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Identification of p53 Mutations by Means of Single Strand Conformation Polymorphism Analysis in Gynaecological Tumours: Comparison With the Results of Immunohistochemistry

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The tumour-suppressing gene p53 may undergo mutation by a variety of mechanisms, thus losing its tumour-suppressing activity, and ultimately behaving like an oncogene. The PAb 1801 monoclonal antibody is known to recognise both wild type and mutated p53, although in practice it seems to show a higher reactivity with the mutated gene product in several human tumours. We studied p53 overexpression in a series of 36 human tumours (17 mammary ductal infiltrating carcinomas, 11 endometrial carcinomas and 8 uterine cervical carcinomas) by means of immunohistochemistry using the PAb 1801 antibody and the streptavidin–biotin peroxidase technique. Furthermore, all tumours were screened for mutations in the "hot spot" regions of the p53 gene (exons 5 to 8) by means of SSCP (single strand conformation polymorphism) DNA analysis following amplification of the target exons using the polymerase chain reaction. A good correlation (75–100%) between positive immunohistochemistry and p53 mutations was observed in mammary and endometrial cancer, whereas mutations were detected in only two out of seven immunoreactive cervical carcinomas. Following these results, immunohistochemistry with the PAb monoclonal antibody may be safely used as a screening tool for the detection of mutated p53 in clinical samples of mammary and endometrial cancer, whereas it should be complemented with DNA analysis in cervix carcinoma.

Key words: p53, SSCP, immunohistochemistry, mammary carcinoma, endometrial carcinoma, cervix carcinoma
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INTRODUCTION

THE TUMOUR suppressor gene p53 has been associated with a variety of human tumours, including mammary, endometrial and cervical carcinomas [1–3]. The wild type p53 gene product has inhibiting effects on cell proliferation and on oncogene-mediated transformation [4, 5]. However, various mechanisms may result in the loss or impairment of normal, wild type p53 tumour suppressing activity. Among these are deletions, rearrangements or loss of heterozygosity in chromosome 17p (p53 is located on the 17p short arm), or point mutations which lead to a slightly modified end product with paradoxical functions, ultimately resulting in a malignant phenotype [5].

Monoclonal antibodies against the p53 protein allow for a rapid identification of p53 overexpressing tumours in clinical material, while preserving the histological architecture of the analysed tissues [6]. However, these antibodies in principle recognise both the wild type and the mutated p53 product, which poses the question of the utility of the method. For some tumours, this seems to be less of a problem, since the visible positive reaction is almost exclusively limited to the expression of the mutated gene. In the case of breast cancer, for instance, in the largest study published [1], immunohistochemically detected p53 overexpression was associated to the presence of negative prognostic factors, and correlated negatively in a significant way with disease-free and overall survival. Positive cases of this study analysed for mutations of the p53 gene invariably showed them. Moreover, the technique employed, using the PAb 1801 antibody, seems applicable, with excellent results, to archival, paraffin-embedded material. It remains to be shown, however, if this high degree of coincidence between the results obtained by immunohistochemistry and the presence of mutations detected by DNA analysis is constantly reproducible and, which is more important, if it is common to every tumour type analysed.

With this in mind, we have studied a series of gynaecological

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tumours for p53 overexpression and mutation, using both immunohistochemistry and DNA analysis, and have compared the results obtained by means of the two methods. The ultimate goal was to develop a reliable and simple technique to be applied in the future as a routine method in the clinical setting.

MATERIALS AND METHODS

Tumours

The tumours were obtained from patients operated upon at Hospital de Cruces (Universidad del Pais Vasco, Bilbao, Spain). An aliquot from each specimen was immediately snap-frozen in liquid nitrogen upon sampling and kept at −70°C until immunohistochemical processing. The rest was routinely fixed in formalin and paraffin embedded for pathological diagnosis. It was from this routinely processed material that the DNA was later extracted for SSCP (single strand conformation polymorphism) analysis. In all, 36 tumours were studied: 17 mammary ductal infiltrating carcinomas, 11 endometrial carcinomas and 8 invasive uterine cervical carcinomas.

