A novel quantitative immunoassay system for p53 using antibodies selected for optimum designation of p53 status

M D Thomas, G G McIntosh, J J Anderson, D M McKenna, A H Parr, R Johnstone, T W J Lennard, C H W Horne, B Angus

Abstract

***Aim*—To develop a highly sensitive and specific enzyme linked immunosorbent assay (ELISA) system for analysis of p53 protein in cancer lysates.**

***Methods*—The anti-p53 monoclonal antibodies DO7, 1801, BP53.12, and 421, and anti-p53 polyclonal antiserum CM1 were assessed by immunohistochemistry and western blot analysis to identify those most suitable for determining p53 status of cancer cells. Antibodies with desired characteristics were used to develop a non-competitive sandwich type ELISA system for analysis of p53 expression in cancer cytosols. Using the ELISA, p53 protein concentrations were measured in a small series of breast cancers, and the quantitative values compared with p53 immunohistochemical data of the same cancers.**

***Results*—DO7 and 1801 gave the most specific and reliable results on immunohistochemisry and western blot analysis. Using these two antibodies, a non-competitive sandwich type ELISA system was developed to analyse p53 quantitatively. Analysis of the breast cancer series showed a good correlation between immunohistochemistry and the ELISA—tumours were generally positive using both techniques. Discrepancies were noted however: some cancers were immunohistochemically negative but ELISA positive. One explanation for this may be that the ELISA is more sensitive than immunohistochemistry.**

***Conclusion*—The p53 ELISA system is a non-competitive double monoclonal antibody sandwich method, using DO7 and 1801 which have been shown to be highly specific for p53 protein by immunohistochemistry and western blot analysis. The lower threshold of the assay is 0.1 ng/ml analyte in an enriched recombinant p53 preparation. As p53 is now regarded as a protein associated with prognosis in breast and other cancers, the assay may have clinical applications.**

(*J Clin Pathol* 1997;**50**:143–147)

Keywords: p53; p53 antibodies; immunohistochemistry; western blot; quantitative ELISA.

Department of Pathology, Royal Victoria Infirmary, University of Newcastle upon Tyne, Newcastle upon Tyne NE1 4LP
M D Thomas
G G McIntosh
J J Anderson
D M McKenna
A H Parr
R Johnstone
C H W Horne
B Angus

Department of Clinical Surgery
T W J Lennard

Correspondence to:
Dr B Angus.

Accepted for publication
5 November 1996

The prediction of likely outcome in cancer would permit allocation of patients to appropriate aggressive therapeutic regimens in instances where prognosis is poor; and conversely, patients with good prognosis could be spared dangerous and debilitating treatment. Mutations of the tumour suppressor gene p53 can result in dysfunction, with accumulation of the inactive/mutated protein which increases genomic instability leading to the expression and progression of the metastatic phenotype.[1] Accumulation of p53 has been shown in several different cancers.[2–4] In breast cancer, we and others have shown that accumulation of p53 is associated with poor clinical prognosis.[5–7] Although p53 overexpression can be detected immunohistochemically, only a qualitative estimation can be made. It has, however, been proposed that quantitative analysis of p53 protein concentrations in cancer will provide additional objective prognostic information.[8] Several groups have described non-competitive sandwich type immunological based assay systems, using monoclonal/polyclonal antibody combinations, for quantitative analysis of p53 in tumour cytosol.[9–11] Using immunohistochemistry, we have recently reported that assessment of p53 status in breast cancer tissue sections is critically dependent upon the antibodies used for screening, and influences the significance of the prognostic data generated.[5] Similarly, the choice of antibodies to be applied in quantitative p53 assays may exert a notable effect on the final estimate of tumour p53 concentrations. Selection of the most appropriate antibodies will assure the specificity and reproducibility of the data on which subsequent assessments of patient prognosis may be based.

Methods

TUMOUR TISSUE

Samples of mammary carcinoma tissue were collected at surgery. The tumour was dissected free of superfluous tissue and representative samples processed for the production of frozen sections and cytosols.

