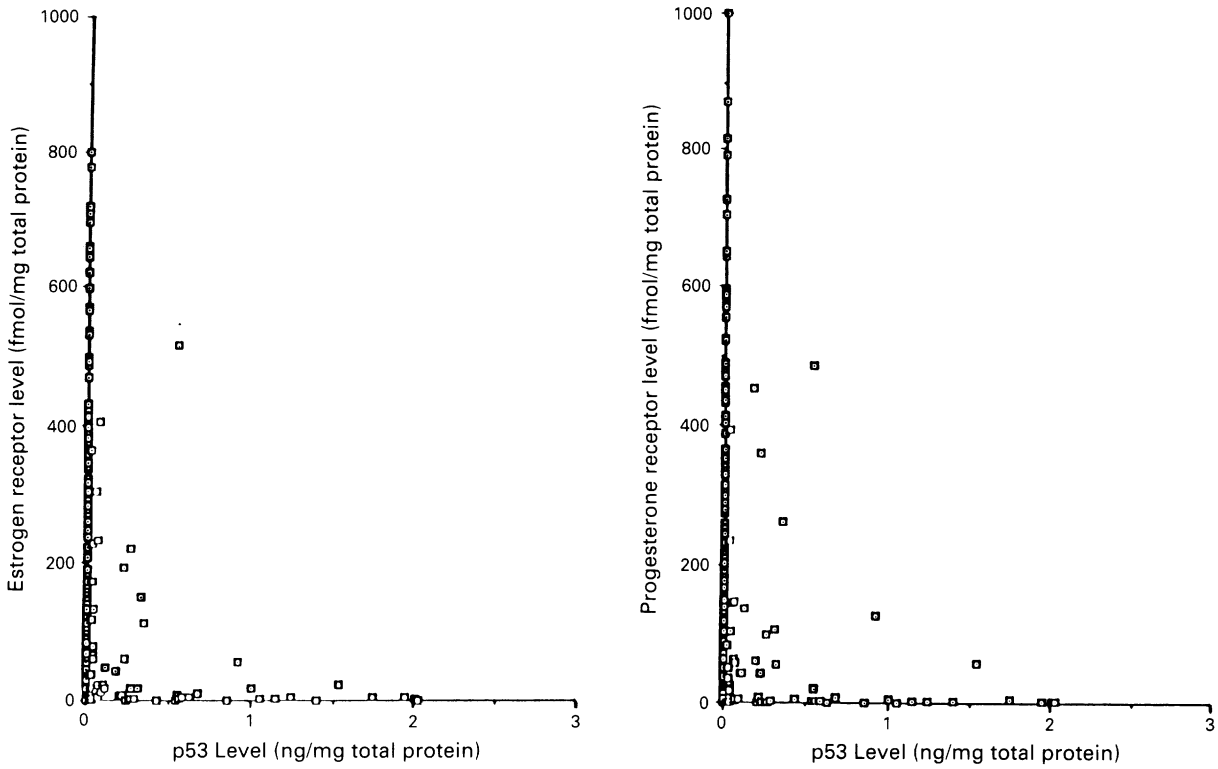

Figure 4 Estrogen receptor level (left panel) and progesterone receptor level (right panel) as a function of the level of mutant p53 protein in 264 breast tumor extracts. Tumors positive for estrogen and/or progesterone receptors are predominantly p53-negative. Most of the p53-positive tumors are steroid hormone receptor-negative.

identification of new tests which are either prognostic indicators or can predict the likelihood that a patient will benefit from a specific therapy (Koenders *et al.*, 1991). One of the most promising new prognostic factors in breast cancer and other malignancies is the p53 tumor suppressor gene product. The accumulation of the mutant p53 protein in tumor tissues seems to be a fundamental change permissive of the neoplastic phenotype in several tumors, and not merely a secondary consequence of disordered cell growth. The accumulation of the mutant protein generally reflects a mutational event in the p53 tumor suppressor gene (Thor *et al.*, 1992). Recently, it has been clearly pointed out that the monitoring of mutant p53 protein levels may not always allow accurate assessment of the mutational status of the p53 gene because in some cases mutations or deletions may abolish the production of p53 protein or may lead to a protein that is not stable (Winford-Thomas, 1992). Additionally, there is evidence of non-mutational stabilization of wild-type p53 protein.