Immunohistochemistry

The immunohistochemical technique employed (streptavidin–biotin peroxidase) was essentially the same as one described previously for the detection of P-glycoprotein in similar human tumour material [7–9]. Briefly, 6-μm cryostat sections were made from each frozen tissue sample. They were air dried and subsequently fixed for 10 min in cold acetone at −20°C. The sections were then pre-incubated in successively, phosphate buffered saline (PBS) for 10 min, mouse pre-immune IgG1 at the same concentration as the primary monoclonal antibody for 15 min and, finally, normal sheep serum at 1:10 dilution for 15 min. The primary anti-p53 monoclonal antibody, PAb 1801 (Cambridge Research Biochemicals, Valley Stream, New York, U.S.A.), was then applied at a dilution of 1:400 in PBS for 18 h (overnight) at 4°C. The secondary, sheep anti-mouse biotinylated antibody at 1:50 dilution for 45 min and the streptavidin–horseradish peroxidase complex at 1:100 dilution, also for 45 min, were then applied successively, with three 5-min washes in PBS between each step. The immunohistochemical reaction was finally developed by means of 3-amino-9-ethylcarbazole (AEC), after which the slides were rinsed in tap water and counterstained with Mayer's haematoxylin before mounting with aqueous medium. As positive controls, we used MDA-MB-231 cells (a kind gift from Prof. Anne Thor's Laboratory, Massachusetts General Hospital, Boston, Massachusetts, U.S.A.), known to have p53 mutations [10]. Negative controls, to rule out unspecific staining, were carried out in parallel for each slide following the same technique described, but omitting the primary antibody. According to Thor and colleagues [1], tumours were scored as positive if any of the malignant cells showed nuclear immunoreactivity. However, there were differences in the number of reactive tumour cells between samples, ranging from less than 10% to more than 50%.

SSCP DNA analysis

The protocol used for SSCP analysis was basically the one described by Orita and colleagues [11,12] with slight modifications introduced by Bernd Seizinger's Laboratory of Molecular Neurooncology at Massachusetts General Hospital.

Briefly, DNA was extracted from 6 μm tumour tissue paraffin sections placed into 1-ml Eppendorf tubes. A process of rehydration was accomplished by adding xylenes, decreasing graded ethanols and acetone to the tubes, and centrifuging to decant inbetween. Then, 20–100 μl of TE-5% Tween 20 and proteinase K, to make a final concentration of 200 μg/ml, were added to the tubes. The mixture was incubated at 55°C for 2 h, shaking the tubes from time to time, and at 95°C for 10 min to inactivate the proteinase K. Afterwards, 1 min of centrifugation at full speed produced a pellet with non-digested debris. An aliquot of 1–10 μl of the liquid was used to start the PCR (polymerase chain reaction) experiments. The remainder was kept refrigerated.

SSCP analysis was performed after amplification of exons 5 to 8 of the p53 gene, using five sets of primers (Table 1). The reaction mixtures contained 0.5–1 μg DNA, 200 μM dATP, 200 μM dTTP, 200 μM dGTP, 20 μM dCTP, 1 μCi of alpha^{-32} PdCTP (Amersham; specific activity 3000 Ci/mmol), 50 mM KCl, 20 mM Tris pH 8.4, 2 μg/μl bovine serum albumin (BSA), 1–2 mM $MgCl_2$, 0.5 U Taq-polymerase (Perkin Elmer Cetus) and 100 ng of each primer in a total volume of 20 μl. Thirty cycles of denaturation (94°C, 1 min), annealing (60°C, 2 min) and extension (72°C, 3 min) were performed with an automated thermal cycler (MJ Research, Inc.). Amplification products were diluted 9-fold with a buffer containing 0.1% sodium dodecyl sulphate (SDS) and 10 mM EDTA. Ten microlitres of loading buffer containing 850 μl deionised formamide, 50 μl saturated bromophenol blue and 50 μl 20X TBE per ml of buffer were added to the diluted PCR products. The mixture

Table 1. Sequence, location information and amplification conditions of the primers used to amplify the p53 gene