CYTOSOL PREPARATION

p53 was quantified in cytosol extracts prepared for routine estimation of oestrogen/progesterone receptor analysis. Frozen tumour tissue (approximately 5 mm^2) was powdered using a micro-dismembranator and resuspended in 1 ml HEPES/EDTA buffer (20 mM

HEPES, 1.5 mM EDTA, pH 7.4) containing protease inhibitors: aprotinin (2 μg/ml), leupeptin (2 μg/ml), phenylmethylsulphonyl fluoride (PMSF) (50 μg/ml), and pepstatin (1 μg/ml). Further disruption was achieved using sonication. Owing to the heat lability of the proteins to be assayed, the samples were maintained on ice throughout the process. The tissue/cell lysate was clarified by ultracentrifugation, 50 000 × g at 4°C for 40 minutes. Cytosol fractions were collected, and stored at −70°C prior to analysis for p53. Samples were analysed within two weeks of preparation.

ESTIMATION OF TOTAL PROTEIN CONCENTRATION

Total protein concentration of the cytosol preparation was determined in relation to bovine serum albumin (BSA) using bicinchoninic acid (BCA) protein assay kits (Pierce and Warriner Ltd).

p53 ANTIBODIES

Anti-p53 monoclonal antibodies DO7, 1801 and BP53.12, and the rabbit anti-p53 polyclonal antiserum CM1 were all obtained from Novocastra Laboratories Ltd, Newcastle upon Tyne, UK. Anti-p53 monoclonal antibody 421 was obtained from Oncogene Science Ltd (Sera Laboratories Ltd, Crawley Down, UK). The concentration of monoclonal antibodies 1801, DO7, and BP53-12 was determined using IgG radial immunodiffusion.

IMMUNOHISTOCHEMISTRY

To assess the immunohistochemical performance of antibodies directed against p53 for the assignment of p53 status, we stained serial cryostat sections of breast cancers with test monoclonal antibodies DO7, 1801, BP53.1 and 421, and the rabbit polyclonal antiserum CM1. Sections, 3 μm thick, of cryopreserved tumour tissue were cut and air dried onto microscope slides. Endogenous tissue peroxidase activity was quenched using 0.6% hydrogen peroxide in methanol, for five minutes. Subsequently, non-specific immunostaining was blocked by incubating the sections with 5% lamb serum/phosphate buffered saline (PBS) for 30 minutes. Test p53 monoclonal antibodies at 0.5 μg/ml, or rabbit anti-serum CM1 at a dilution of 1 in 1000 were applied and incubated with sections for one hour at room temperature. Binding of p53 antibody was detected using biotinylated goat anti-mouse or goat anti-rabbit polyclonal antiserum and streptavidin/biotinylayed horseradish peroxidase complex (Dako, High Wycombe, UK). Sections were developed with 3,3-diaminobenzidine tetrahydrochloride (Sigma, Poole, Dorset, UK) in Tris buffered saline (TBS) containing 0.3% hydrogen peroxide, counterstained with Meyer's haematoxylin, dehydrated stepwise in ethanol, cleared in xylene, and mounted. Tumours were scored according to the proportion of cells showing nuclear reactivity: −, no reactivity; +, <25% reactivity; ++, 25–50% reactivity; and +++, >50% reactivity.

WESTERN BLOT ANALYSIS

Specificity of the p53 antibodies was assessed using western blot analysis against a cell lysate of the breast adenocarcinoma cell line MDA-MB-231. Cells mechanically harvested from confluent 250 ml tissue culture flasks were washed three times with PBS and lysed in 1.5 ml ice cold Laemmli buffer (0.125 M Tris, 4% SDS, 40% glycerol, 1% bromophenol blue, and 5% mercaptoethanol, pH6.8). The lysate was held on ice, sonnicated using a single five second burst, and stored at −70°C until required. Samples of lysate were fractionated by discontinuous SDS-polyacrylamide gel electrophoresis, using a 10% T, 2.6% C, polyacrylamide gel. Proteins were transferred onto nitrocellulose membrane using a semi-dry electroblotting system. Blots were probed with p53 antibodies DO7, 1801, BP53.12, 421, each at 0.5 μg/ml and with CM1 at dilutions of 1 in 1000 and 1 in 2000. Reacted p53 antibodies were detected using alkaline phosphatase conjugated goat anti-mouse immunoglobulin or goat anti-rabbit immunoglobulin polyclonal anti-sera. Blots were developed with 0.1 mg/ml nitroblue tetrazolium/0.15 mg/ml BCIP (5-bromo-4-chloro-3-indolyl phosphate), in development buffer (0.1 M Tris buffer, 0.1 M NaCl and 50 mM $MgCl_2$, pH 9).