A number of recent reports have examined the prognostic significance of p53 analysis in tumor tissue (Cattoretti *et al.*, 1988; Davidoff *et al.*, 1991; Ostrowski *et al.*, 1991; Thor *et al.*, 1992; Visakorpi *et al.*, 1992). There is general agreement that p53 is associated with steroid hormone receptor-negative, epidermal growth factor receptor-positive, high-grade tumors which have increased potential to metastasize. Additionaly, p53-positive tumors were associated with poorer survival rates and p53 was found to be an independent risk factor. While most studies deal with breast tumors, a recent study of prostatic carcinomas also revealed an association between p53-positive tumors and unfavorable prognostic factors as well as decreased disease-free intervals and survival rates (Visakorpi *et al.*, 1992).

All of the above studies have been carried out using immunohistochemical techniques for mutant p53 analysis. This technique, although useful for p53 detection, may give variable results among investigators because of differences in tumor fixation methodologies and variability of monoclonal or polyclonal antibodies and detection reagents used (Cattoretti *et al.*, 1988; Callahan, 1992). Moreover, the method is qualitative, labour intensive, expensive, subjective and requires skilled personnel for interpretation. In this paper, we have used the highly sensitive immunofluorometric procedure for mutant p53 quantification. Using this technique, we have identified that 24% of breast tumors are p53-positive using PAB 240 (Gannon *et al.*, 1990). This positivity rate is almost identical to rates reported by others using immunocytochemistry (Cattoretti *et al.*, 1988; Thor *et al.*, 1992). Because our technique is quantitative we were able, unlike all previous studies, to separate tumors with low, intermediate and high p53 levels and establish a clear quantitative negative correlation between p53 and estrogen and/or progesterone receptor levels (Figures 4 and 5). These data suggests that the amount of p53 present in tumor tissue may be a better prognostic indicator than the qualitative information for its presence or absence. This is also suggested by the report in the prostatic carcinoma series (Visakorpi *et al.*, 1992) which suggests that low-level p53 accumulation had no prognostic significance, contrary to the high-level p53 staining which was associated with poor patient prognosis.

Although as shown in Figure 4 in most cases p53-negative tumors are steroid hormone receptor-positive