Primers	Exon	Base pairs	Mg^{2+}	Temperature
918: 5′-TTA TCT GTT CAC TTG TGC CC-3′ 919: 5′-TCA TGT GCT GTG ACT GCT TG-3′	5	198	1.5 mM	60°C
920: 5′-TTC CAC ACC CCC GCC CGG CA-3′ 921: 5′-ACC CTG GGC AAC CAG CCC TG-3′	5	163	1 mM	60°C
930: 5′-ACG ACA GGG CTG GTT GCC CA-3′ 933: 5′-CTC CCA GAG ACC CCA GTT GC-3′	6	201	1 mM	60°C
924: 5′-GGC CTC ATC TTG GGC CTG TG-3′ 925: 5′-CAG TGT GCA GGG TGG CAA GT-3′	7	171	1 mM	60°C
927: 5′-CTG CCT GCT TCT CTT TT-3′ 928: 5′-TCT CCT CCA CCG CTT CTT GT-3′	8	204	2 mM	60°C

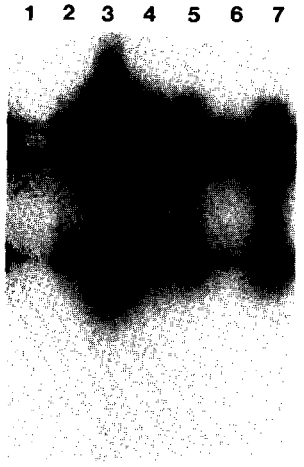

Figure 1. SSCP analysis of p53 exon 6, mammary carcinoma. One abnormal band pattern (lane 3), compatible with mutation.

was heated at 95°C for 10 min, quickly chilled on ice and applied to a 6% acrylamide nondenaturing gel containing 10% glycerol. Gels were run at 3 W for 15 h at room temperature, dried on a gel drier at 80°C for 2 h and exposed to X-ray film at room temperature for 24 h.

Samples containing mutations or polymorphisms of the sequence studied are evident by an altered band pattern compared to the wild pattern (Figure 1).

Table 2. Mammary carcinoma. Pathological features, p53 overexpression and SSCP results of the tumours studied

Patient	Nodes	p53 (immunohistochemistry)	p53 (SSCP)
1	+	+	+ (exon 6)
2	+	+	+ (exon 8)
3	−	−	−
4	+	−	−
5	+	−	−
6	+	+	+ (exon 7)
7	+	−	+ (exon 6)
8	+	−	−
9	−	−	−
10	−	−	−
11	−	−	−
12	−	−	−
13	−	+	+ (exon 6)
14	−	−	−
15	−	−	−
16	+	+	+ (exon 8)
17	+	+	+ (exon 6)

RESULTS

Thirty-six human gynaecological tumours were studied for the overexpression of p53 protein by means of immunohistochemistry and for p53 mutations in exons 5, 6, 7 and 8 by means of SSCP DNA analysis (Tables 2–4).

Table 3. Endometrial carcinoma. Pathological features, p53 overexpression and SSCP results of the tumours studied

Patient	Histology	p53 (immunohistochemistry)	p53 (SSCP)
1	Endometrioid	+	+ (exon 5)
2	Papillary serous	−	−
3	Endometrioid	−	−
4	Endometrioid	−	−
5	Adenosquamous	+	+ (exon 5)
6	Endometrioid		−
7	Endometrioid	+	−
8	Papillary serous	+	+ (exon 7)
9	Endometrioid	−	−
10	Endometrioid	−	−
11	Endometrioid	−	+ (exon 8)

Table 4. Cervical carcinoma. Pathological features, p53 overexpression and SSCP results of the tumours studied

Patient	Histology	p53 (immunohistochemistry)	p53 (SSCP)
1	Squamous	−	−
2	Squamous	+	−
3	Adenocarcinoma	+	+ (exon 8)
4	Squamous	+	−
5	Squamous	+	−
6	Adenocarcinoma	+	−
7	Squamous	+	−
8	Squamous	+	+ (exon 6)