SELECTION OF p53 ANTIBODIES FOR ELISA DEVELOPMENT

p53 antibodies for the development of the ELISA were selected on the basis of their performance in immunohistochemistry, for optimum assignment of p53 status, and their specificity for p53 in western blot analysis. Using these criteria, monoclonal antibodies 1801 and DO7 were selected for use in assay development.

PREPARATION OF BIOTINYLATED DO7

Monoclonal antibody DO7 was purified from hybridoma culture supernatant by absorption with protein A (Sigma). Antibody was eluted at pH 3 and dialysed against 0.1 M borate buffer, pH 8.8. Purified antibody was then reacted with ester of biotinamidocaproate N-hydroxysuccinimide, in dimethyl sulphoxide (DMSO), (100 μg ester/mg monoclonal antibody) for four hours at room temperature. The conjugation reaction was terminated by the addition of 1 M ammonium chloride (25 μl/mg monoclonal antibody), and the products extensively dialysed against 0.1 M Tris/HCl, pH 7.4.

ELISA PROCEDURE

A sandwich type ELISA system was developed for the quantitative analysis of p53. Solid phase monoclonal antibody 1801 was used as the p53 immobiliser, and biotinylated DO7 as the p53 detector. In brief, Immulon-1 96-well ELISA trays (Dynatech Laboratoies, Billinghurst, UK) were coated overnight at 4°C with 50 μl/well 10 μg/ml 1801 in PBS. Unbound antibody was removed by three washes with PBS (this buffer was used for all subsequent washing steps), and the wells incubated for one hour at 37°C with 200 μl 5% BSA in PBS, to block non-specific immunoreactivity. Wells were then washed

once and incubated for three hours at 37°C with 50 µl standard recombinant p53 protein, tumour cytosol at 2 mg/ml, 1 mg/ml, 0.5 mg/ml, and 2.5 mg/ml, or patient serum diluted 1 in 10 and 1 in 100. In each instance, 2% BSA in PBS was used as the diluent. After washing three times, the wells were next incubated for two hours at 37°C with 50 µl biotinylated DO7 diluted 1 in 10 in 2% BSA in PBS (this buffer was used for all subsequent antibody dilutions). The wells were washed again three times and then incubated for one hour at 37°C with 50 µl sheep anti-biotin polyclonal antiserum (The Binding Site, Birmingham, UK) diluted 1 in 1000. Specificity of this reagent was assured by extensive pre-absorption with mouse IgG-agarose. After washing three times, the wells were incubated for one hour at 37°C with 50 µl horseradish peroxidase conjugated donkey anti-sheep immunoglobulin anti-serum (Stratech Scientific Ltd, Luton, UK) diluted 1 in 4000. The wells were washed a final four times and developed with 50 µl 3,3' 5,5'-tetramethylbenzidine substrate. The reaction was stopped with 0.2 M sulphuric acid and results recorded at 250 nm using a Titertek multi-scan plate reader. The assay was standardised using an enriched preparation of recombinant p53 protein (kindly supplied by Professor D Lane). Test absorbance values were corrected against control values derived from wells processed in the absence of biotinylated DO7. p53 concentrations were calculated with reference to the linear range of standard recombinant p53 titration curves. Cytosol p53 values were expressed as p53 ng/mg total cytosol protein, and serum values as p53 ng/ml serum.

