

p53 Mutation and *MDM2* Amplification in Human Soft Tissue Sarcomas¹

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Abstract

The *p53* and *MDM2* genes were analyzed in 24 human soft tissue sarcomas (11 malignant fibrous histiocytomas and 13 liposarcomas). Alterations of *p53*, consisting of point mutations, deletions, or overexpression, were detected in one-third (8 of 24) of the sarcomas. *MDM2* gene amplification was detected in another 8 tumors, but no tumor contained an alteration of both genes. Monoclonal antibodies reactive with the human *MDM2* gene product were developed, and immunohistochemical analysis revealed nuclear localization and overexpression of *MDM2* in those tumors with amplified *MDM2* genes. These data support the hypothesis that *p53* and *MDM2* genetic alterations are alternative mechanisms for inactivating the same regulatory pathway for suppressing cell growth.

Introduction

The *p53* gene is mutated in many, but not all, human malignancies (1). What is the role of *p53*, if any, in those tumors without evident *p53* mutation? Three possibilities can be considered. First, *p53* mutations could exist but be undetectable by standard methods (e.g., mutations in introns which affect expression). Second, mutations could exist in other genes that interact with *p53* or are downstream of *p53* and result in an identical physiological defect within the cell. Third, mutations in other genes, totally unrelated to *p53*, could occur in some tumors, resulting in a transformation process that is qualitatively different from that occurring in *p53*-mutant cells.

In the current study, we sought to address aspects of this important question in human soft tissue sarcomas. Previous analyses of these tumors have revealed that *p53* abnormalities are relatively frequent (30–60%) (2–6). Moreover, a gene (*MDM2*), the product of which binds to *p53* (7), has been shown to be amplified in a subset of such tumors (8). If a major effect of *MDM2* amplification were to inactivate the *p53* gene product, one would expect that those tumors with *MDM2* gene amplification would be devoid of *p53* mutations and vice versa—double mutations in both *p53* and *MDM2* would be redundant for the neoplastic process and would provide no selective advantage over that conferred by mutation in only one of the two genes. This hypothesis was tested in the current study using genetic and immunohistochemical methods.

Materials and Methods

Tumors. Ten primary malignant fibrous histiocytomas and 13 primary liposarcomas from 23 patients were frozen immediately after surgery. The OsA-CL cell line was derived from a sarcoma that occurred in bone (9) but had histological features characteristic of a malignant fibrous histiocytoma and is considered here to be a tumor of the latter type. Most of these tumors have

previously been evaluated for *MDM2* gene amplification (8) but not for expression of *MDM2* or alteration of *p53*.

DNA Analysis. *p53* exons 5, 6, 7, and 8 were amplified using the polymerase chain reaction as previously described (10) except that the 5' primer contained an artificial *Bam*HI site and the 3' primer contained an artificial *Eco*RI site. The resultant 1.8-kilobase PCR³ product was digested with *Eco*RI and *Bam*HI, gel purified, and cloned between the *Bam*HI and *Eco*RI sites of pBluescript (Stratagene). DNA purified from pools of at least 100 clones was sequenced with primers specific for each exon (10). Southern blot analysis was performed by digesting 4 µg of DNA with *Eco*RI, separating the fragments by gel electrophoresis, and transferring them to nylon filters. The DNA on filters was then sequentially hybridized with probes for *MDM2* (clone C14-2; Ref. 8), for *p53* (1.8-kilobase complementary DNA containing all coding exons; Ref. 11), and for control sequences on chromosome 17p12 (EW503; Ref. 12). Probe labeling and hybridization were performed as previously described (8, 13).

Monoclonal Antibody Production. Female (BALB/C × C57BL/6) F1 mice were immunized and boosted by i.p. injection of purified GST-*MDM2* fusion protein in Ribi adjuvant (Ribi Immunochem Research, Inc.). The fusion protein, containing amino acids 27 to 168 of *MDM2*, was expressed in *Escherichia coli* and purified using glutathione Sepharose. Hybridomas were produced as described (14, 15), except that test bleeds and hybridomas were screened for anti-*MDM2* reactivity using trpE-*MDM2* and purified GST-*MDM2*. Two hybridomas were isolated which appeared to react specifically with *MDM2*. One of them, mAb IF-2, was found to be particularly useful since it was reactive with human *MDM2* in Western blots, immunoprecipitation, and immunohistochemical assays (on frozen, but not paraffin, sections).