Of 17 mammary carcinomas, p53 overexpression was found in six tumours (35%). An example of the typical positive immunohistochemical reaction for mammary carcinoma is given in Figure 2a. As can be seen from Figure 2, the quality of the staining reaction was less than optimal, although positive cases were clearly discernible from negative ones. To our surprise, we obtained much better results later on in similar tumours after formalin fixation and paraffin embedding, so that we attribute the loss in image quality of the samples of this study to the fixation procedure of the cryostat sections in acetone. Other authors, nevertheless, report that they have obtained similar results with both fixation and preservation techniques [1]. All of our six positive cases also showed altered band patterns characteristic of mutations in the SSCP analysis, for a 100% correlation between immunohistochemistry and SSCP in the detection of mutated p53 overexpression. This correlation was nearly as good for negative results, since only one of the 11 tumours with negative immunohistochemistry showed a band pattern compatible with mutations on SSCP (91% negative correlation). Furthermore, positive cases were almost exclusively restricted to patients with an ominous prognosis on clinical grounds. In fact, if stratified for axillary node invasion, which is the most powerful clinical prognostic factor, six of the seven tumours with p53 mutations belonged to patients with three or more affected axillary nodes, which is the criterion of worst clinical prognosis currently used. This result was highly significant, despite the small size of the series (Fisher's exact test, $P< 0.02$).

For endometrial carcinoma, the results were rather similar to those obtained with breast tumours (Table 3). Of 11 endometrial cancers, four were positive on immunohistochemistry (36%), with three disclosing mutations by means of SSCP analysis (75% correlation). Again, like mammary carcinoma, one case with negative immunohistochemistry showed an altered band pattern on SSCP (86% negative correlation).

Cervical carcinoma, finally, yielded strikingly different results (Table 4). All but one of the eight tumours studied were positive for p53 overexpression on immunohistochemistry (Figure 2b), but only two of these seven tumours were found to have p53 mutations on SSCP analysis.

DISCUSSION

Accumulation of the p53 protein has been previously studied in mammary carcinoma by Thor and colleagues [1] by means of immunohistochemistry on archival, paraffin-embedded material, and on fresh-frozen tissue using the same antibody (PAb 1801). In addition, they screened 15 cases for p53 mutations by means of PCR amplification and direct sequencing of the "hot spot" regions from codon 128 to 331, largely encompassing the same exons (5, 6, 7 and 8) as in this study (codons 126 to 306). They found a complete concordance of results between immunohistochemistry and DNA analysis: 10 immunohistochemically negative tumours did not show any mutations, whereas mutations were detected in all five cases with immunohistochemically verified accumulation of p53 protein. Our results corroborate those. Of our 17 mammary carcinomas, the six with positive immunohistochemistry also showed an altered band pattern characteristic of mutations in the SSCP DNA analysis. However, one out of 11 cases with negative immunohistochemistry also had an SSCP compatible with mutations.

Overexpression and mutation of p53 have been recently investigated in endometrial carcinoma by Kohler and colleagues [2]. They also evaluated the correlation between positive immunohistochemistry and mutations of the p53 gene by means of PCR amplification of exons 4 to 10 and direct sequencing of exons 5 to 8 in eight tumours of their series (five with positive and three with negative immunohistochemistry). They found mutations in exons 5, 7 and 8 in the five tumours with immunohistochemically detected accumulation of p53 protein, whereas the three tumours with negative immunohistochemistry showed the wild type sequence. We also found a good correlation