Results

IMMUNOHISTOCHEMISTRY

Using a series of breast carcinomas, we assessed the immunohistochemical performance of p53 antibodies for the assignment of tumour p53 status. Each antibody was assessed on consecutive sections cut from each tumour sample. For immunohistochemistry, cryopreserved tissue was used in preference to formalin fixed, paraffin wax embedded tissue as the results generated would best reflect the performance of the antibodies in ELISA for the analysis of p53 in cancer cytosol. Positive staining with any of the monoclonal antibodies tested was restricted to the nucleus, and was consistent with the nuclear localisation of p53. In comparison, however, CM1 commonly exhibited reactivity within the cell cytoplasm which was independent of nuclear staining, suggesting reactivity with cellular antigens in addition to p53. On this basis, cancers assessed for p53 using CM1 were scored p53 positive only if nuclear staining was apparent.

Table 1 shows the percentage of cancers scored as p53 positive using each of the antibodies tested. A higher percentage of cancers were scored p53 positive with monoclonal antibodies DO7 and 1801 than any of the other antibodies tested, indicating that DO7 and 1801 identify most p53 positive cancers. All samples scored positive using BP53.12, 421 or CM1 were also positive on staining with DO7 and 1801.

Table 1 Percentage of breast cancers immunohistochemically scored as p53 positive using antibodies DO7, 1801, 421, BP53.12, and CM1

Antibody	*Type/species*	*Per cent positive cases*
DO7	Monoclonal/mouse	46
1801	Monoclonal/mouse	45
421	Monoclonal/mouse	42
CM1	Polyclonal/rabbit	17
BP53.12	Monoclonal/mouse	16

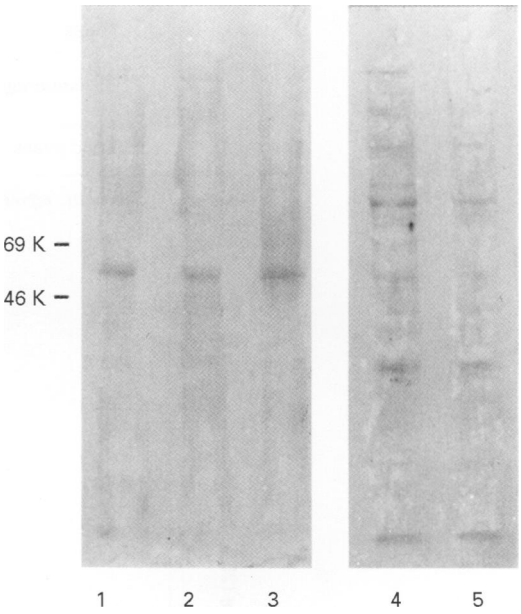

Figure 1 Western blot analysis of p53 antibodies against a cell lysate of the breast carcinoma cell line MDA-MB-231. Lane 1, DO7; lane 2, 1801; lane 3, BP53.12; lane 4, CM1 (diluted 1 in 1000); lane 5, CM1 (diluted 1 in 2000).

WESTERN BLOT ANALYSIS

Specificity of the p53 antibodies for p53 protein was assessed by western blot analysis against lysate of the breast adenocarcinoma cell line MDA-MB-231. Figure 1 shows that monoclonal antibodies DO7, 1801 and BP53.1 exhibit specificity for a single protein, the molecular weight of which is consistent with that of p53 (monoclonal antibody 421 at 1 µg/ml failed to detect p53). In comparison, CM1, at different concentrations, reacted with several cell proteins in addition to a weak reaction with p53. We suggest that this polyreactivity is analogous to CM1 cytoplasmic staining of breast cancers which are p53 negative—that is, no nuclear reactivity with CM1, DO7, 1801, and 421.