Immunohistochemistry. Frozen sections of 6-µm thickness were fixed with Histochoice (Amresco) for 10 min following air drying. After blocking endogenous peroxidase activity with 0.3% H₂O₂ in methanol, the section was incubated with goat serum for 30 min at room temperature and then incubated with antibodies diluted in goat serum for 2 h at room temperature in a humidified chamber. The antibodies used were IF-2 (specific for *MDM2*, used at 5 µg/ml), 1801 (specific for *p53*, used at 0.5 µg/ml; Oncogene Science), and CF-11 (same Ig isotype as IF-2, generated against an irrelevant protein, Ref. 15, and used at 10 µg/ml as a negative control; no staining was observed in sections adjacent to those shown in Fig. 5). Following washing with phosphate-buffered saline, the sections were incubated with biotinylated goat anti-mouse Ig and developed with a horseradish peroxidase system (Vectastain Elite, Vector Labs).

Results

To search for gross alterations of the *p53* gene, DNA from each of the 24 sarcomas was digested with *Eco*RI, and Southern blot analysis was performed with the *p53* probe. In three of the tumors, a deletion of the normal-sized 18-kilobase fragment was observed, as evidenced by the very faint bands at this position (Fig. 1, Lanes 1, 4, and 6). Ethidium bromide staining demonstrated that all lanes contained equal quantities of undegraded DNA (not shown). To demonstrate the specificity of the *p53* deletions, the blots were rehybridized with another probe for chromosome 17p. Fig. 1 (bottom) shows that the EW503 probe, detecting sequences on chromosome 17p near *p53* (12), efficiently hybridized to a 3.3-kilobase DNA fragment from all tumors,

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³ The abbreviations used are: PCR, polymerase chain reaction; mAb, monoclonal antibody; HPV, human papillomavirus.

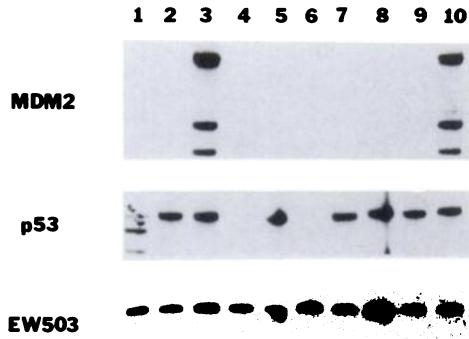


Fig. 1. Southern blot analysis of the *MDM2* and *p53* genes in human sarcomas. Southern blotting was performed using probes for *p53*, *MDM2*, and *EW503*, as described in "Materials and Methods." The hybridizations were sequentially performed with the same blot. Lanes 1 to 10, tumors 1 to 10, respectively (Table 1). The *MDM2* fragments migrated at 8, 4, and 3 kilobases, the *p53* fragment at 18 kilobases, and the *EW503* fragment at 3.3 kilobases.

including tumors 1, 4, and 6. Of the three tumors with deletion, two showed a total absence of signal (Lanes 4 and 6), while one showed two smaller-size bands reactive with the *p53* probe (Lane 1). These bands were not the result of contamination with plasmid DNA, as shown by the absence of hybridization to a radiolabeled probe containing only vector sequences (not shown). Thus, deletion of *p53* in tumor 1 was associated with at least one intragenic rearrangement, whereas the deletions observed in tumors 4 and 6 were the result of rearrangements the borders of which were outside the region detected by the *p53* probe.

Fig. 1 also shows examples of *MDM2* amplification, noted in 8 of the tumors studied (Fig. 1, Lanes 3 and 10; Table 1). Each of these 8 tumors contained at least 10 copies of the *MDM2* gene/cell. The *p53* and *EW503* probes served as controls for DNA loading and transfer. No rearrangements were noted in tumors with or without amplification, since only the expected fragments of 8, 4, and 3 kilobases were observed. Longer exposures revealed the same size fragments in the tumors without amplification of *MDM2* (not shown).