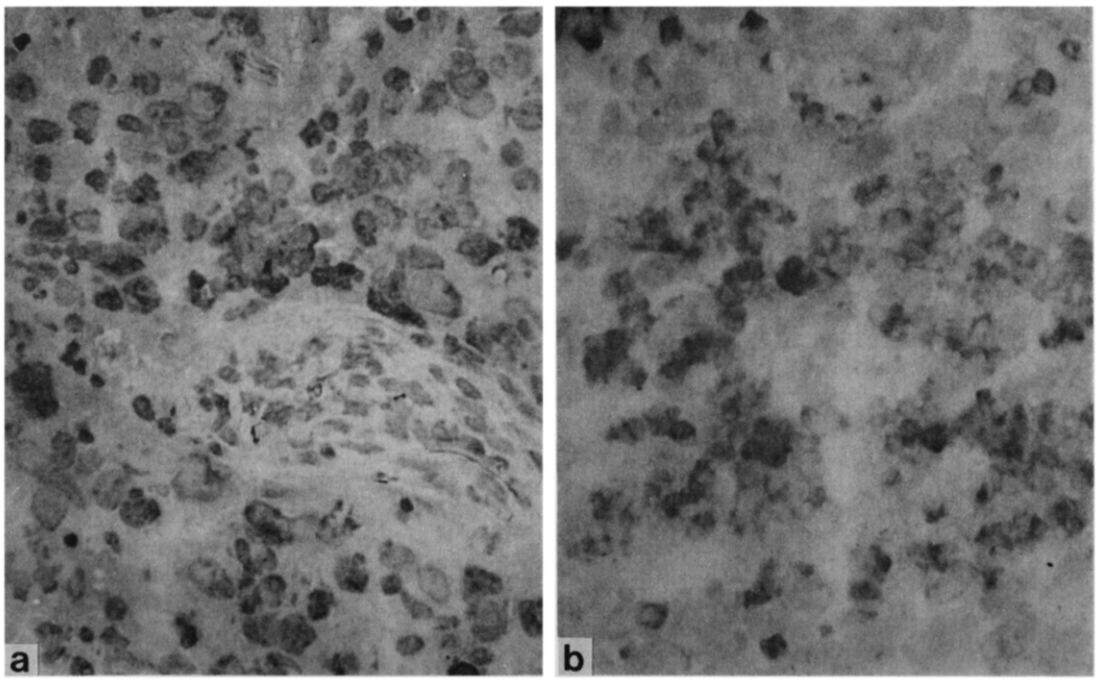

Figure 2. Immunohistochemical detection of p53 overexpression. Streptavidin biotin peroxidase × 250. (a) Mammary carcinoma. Staining in tumour cell nuclei, as opposed to stromal cells in the middle of the picture. (b) Cervix carcinoma.

between the two methods for this tumour type, although not as excellent as the one reported by Kohler and colleagues which was 100%. Of the 11 endometrial carcinomas examined in this study, using similar methods, four were positive on immunohistochemistry. Three showed mutations in exons 5 (two cases) and exon 7 (one case) on SSCP, and one did not. On the other hand, one of the seven immunohistochemically negative tumours showed mutation in exon 8 on SSCP.

The greatest discordance between positive immunohistochemistry and p53 mutations was observed in this study in cervical carcinoma. Eight of our nine cases were positive on immunohistochemistry, while only two of them had an SSCP compatible with mutations in exons 6 and 8. Fujita and colleagues have previously studied 36 cervical carcinomas for p53 mutations by means of PCR amplification and SSCP, followed by direct sequencing [3]. They found only two cases of mutation (exons 5 and 7), which were correctly detected by SSCP and confirmed by sequencing. Unfortunately, they did not perform immunohistochemistry on their material, so that both methods cannot be compared from their report.

It is difficult to find an explanation for the fact that there appears to be such a good correlation between p53 accumulation, as detected by immunohistochemistry, and p53 mutation in breast and endometrial carcinoma, and such a poor one in cervical carcinoma. A possible explanation could be that mutations, if present, are located in other exons as the one studied by us in this particular tumour. It could also be that wild type p53 protein is overexpressed in cervical carcinoma in such increased amounts that it is detectable by the PAb 1801 antibody, which is known to react with both isoforms of the protein. Genotoxic stress, as produced by chemotherapeutic drugs or by radiation, for example, has been shown to induce abnormally increased nuclear accumulation of wild type p53 protein, which remains detectable for a long period (at least 20 days). Accumulation in this case seems to occur because of increased p53 protein stability, and not because of mutation of the p53 gene, since it takes place in normal diploid cells within hours of exposure to the causating agent [13]. Moreover, mutation of the p53 gene seems not to be a prerequisite for malignant transformation of uterine cervical cells, because the normal function of wild type p53 protein may be altered in them by other mechanisms. One of these is mediated by the E6 protein produced by human papillomavirus type 16, which has been epidemiologically related to the appearance of cervix carcinoma. The HPV16 E6 oncoprotein has been shown in several studies [14,15] to form a complex with the wild type p53 protein, thereby promoting its degradation. Other similar mechanisms which also inhibit normal p53 function have been described. Momand and colleagues [16] have shown that the mdm-2 oncogene product p90 is also able to form a complex with the wild type p53 protein, thus blocking p53-mediated transactivation.