ELISA

Results generated from immunohistochemical and western blot analysis of the p53 antibodies suggest that ELISA systems incorporating combinations of monoclonal antibodies 1801 and DO7 would provide a highly specific system for the analysis of p53 in most cancers. Therefore, an ELISA system was developed using solid phase 1801 as the p53 immobiliser and biotinylated DO7 as the p53 detector. Subsequent detection and amplification of

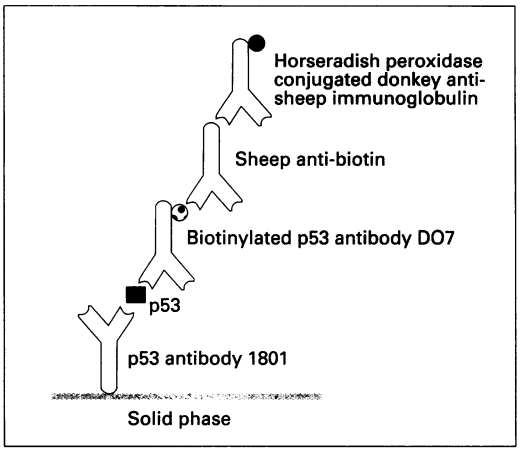

Figure 2 Schematic representation of the p53 quantitative ELISA.

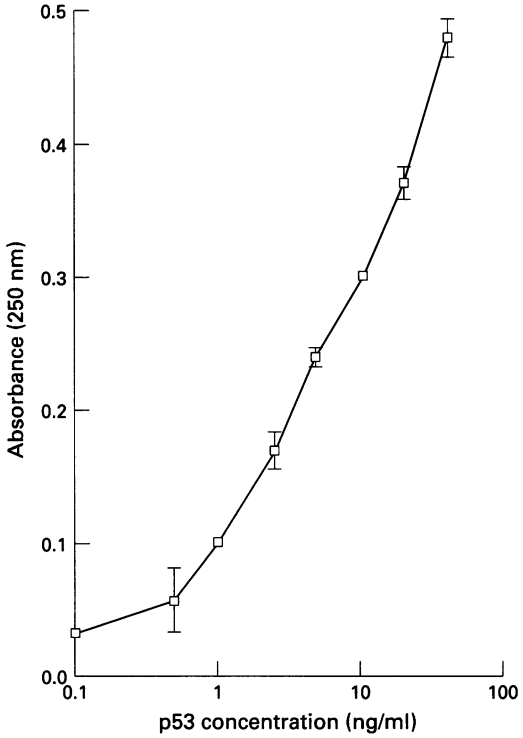

Figure 3 Typical titration curve for detection of recombinant p53 using the quantitative ELISA.

DO7 binding was achieved using sheep anti-biotin and horseradish peroxidase conjugated donkey anti-sheep immunoglobulin polyclonal anti-sera (fig 2). A typical calibration curve for the p53 assay using an enriched preparation of standard recombinant p53 is shown in fig 3. The detection limit, the concentration of p53 which could be distinguished from the zero value with 95% confidence, was 0.1 ng/ml of the enriched p53 protein.

Concentrations of p53 were measured using the ELISA system in a small series of breast cancer cytosols and in serum samples from patients with breast cancer. Cytosol values ranged from zero (values which could not be distinguished from the control with statistical significance) to 14.9 ng/mg cytosol protein. Comparisons of the quantitative values with immunohistochemical staining of frozen sections of the same cancer tissue, using antibodies 1801 and DO7, are shown in fig 4. Good correlation between cancer immunohistochemically scored as p53 positive, using DO7/1801, and corresponding positive cytosol values was shown. In no case were any cancers scored positive by immunohistochemistry which did not have a cytosol p53 value statistically distinguishable from the background value. Several cancers immunohistochemically scored p53 negative were, however, positive for p53 in ELISA. p53 was not detected in any of the serum samples tested.

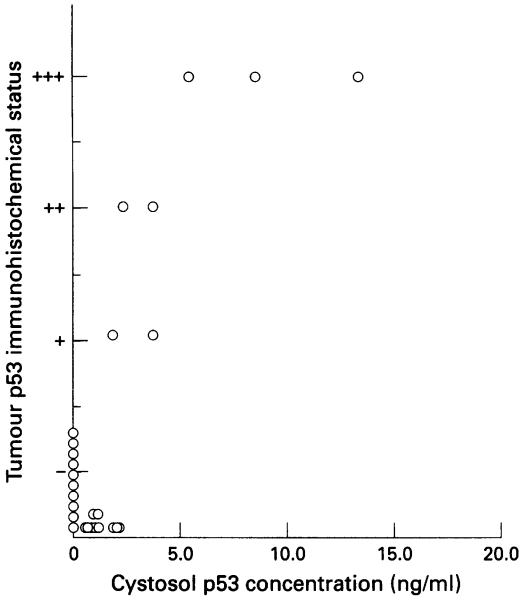

Figure 4 Comparison between immunohistochemical p53 staining (using 1801 and DO7) of breast cancer tissue and corresponding cytosol p53 values (assessed using the quantitative ELISA).