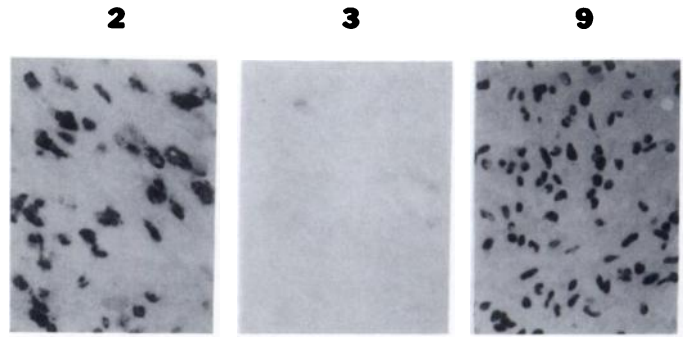


Fig. 2. *p53* expression in primary soft tissue sarcomas. Cryostat sections of human sarcomas were incubated with the mAb 1801 antibody specific for *p53* as described in "Materials and Methods." Tumors 2 and 9 showed overexpression of *p53*, while no staining was observed in tumor 3.

To detect subtle sequence alterations of *p53*, exons 5–8 were sequenced. These exons have been shown to harbor most of the point mutations observed in human tumors (reviewed in Ref. 1). In each case, a PCR product containing exons 5–8 was cloned, and a pool of at least 100 clones was sequenced. Four sarcomas contained point mutations demonstrable in this assay. Two of these (tumors 2 and 12) contained missense mutations; one tumor (tumor 15) contained a nonsense mutation; and another (tumor 18) contained a mutation altering a consensus splice site (Table 1). In these four cases, the signal corresponding to the normal nucleotide sequence of *p53* was weaker than that of the mutant nucleotide, suggesting that the mutation was accompanied by a loss of the wild-type allele, with the residual signal contributed by nonneoplastic cells within the tumor (not shown). In each of the four cases, an independent PCR and sequencing reaction were performed to confirm the mutation.

For seven of the tumors used in this study, frozen samples were available for immunohistochemical analysis. In five of the tumors, little or no reactivity with the *p53*-specific antibody was observed, a result consistent with the absence of *p53* mutation. In contrast, two tumors (tumors 2 and 9) showed strong nuclear staining with the

Table 1 Profile of tumors and mutations

Tumor no.	Tumor ID	Type ^a	<i>MDM2</i> amplification ^b	<i>p53</i> alteration ^c	Over expression ^d
1	M-2	MFH	Absent	Deletion/rearrangement	None
2	M-5	MFH	Absent	CGC-CUC mutation; Arg(158)-His	<i>p53</i>
3	M-7	MFH	Present	None observed	<i>MDM2</i>
4	M-8	MFH	Absent	Deletion	None
5	M-14	MFH	Absent	None observed	NT
6	M-15	MFH	Absent	Deletion	NT
7	M-16	MFH	Absent	None observed	None
8	M-17	MFH	Absent	None observed	NT
9	M-18	MFH	Absent	Overexpressed	<i>p53</i>
10	M-20	MFH	Present	None observed	<i>MDM2</i>
11	L-5	Liposarcoma	Absent	None observed	NT
12	L-7	Liposarcoma	Absent	AAC-AGC mutation; Asn(239)-Ser	NT
13	L-9	Liposarcoma	Present	None observed	NT
14	L-11	Liposarcoma	Absent	None observed	NT
15	KL5B	Liposarcoma	Absent	CAG-UAG mutation; Gln(144)-stop	NT
16	KL7	Liposarcoma	Present	None observed	NT
17	KL10	Liposarcoma	Absent	None observed	NT
18	KL11	Liposarcoma	Absent	GGT-GAT mutation; exon 5 splice donor site	NT
19	KL12	Liposarcoma	Absent	None observed	NT
20	KL28	Liposarcoma	Present	None observed	NT
21	KL30	Liposarcoma	Present	None observed	NT
22	S189	Liposarcoma	Present	None observed	NT
23	S131B	Liposarcoma	Absent	None observed	NT
24	OSA-CL	MFH	Present	None observed	<i>MDM2</i>

^a MFH, malignant fibrous histiocytoma.