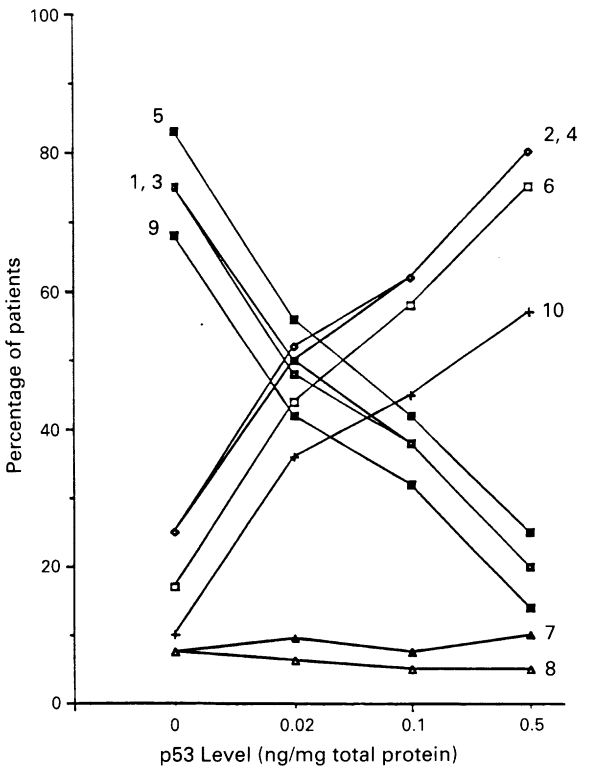

Figure 5 Quantitative correlation beween percentages of patients and p53 levels in breast tumor extracts. Data were obtained from Table 1. The p53 level on the x-axis was either 0, >0.02, >0.1 and >0.5 ng of p53 protein per mg of total protein in the extract. Lines represent percentages of patients who are estrogen receptor-positive (1), progesterone receptor-positive (3), estrogen and/or progesterone receptor-positive (5), estrogen and progesterone receptor-positive (9), estrogen receptor-negative (4), progesterone receptor-negative (2), estrogen or progesterone receptor-negative (6), estrogen and progesterone receptor negative (10), estrogen receptor-positive but progesterone receptor-negative (7) and estrogen receptor negative but progesterone receptor-positive (8)

and vice-versa, some tumors were p53-positive and estrogen or progesterone-receptor positive. These findings suggests that the two parameters are indicators of a more aggressive tumor type but that they are individually distinct. One would expect an association of p53 with a known tumor prognostic factor but anticipate an expression in some receptor positive tumors that can also progress rapidly. This notion is supported by the recent report that p53 is an independent risk factor, operating separately from steroid hormone receptors (Thor *et al.*, 1992). Another study (Cattoretti *et al.*, 1988) also reported that about 25% of patients positive for p53 by immunocytochemistry were also strongly positive for estrogen receptors, in agreement with our results (Table 3), although the older results are only qualitative.

Based on the recent clinical reports and our data it is conceivable that p53 in tumor tissue will become a routine test for prognosis, mainly for breast cancer (Thor *et al.*, 1992) but for other malignanices as well (Visakorpi *et al.*, 1992). In this case, the proposed immunofluorometric analysis has many advantages over immunohistochemistry. This method is quantitative and will allow upper reference intervals to be set which can be used to assess the clinical significance of p53 presence. The concentration of p53, rather than just the qualitative information, may also by itself be a prognostic indicator of the malignant process and aggressiveness.

Apart from its objectiveness, the method is also fast, potentially automatable, economical and can be carried out by technical personnel by utilising established, widely used immunological methodologies. We have demonstrated that p53 and steroid hormone receptor levels can be assessed in the same tumor extract. This is very useful because of the savings in labour and tumor tissue requirements. We suggest that the proposed methodology is superior to immunohistochemical analysis in many respects and should be considered as a candidate method in prospective clinical trials and routine use.

In conclusion, our new method is, to our knowledge, the most sensitive tool for monitoring levels of wild-type and mutant p53 protein. As such, it may find different applications e.g. monitoring levels of p53 during cell cycle (S. Benchimol *et al.*; manuscript in preparation) and analysis of body fluids such as urine, feces, cerebrospinal or ascites fluid. As we and others (Bartek *et al.*, 1991) have shown, this assay can quantitatively measure p53 in tumor tissue extracts and tumor cell line lysates. The strong quantitative negative correlation of p53 levels and steroid hormone receptors that we have established, in combination with the suggestion that these two parameters are independent risk factors, calls for the consideration of p53 as a routine prognostic indicator for breast and other malignancies. In this respect, our method would be very useful.

Materials and methods

Cell lines and culture conditions

The cell lines used are shown in Tables 1 and 2. All cell lines were grown in suspensions or monolayers to a density of approximately 10^6 cell ml^{-1} or 70–90% confluence, respectively, at 37°C with 5% CO_2. Cells were spun at 3000 r.p.m., washed twice with isotonic saline and either lysed or stored at −70°C until lysis was performed.