The results of Fujita and colleagues, who only found two point mutations among their 36 cervical carcinomas using the same approach as this study, plus a final sequencing analysis that completely corroborated their results of SSCP, are similar to our results (two mutations in eight tumours), and seem to exclude a technical error from our part with the SSCP analysis. This was repeated by us, because of the suspicion which arose from the difference in the results when compared to the other two tumour types, and the repeat gels showed identical results.

In conclusion, immunohistochemistry may be a valuable tool in practice to screen for p53 overexpression and mutation in clinical samples from mammary and endometrial carcinomas. For cervical carcinoma, however, DNA analysis seems mandatory, at least when using the PAb 1801 antibody. In this case, SSCP is a relatively simple procedure for this purpose, and its results, from our own experience and that of other investigators [1, 2] seem to be reliable for the detection of mutations in these tumours. It must be borne in mind, however, that SSCP, like any other screening method, will necessarily have its own (albeit small) failure rate. The results presented in this paper, where no 100% concordance between both screening methods tested could be reached, are a good corollary to this statement.

1. Thor AD, Moore DH, Edgerton SM, *et al.* Accumulation of p53 tumor suppression gene protein: an independent marker of prognosis in breast cancers. *J Natl Cancer Inst* 1992, **84**, 845–855.
2. Kohler MF, Berchuk A, Davidoff AM, *et al.* Overexpression and mutation of p53 in endometrial carcinoma. *Cancer Res* 1992, **52**, 1622–1627.
3. Fujita M, Inoue M, Tanizawa O, Iwamoto S, Enomoto T. Alterations in the p53 gene in human primary cervical carcinoma with and without human papillomavirus infection. *Cancer Res* 1992, **52**, 5323–5328.
4. Vogelstein B. Cancer: a deadly inheritance. *Nature* 1990, **348**, 681–682.
5. Levine AJ, Momand J, Finlay CA. The p53 tumor suppressor gene. *Nature* 1991, **351**, 453–456.
6. Banks L, Matlashewski G, Crawford L. Isolation of human p53-specific monoclonal antibodies and their use in the studies of human p53 expression. *Eur J Biochem* 1986, **159**, 529–534.
7. Schneider J, Bak M, Efferth T, Kaufmann M, Mattern J, Volm M. P-glycoprotein expression in treated and untreated human breast cancer. *Br J Cancer* 1989, **60**, 815–819.
8. Schneider J, Efferth T, Mattern J, Rodriguez-Escudero FJ, Volm M. Immunohistochemical detection of the multidrug-resistance marker P-glycoprotein in uterine cervical carcinomas and normal cervical tissue. *Am J Obstet Gynecol* 1992, **166**, 825–829.
9. Schneider J, Efferth T, Centeno MM, Mattern J, Rodriguez-Escudero FJ, Volm M. High rate of expression of multidrug resistance-associated P-glycoprotein in human endometrial carcinoma and normal endometrial tissue. *Eur J Cancer* 1993, **29A**, 554–558.
10. Cattoretti G, Rilke F, Andreola S, *et al.* p53 expression in breast cancer. *Int J Cancer* 1988, **41**, 178–183.
11. Orita M, Suzuki Y, Sekiya T, Hayashi K. Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. *Genomics* 1989, **5**, 874–879.
12. Orita M, Iwahana H, Kanazawa H, Hayashi K, Sekiya T. Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. *Proc Natl Acad Sci USA* 1989, **86**, 2766–2770.
13. Fritsche M, Haessler C, Brandner G. Induction of nuclear accumulation of the tumor-suppressor protein p53 by DNA-damaging agents. *Oncogene* 1993, **8**, 307–318.
14. Meitz JA, Unger T, Huibregtse JM, Howley PM. The transcriptional transactivation function of wild-type p53 is inhibited by SV40 large T-antigen and by HPV-16 E6 oncoprotein. *EMBO J* 1992, **11**, 5013–5020.
15. Huibregtse JM, Scheffner M, Howley PM. Cloning and expression of the cDNA for E6-AP, a protein that mediates the interaction of the human papillomavirus E6 oncoprotein with p53. *Mol Cell Biol* 1993, **13**, 775–784.
16. Momand J, Zambetti GP, Olson DC, George D, Levine AJ. The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. *Cell* 1992, **69**, 1237–1245.

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