^b As assessed by Southern blot.

^c As assessed by Southern blot, sequencing of exons 5–8, or immunohistochemical analysis.

^d As assessed by immunohistochemical analysis. NT, not tested.

antibody (Fig. 2). In tumor 2, this reactivity was expected because the tumor contained a missense mutation (Table 1). In tumor 9, no mutation in exons 5–8 was detected, and the mutation giving rise to the overexpression was presumably outside the region sequenced. There is ample precedent for occasional *p53* mutations outside exons 5–8 in other tumor types (1, 16).

To evaluate *MDM2* expression at the cellular level, we produced monoclonal antibodies against bacterially generated fusion proteins containing residues 27 to 168 of *MDM2*. Of several antibodies tested, mAb IF-2 was the most useful, since it detected *MDM2* in several assays (see "Materials and Methods"). For initial testing, we compared proteins derived from OsA-CL, a sarcoma cell line with *MDM2* amplification but without *p53* mutation (Table 1) and proteins from SW480, a colorectal cancer cell line with *p53* mutation (11) but without *MDM2* amplification (data not shown). Fig. 3 shows that the mAb IF-2 detected an intense M_r 90,000 band plus several other bands of lower molecular weight in OsA-CL extracts, and a much less intense M_r 90,000 band in SW480 extracts. We could not distinguish whether the low-molecular-weight bands in OsA-CL were due to protein degradation or alternative processing of *MDM2* transcripts. The more than 20-fold difference in intensity between the signals observed in OsA-CL and SW480 is consistent with the greater than

20-fold difference in *MDM2* gene copy number in these two lines. Conversely, the M_r 53,000 signal detected with *p53*-specific mAb 1801 was much stronger in SW480 than in OsA-CL, consistent with the presence of a mutated *p53* in SW480 (Fig. 3).

Cells grown on coverslips were then used to assess the cellular localization of the *MDM2* protein. A strong signal, exclusively nuclear, was observed in OsA-CL cells with the IF-2 mAb, and a weaker signal, again strictly nuclear, was observed in SW480 (Fig. 4). The nuclear localization of *MDM2* is consistent with previous studies of mouse cells (17) and the fact that human *MDM2* contains a nuclear localization signal at residues 179 to 186 (8). Reactivity with the *p53*-specific antibody was also confined to the nuclei of these two cell lines (Fig. 4), with the relative intensities consistent with the Western blot results (Fig. 3).

The IF-2 mAb was then used to stain the seven primary sarcomas noted above. The nuclei of two of them (tumors 3 and 10) stained strongly (Fig. 5). Both of these tumors contained *MDM2* gene amplification (Table 1). In the five tumors without amplification, little or no *MDM2* reactivity was observed (example in Fig. 5).

Discussion

The results of this study show that at least two-thirds of the soft tissue sarcomas analyzed contained alterations of *p53* or *MDM2*. Importantly, tumors contained either a *p53* alteration or an *MDM2* alteration, but not both. This distribution was significant ($P < 0.013$, χ^2) and supports the hypothesis that the major effect of *MDM2* amplification is identical to that resulting from *p53* mutation; otherwise, *p53* gene mutations would be expected to occur at equal frequencies in tumors with or without *MDM2* amplification.

This interpretation is consistent with biochemical and physiological data documenting *p53*-*MDM2* interaction. *MDM2* is an oncogene (18, 19) which binds to *p53* *in vivo* and *in vitro* (7, 8). *p53* is thought to function by transcriptionally activating target genes through an acidic activation domain located at codons 20–42 (reviewed in Ref. 20). These target genes contain two copies of a 10-base pair *p53*-specific DNA binding motif within their controlling regions. Overexpression of *MDM2* has been shown to inhibit the ability of *p53* to stimulate expression from such target genes (7, 21). Moreover, it has recently been shown that this inhibition is likely to result from *MDM2* binding directly to the acidic activation domain of *p53*, concealing it from the transcriptional machinery (21). Thus, the biochemical data are in accord with the results presented here; one would expect that either *p53* or *MDM2* would be altered in a given sarcoma but that mutations in both genes would be functionally redundant and should not be observed.