Solutions and reagents

Lysis buffer: 150 mM NaCl, 20 mM Tris, 1% Nonidet P-40, 0.5 mM phenylmenthylsulfonylchloride (PMSF), 1 $\mu g\ ml^{-1}$ leupeptin, 50 $\mu g\ ml^{-1}$ aprotinin. Sample diluent (diluent for cell lysates, serum, polyclonal anti-p53 rabbit antiserum and alkaline phosphatase-conjugated goat anti-rabbit antibody): 50 mM Tris, pH 7.40, containing 60 g bovine serum albumin (BSA) and 1 g sodium azide per litre. Monoclonal anti-p53 antibody diluent: 50 mM Tris, pH 7.40, containing 60 g bovine serum albumin, 1 g sodium azide and 0.5 mol KCl per litre.

Substrate buffer: 0.1 M Tris, pH 9.1, 0.15 M NaCl, 1 mM $MgCl_2$. Developing solution: 2×10^{-3} M $TbCl_3$, 3×10^{-3} EDTA, 0.4 M NaOH, 1 M Tris base (no pH adjustment). Prepare as described elsewhere (Christopoulos & Diamandis, 1992). Wash solution: Distilled water. Coating buffer: 50 mM Tris, pH 7.80, containing 1 g of sodium azide per litre. The phosphate ester of 5-fluorosalicylic acid (FSAP) was obtained from Cyberfluor Inc., Toronto, Canada. It is stored as a 10 mM stock solution in 0.1 M NaOH at 4°C for many months. This stock is diluted 10-fold in the substrate buffer just before use. All other chemicals were from Sigma Chemical Co., St. Louis, MO, USA, except Nonidet P-40 (Boehringer Mannheim, Indianopolis, IN, USA), $TbCl_3 \cdot 6H_2O$

(GFS Chemicals, Columbus, OH, USA) and the biotinylation reagent NHS-LC-Biotin (Pierce Chemical Co., Rockford, IL, USA).

Instrumentation

Microtiter plate washing was performed with an automatic washer from Cyberfluor Inc. For measuring liquid-phase, Tb^{3+} – specific delayed fluorescence in white microtitre wells we used the Cyberfluor 615 Immunoanalyser, a time-resolved fluorometer, as described elsewhere (Christopoulos & Diamandis, 1992).

p53 standards

Recombinant mutant human p53 protein standards in the range from 0.25–4 ng ml^{-1} were obtained from Oncogene Science, Inc. Uniondale, NY, USA and were considered the primary standards. These standards were used to optimize the assay and standardize cell lysates for subsequent studies. Another human wild-type recombinant p53 solution, prepared as described elsewhere (Wang *et al.*, 1989) was a gift to us by Dr C. Prives, Columbia University. This p53 preparation was diluted in the sample diluent to make standard solutions.

Antibodies

The mouse anti-p53 monoclonal antibodies, PAb 421 and PAB 240 were kindly provided by Dr S. Benchimol, Ontario Cancer Institute. These are tissue culture supernatants containing approximately 30 μg ml^{-1} antibody. The rabbit polyclonal anti-p53 antibody, CM-1, was obtained from Dimension Labs, Mississauga, Ontario, Canada. The goat anti-rabbit antibody, conjugated to alkaline phosphatase, and the goat anti-mouse antibody, F_c specific, both approximately 1 mg ml^{-1}, were obtained from Jackson Immunoresearch, West Grove, Pennsylvania, USA.

Methods

Cell lysis Cells from each cell line were grown until they reached approximately 10^6 cells ml^{-1}. The cell pellet from a 10 ml culture was lysed in 300 μl lysis buffer, for 30 min, on ice. The cell extract was centrifuged at 12 000 *g* for 10 min and the pellet discarded. The lysate was used within 2 h. Total protein was measured in the lysates with the bicinchoninic acid (BCA) assay, commerically available by Pierce. Lysates typically contained 1–3 mg of protein per ml.