Discussion

Mutation of the tumour suppressor gene p53 is a common event in carcinogenesis, and can lead to accumulation of the mutated protein.[1-4] Accumulation of the p53 protein has been assessed qualitatively using immunohistochemistry and recently quantitatively using immunoassay based systems. Both qualitative and quantitative analyses have shown that p53 overexpression in breast cancers is commonly associated with poor prognosis.[5-7] If assessment of p53 overexpression is to have any important clinical implications in cancer prognosis, we believe it is necessary to have screening systems which are highly sensitive and specific for the analyte. For this reason, we have developed a highly specific and sensitive immunoassay system which can be used to analyse quantitatively p53 concentrations in most cancers. The assay system incorporates two monoclonal antibodies directed against p53 which exhibit high specificity for the antigen, and detect immunohistochemically a high proportion of p53 positive cancers.

In a recent paper, we have shown that the immunohistochemical assessment of p53 status in formalin fixed, paraffin wax embedded cancer tissue is critically dependent on the p53 antibodies used for screening.[5] In the present study, using cryopreserved, non-fixed breast cancer tissue, immunohistochemical assessment of p53 has shown that a higher percentage of cancers are scored positive using monoclonal

antibodies 1801 and DO7, in comparison with 421 and BP53.12, and that polyclonal antibody CM1 performs poorly. These findings are similar to those reported in routinely processed tissue.[5] In addition to the immunohistochemical assessment of p53 antibodies, we have also analysed their specificity for p53 by western blotting. CM1 was again found to perform poorly, exhibiting only weak reactivity with p53 and stronger reactivity with other cellular antigens. In comparison, monoclonal antibodies 1801, DO7 and BP53.12, exhibit specificity for a single protein with molecular weight indicative of p53. From analysis of these data, we suggest that quantitative immunoassay systems for p53 using CM1 may not produce consistently specific and reproducible results. As monoclonal antibodies DO7 and 1801 were preferentially selected for both their detection of p53 in immunohistochemistry and their specificity for p53 in western blot analysis, we suggest that the quantitative immunoassay system we describe has advantages over other systems for analysis of tumour p53 concentrations.

DO7 and 1801 bind epitope regions located at the N-terminus of the p53 molecule: 1801 recognises an epitope mapping between amino acid residues 32–79[12] and DO7 within the region defined by amino acids 20–25.[13] Both regions are distant from the central portion of the p53 molecule which is most commonly affected by point mutations.[14] We, therefore, suggest that these epitope regions will be structurally conserved in a high proportion of cancers, and, thus, it is expected that in most cancers p53 protein concentrations can be measured using this antibody combination. No competition of p53 binding was observed between the two antibodies when using solid phase 1801 and DO7 as the p53 detector. To accommodate the reduction in amplification accompanying the use of a monoclonal antibody as p53 detector in comparison with a polyclonal anti-serum—for example, CM1, we labelled DO7 with biotin and used sheep anti-biotin anti-sera and horseradish peroxidase conjugated donkey anti-sheep immunoglobulin anti-sera. This approach permitted the detection of 0.1 ng/ml of p53 in an enriched recombinant p53 preparation. This is less than that described by other assays using CM1.[9 10] We are, however, confident in suggesting that the analyte measured in our system is p53.