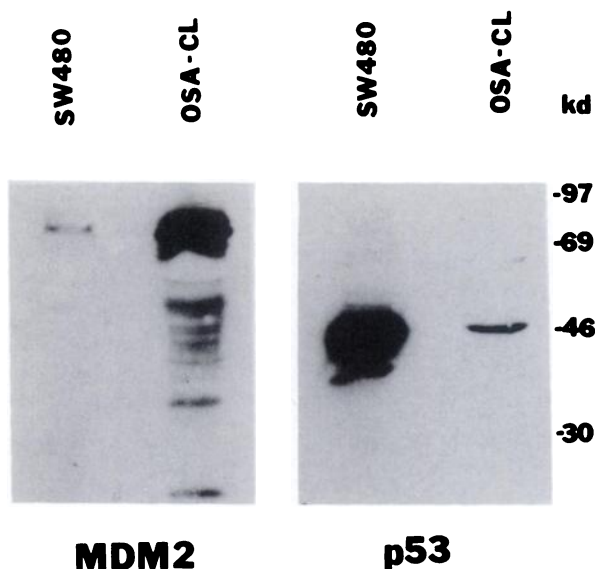


Fig. 3. Western blot analysis using monoclonal antibodies to *MDM2* or *p53*. Fifty μ g of total cellular proteins from OsA-CL or SW480 cells were used for Western blot analysis, as described in "Materials and Methods." The position of molecular weight markers, in kilodaltons, is given on the right.

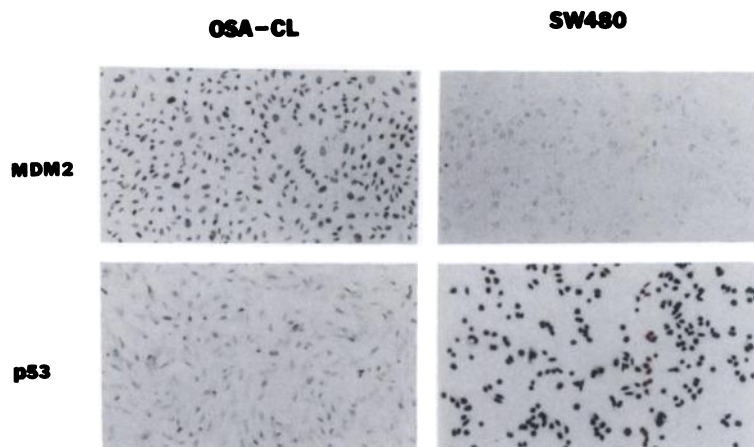


Fig. 4. Immunocytochemical analysis of OsA-CL and SW480 cells grown *in vitro*. Monoclonal antibody IF-2, specific for *MDM2*, and mAb 1801, specific for *p53*, were used as described in "Materials and Methods." The exclusively nuclear localization of both proteins is evident, as is the higher expression of *MDM2* protein in OsA-CL cells than in SW480 cells, the reverse of the pattern observed for *p53*.

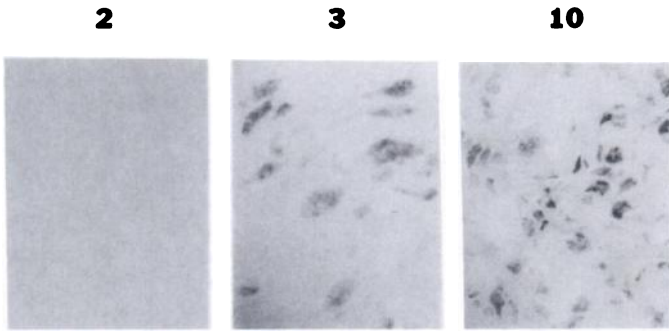


Fig. 5. *MDM2* expression in primary soft tissue sarcomas. Cryostat sections of human sarcomas were incubated with the IF-2 antibody specific for *MDM2* as described in "Materials and Methods." Tumors 3 and 10 showed nuclear expression of *MDM2*, while tumor 2 showed no staining.