Immunoassay of p53 White, opaque, 12-well mitrotiter strips (Dynatech Labs, Alexandria, VA, USA) were coated with a goat anti-mouse antibody by pipetting 100 μl 500 ng per well of the antibody solution in the coating buffer. After overnight incubation at room temperature, the wells were washed four times with distilled water. The wells were then blocked by pipetting 200 μl per well of the sample diluent, incubating for 1 h and washing as above. The wells were then used for the assay as follows: We add 100 μl per well of mouse monoclonal anti-p53 antibody (PAb 421 or PAb 240) and 50 μl of sample (p53 standards of cell lysates). The antibodies are cell culture supernatants containing about 30 μg ml^{-1} of antibody and they were diluted × 20 in the monoclonal anti-p53 antibody diluent. The cell lysates were used in different dilutions in the sample diluent, varying from 10–1000-fold. After 3 h incubation with shaking at 37°C, the plates were washed × 4. We then added 100 μl per well of the polyclonal rabbit anti-p53 antibody (diluted 5000-fold in the sample diluent) and incubated with shaking for 1 h at room temperature. After washing × 4, we added 100 μl per well of the goat anti-rabbit alkaline phosphatase conjugate solution (diluted 5000-fold in the sample diluent) and incubated with shaking for 1 h at room temperature. The strips were washed again × 4 and 100 μl per well of the FSAP solution (10^{-3} M in the substrate buffer) were adeed and incubated for 10 min with shaking at room temperature. The fluorescent complex was then formed by adding 100 μl per well of the developing solution followed by brief mixing for 1 min. Time-resolved fluorometric measurements at 615 nm were performed on the Cyberfluor 615 Immunoanalyser. Data reduction and plotting of calibration curves was automatic through the analyser software.

Control of possible non-specific binding effects Each assay run was always accompanied by a parallel run which assesses any non-specific binding effects. This run is identical to the assay described above but instead of using the specific monoclonal anti-p53 antibody we used a mouse IgG_1 antibody against an irrelevant protein (alpha-fetoprotein).

Analysis of patient sera for p53 Eight hundred sera collected from patients with diverse malignancies and disease stages as well as 300 sera from non-cancer patients, were diluted 10-fold in the sample diluent and tested for p53 immunoreactivity with the proposed assay as described above.

Extracts of breast tumor tissue Breast tumor tissue was immediately stored in liquid nitrogen after resection, transported to the laboratory and stored subsequently at −70°C until extraction was performed. Approximately 0.5 g of tumor tissue is weighed out, smashed with a hammer if necessary, and pulverized in a Thermovac tissue pulverizer with liquid N_2. The resulting powder is transferred into 50-ml plastic tubes along with 10 ml of extraction buffer (0.01 mol l^{-1} Tris, 1.5 mmol l^{-1} ethylenediaminetetraacetic acid, 5 mmol l^{-1} sodium molybdate, pH adjusted to 7.40 with 5 mol l^{-1} HCl). If the tumor tissue is less than 0.5 g the volume of the buffer used is reduced proportionally. The tissue powder is homogenized on ice with a single 5 s burst of a Polytron homogenizer at setting 6. The particulate material is pelleted by 1 h contrifugation at 105 000 *g*.

The intermediate layer (cytosol extract) is collected without disturbing the lipid or particulate layers. Protein concentration of the resulting cytosol extract is determined by the BioRad method, based on the procedure described by Bradford (Bradford, 1976). If the protein concentration is greater than 4 mg ml^{-1}, the cytosol is diluted to a protein concentration of approximately 2 mg ml^{-1}. The exact protein concentration of all extracts is then determined by the method of Lowry *et al.* (1951). The extracts were stored at −70°C until analysis.

Steroid hormone receptor assays For quantitative analysis of estrogen and progesterone receptors (ER, PR) we have used the Abbott enzyme immunoassay kits (Abbott Laboratories, North Chicago, IL 60064, USA). The kits were used according to the manufacturer's instructions.

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