The ELISA system was used to assess quantitative p53 values in a small series of breast cancers, and the results compared with immunohistochemical staining of frozen sections from representative samples of the same cancer tissue, using antibodies 1801 and DO7. All cancers with positive immunohistochemical status were similarly positive in ELISA, with p53 values ranging from 1 to 14.9 ng/mg. In general, high immunohistochemically p53 positive cancers had corresponding high quantitative p53 values. About 38% of the series scored immunohistochemically negative had positive values in ELISA, with values ranging from 0.3 to 2 ng/mg. Owing to the high specificity of the antibodies used in the ELISA, we suggest that these cancers are indeed p53 positive, but the levels expressed are below the threshold value for immunohistochemical detection. Alternatively, the variation between cytosol and immunohistochemical results may simply reflect heterogeneity within a tumour, suggesting that more relevant cancer p53 data will be obtained from assessment of samples taken from several different sites of the primary cancer. In line with previous findings, p53 was not detected in the serum samples.[11]

In conclusion, we suggest that our method is a precise and sensitive immunologically based system for the quantitative analysis of p53 protein. Whether the precise estimation of p53 will provide more significant prognostic data will be assessed by the analysis of a larger series of cancer cytosols.

1 Lane DL. P53 guardian of the genome. *Nature* 1992;**358:**15–16.
2 Hollstein M, Sidransky D, Vogelstein B, Harris CC. P53 mutations in human cancers. *Science* 1991;**253:**49–53.
3 Nigro JM, Baker SJ, Preisinger AC, Jessop JM, Hostetter R, Cleary K, *et al.* Mutations in the p53 gene occur in diverse human tumour types. *Nature* 1989;**342:**705–8.
4 Bartek J, Bartkova J, Voijesek B, Staskova Z, Lukas J, Rejthar A, *et al* Aberrant expression of the p53 onco-protein is a common feature of a wide spectrum of human malignancies. *Oncogene*1991;**6:**1699–703.
5 Horne GM, Anderson JJ, Tiniakos DG, McIntosh GG, Thomas MD, Angus B, *et al* P53 protein as a prognostic indicator in breast carcinoma: a comparison of four antibodies for immunohistochemistry. *Br J Cancer* 1986; **73:**29–35.
6 Elledge RM, Fuqua SAW, Clark GM, Pujol P, Allred G, McGuire WL. Prognostic significance of p53 gene alterations in node negative breast cancer. *Breast Cancer Res Treat* 1993;**26:**225–35.
7 Iwaya K, Tsuda H, Hiraide H, Tamaki K, Takamura S, Fukutomi T, *et al.* Nuclear p53 immunoreaction associated with poor prognosis of breast cancer. *J Cancer Res* 1991;**82:** 835–40.
8 Thomas MD, Parr AH, McIntosh GG, Anderson JJ, Ellis I, Nicholson R, *et al.* Measurement of p53 protein in human breast cancer: comparison between immunohistochemistry and semi-quantitative ELISA values. *J Pathol* 1995; **176(Suppl):**172.
9 Vojtesek B, Fisher CJ, Barnes DM, Lane DP. Comparison between p53 staining in tissue sections and p53 protein levels measured by ELISA technique. *Br J Cancer* 1993;**67:** 1254–8.
10 Levesque MA, Diamandis EP, Yu H, Sutherland JA. Quantitative analysis of mutant p53 protein in breast tumour cytosols and study of its association with other biochemical prognostic indicators in breast cancer. *Breast Cancer Res Treat* 1994;**30:**179–95.
11 Hassapoglidou S, Diamandis EP, Sutherland DJA. Quantification of p53 protein in tumour cell lines, breast tissue extracts and serum with time-resolved immunofluorometry. *Oncogene* 1992;**8:**1501–9.
12 Banks L, Matlashewski G, Crawford L. Isolation of human p53 specific monoclonal antibodies and their use in the study of human p53 expression. *Eur J Biochem* 1986;**159:** 529–34.
13 Stephen CW, Heiminen P, Lane DP. Characterisation of epitopes on human p53 using phage displayed peptide libraries: Insight into antibody peptide interactions. *J Mol Biol* 1995;**248:**58–78.
14 Stephen CW, Lane DP. Mutant conformation of p53. Precise epitope mapping using filamentous phage epitope library. *J Mol Biol* 1992;**225:**577–83.