The closest analogue to the *MDM2/p53* relationship in sarcomas is provided by *E6/p53* in cervical cancers (22). The E6 oncoprotein encoded by HPV types 16 and 18 can functionally inactivate *p53* (23). Accordingly, it has been reported that cervical cancers with HPV infection infrequently contain *p53* gene mutations (24). However, there have been some exceptions to this paradigm, since tumors containing both HPV sequences and *p53* mutations have been discovered (25). It remains to be seen whether similar exceptions regarding *MDM2* and *p53* will be found as additional sarcomas are analyzed, but the present data suggest with high statistical significance that alterations of these two genes are mutually exclusive.

Finally, what about the soft tissue sarcomas (one-third of the total) without evident *p53* mutations or *MDM2* gene amplification? It is possible that more detailed analyses of such tumors will reveal other alterations of *p53* or *MDM2*, such as point mutations outside exons 5–8 in *p53* or increased expression of *MDM2* in the absence of amplification. Alternatively, some of these tumors might progress through genetic events that involve a totally different pathway. It will be of interest in the future to correlate histopathology, disease course, and response to therapy in sarcomas with and without alterations of *p53* or *MDM2*. Additionally, further examination of such tumors might allow the discovery of genes other than *MDM2* that can functionally inactivate *p53* or its downstream effectors.

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References

1. Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C. C. *p53* mutations in human cancers. *Science* (Washington DC), 253: 49–53, 1991.
2. Toguchida, J., Yamaguchi, T., Ritchie, B., Beauchamp, T. L., Dayton, S. H., Herrera, G. E., Yamamuro, T., Kotoura, Y., Sasaki, M. S., Little, J. B., Weichselbaum, R. R., Ishizaki, K., and Yandell, D. W. Mutation spectrum of the *p53* gene in bone and soft

- tissue sarcomas. *Cancer Res.*, 52: 6194–6199, 1992.
3. Soini, Y., Vahakangas, K., Nuorva, K., Kamel, D., Lane, D. P., and Paakko, P. *p53* immunohistochemistry in malignant fibrous histiocytomas and other mesenchymal tumours. *J. Pathol.*, 168: 29–33, 1992.
4. Ueda, Y., Dockhorn-Dworniczak, B., Blasius, S., Mellin, W., Wuisman, P., Bocker, W., and Roessner, A. Analysis of mutant *p53* protein in osteosarcomas and other malignant and benign lesions of bone. *J. Cancer Res. Clin. Oncol.*, 119: 172–178, 1993.
5. Andreassen, Ø., Ørd, T., Hovig, E., Holm, R., Flørenes, V. A., Nesland, J. M., Myklebost, O., Høie, J., Bruland, Ø. S., Børresen, A.-L., and Fodstad, Ø. *p53* abnormalities in different subtypes of human sarcomas. *Cancer Res.*, 53: 468–471, 1993.
6. Dei Tos, A. P., Doglioni, C., Laurino, L., Barbareschi, M., and Fletcher, C. D. M. *p53* protein expression in non-neoplastic lesions and benign and malignant neoplasms of soft tissue. *Histopathology*, 22: 45–50, 1993.
7. Momand, J., Zambetti, G. P., Olson, D. C., George, D., and Levine, A. J. The *MDM2* oncogene product forms a complex with the *p53* protein and inhibits *p53*-mediated transactivation. *Cell*, 69: 1237–1245, 1992.
8. Oliner, J. D., Kinzler, K. W., Meltzer, P. S., George, D. L., and Vogelstein, B. Amplification of a gene encoding a *p53*-associated protein in human sarcomas. *Nature* (Lond.), 358: 80–83, 1992.
9. Roberts, W. M., Douglass, E. C., Peiper, S. C., Houghton, P. J., and Look, A. T. Amplification of the *gli* gene in childhood sarcomas. *Cancer Res.*, 49: 5407–5413, 1989.
10. Baker, S. J., Preisinger, A. C., Jessup, J. M., Paraskeva, C., Markowitz, S., Willson, J. K. V., Hamilton, S. R., and Vogelstein, B. *p53* gene mutations occur in combination with 17p allelic deletions as late events in colorectal tumorigenesis. *Cancer Res.*, 50: 7717–7720, 1990.
11. Baker, S. J., Markowitz, S., Fearon, E. R., Willson, J. K. V., and Vogelstein, B. Suppression of human colorectal carcinoma cell growth by wild-type *p53*. *Science* (Washington DC), 249: 912–919, 1990.
12. Baker, S. J., Fearon, E. R., Nigro, J. M., Hamilton, S. R., Preisinger, A. C., Jessup, J. M., van Tuinen, P., Ledbetter, D. H., Barker, D. F., Nakamura, Y., White, R., and Vogelstein, B. Chromosome 17 deletions and *p53* gene mutations in colorectal carcinomas. *Science* (Washington DC), 244: 217–221, 1989.
13. Feinberg, A. P., and Vogelstein, B. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.*, 132: 6–13, 1983.
14. McKenzie, S. J., Marks, P. J., Lam, T., Morgan, J., Panicali, D. L., Trimpe, K. L., and Carney, W. P. Generation and characterization of monoclonal antibodies specific for the human *neu* oncogene product, p185. *Oncogene*, 4: 543–548, 1989.
15. Smith, K. J., Johnson, K. A., Bryan, T. M., Hill, D. E., Markowitz, S., Willson, J. K. V., Paraskeva, C., Petersen, G. M., Hamilton, S. R., Vogelstein, B., and Kinzler, K. W. The APC gene product in normal and tumor cells. *Proc. Natl. Acad. Sci. USA*, 90: 2846–2850, 1993.
16. Bodner, S. M., Minna, J. D., Jensen, S. M., Damico, D., Carbone, D., Mitsudomi, T., Fedorko, J., Buchhagen, D. L., Nau, M. M., Gazdar, A. F., and Linnoila, R. I. Expression of mutant *p53* proteins in lung cancer correlates with the class of *p53* gene mutation. *Oncogene*, 7: 743–749, 1992.
17. Barak, Y., Juven, T., Haffner, R., and Oren, M. *MDM2* expression is induced by wild type *p53* activity. *EMBO J.*, 12: 461–468, 1993.
18. Fakhrazadeh, S. S., Trusko, S. P., and George, D. L. Tumorigenic potential associated with enhanced expression of a gene that is amplified in a mouse tumor cell line. *EMBO J.*, 10: 1565–1569, 1991.
19. Finlay, C. A. The *MDM2* oncogene can overcome wild-type *p53* suppression of transformed cell growth. *Mol. Cell. Biol.*, 13: 301–306, 1993.
20. Vogelstein, B., and Kinzler, K. W. *p53* function and dysfunction. *Cell*, 70: 523–526, 1992.
21. Oliner, J. D., Pietenpol, J. A., Thiagalingam, S., Gyuris, J., Kinzler, K. W., and Vogelstein, B. *MDM2* conceals the activation domain of *p53*. *Nature* (Lond.), in press, 1993.
22. Werness, B. A., Levine, A. J., and Howley, P. M. Association of human papillomavirus types 16 and 18 E6 proteins with *p53*. *Science* (Washington DC), 248: 76–79, 1990.
23. Mietz, J. A., Unger, T., Huibregtse, J. M., and Howley, P. M. The transcriptional transactivation function of wild-type-*p53* is inhibited by SV40 large T-antigen and by HPV-16 E6 oncoprotein. *EMBO J.*, 11: 5013–5020, 1992.
24. Crook, T., Farthing, A., and Vousden, K. HPV-16 and cervical intraepithelial neoplasia. *Lancet*, 339: 1231, 1992.
25. Fujita, M., Inoue, M., Tanizawa, O., Iwamoto, S., and Enomoto, T. Alterations of the *p53* gene in human primary cervical carcinoma with and without human papillomavirus infection. *Cancer Res.*, 52: 5323–5328, 1992.