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Short Communication

Alterations in the p53 and MDM-2 Genes Are Infrequent in Clinically Localized, Stage B Prostate Adenocarcinomas

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Alterations in the p53 gene have been described in a variety of human malignant neoplasms. We have examined 29 stage B prostate carcinomas for alterations in the p53 gene and for amplification of the MDM-2 gene. No evidence of mutations in the conserved exons 5 to 8 was found by polymerase chain reaction single-stranded conformation polymorphism analysis and no accumulation of p53 protein was found by immunohistochemistry. However, loss of heterozygosity at the p53 locus was observed in 11% of informative cases. Amplification of the MDM-2 gene was not observed by Southern blot hybridization. In contrast, stage C and D prostate carcinomas showed accumulation of p53 protein in 33 to 66% of cases. We conclude that alterations in p53 function are infrequent in clinically localized prostate cancers but are more common in advanced cancers. (Am J Pathol 1994, 145:287–293)

Alterations in the p53 gene are the most common known genetic change in human malignant neoplasms. Many common and uncommon malignant neoplasms have been found to have genetic alterations at the p53 locus including carcinomas of lung,[1-3] breast,[1,4] esophagus,[5] colon, and a variety of other malignant neoplasms.[6,7] The exact role of the p53 protein in normal and transformed cells is still unclear but it probably acts as a transcription factor.[8] Mutations are the most common alteration seen in the p53 gene and may be either missense, nonsense, or splice site mutations. The missense mutations in exons 5 to 8 usually give rise to abnormally stable proteins that can be detected by immunohistochemistry.[2] Mutation of one allele of the p53 gene is often accompanied by loss of the other allele, ie, loss of heterozygosity (LOH), which would lead to complete loss of normal p53 protein. Several instances of LOH near the p53 locus without evidence of mutation in the p53 gene have been reported[3,4,9] and mutations without LOH have been observed,[9] presumably representing dominant negative mutations. Recently, another mechanism of inactivation of the p53 gene has been reported. The MDM-2 gene is a negative regulator of p53 function and is amplified and overexpressed in 35% of sarcomas,[10] presumably resulting in loss of p53 function. Thus, to evaluate the role of alterations p53 function in a given tumor, a variety of genetic and biochemical changes must be analyzed.

There is evidence that alterations in the p53 gene occur in prostate cancer. Most of these studies have used immunohistochemistry to evaluate the level of p53 protein in prostate carcinomas, because increased p53 protein is correlated with the presence of p53 missense mutations in exons 5 to 8. Visakorpi et al[11] observed that 6% of prostatic carcinomas showed high level staining with p53 antibody, defined as >20% positive nuclei, and 11% had low level stain-

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ing (1 to 20% positive nuclei). High level p53 accumulation was associated with aneuploidy, high tumor grade, high proliferation rate, and poor survival. Mellon et al[12] found that 17% of prostate cancers had positive nuclear staining with p53 antibody, ranging from 0.2 to 26% of nuclei. In contrast, Soini et al[1] found evidence of p53 accumulation in only 6% of prostate adenocarcinomas at radical prostatectomy. Recently, Bookstein et al[13] have found accumulation of p53 protein in 12.7% of prostate cancers analyzed by immunohistochemistry with a significantly higher rate of positivity in high stage tumors compared with localized cancers. However, immunohistochemistry does not detect all alterations that may affect p53 function such as LOH at the p53 locus, nonsense, or splice site mutations or amplification of the MDM-2 gene. In addition, there is some uncertainty about the significance of low level staining of p53 protein by immunohistochemistry.[4] Sequence analysis of prostate cancer cell lines revealed that three of five prostate cancer cell lines contained mutations in the conserved p53 exons 5 to 8 as did one of two primary prostate cancers analyzed.[14] Therefore, to comprehensively examine the role of alterations in the p53 gene function in clinically localized carcinoma of the prostate, we have analyzed 29 prostate carcinomas from patients undergoing radical prostatectomy for stage B prostate cancer for mutations in the conserved exons 5 to 8 of the p53 gene, LOH at the p53 locus, and accumulation of p53 protein as well as amplification of the MDM-2 gene.

Materials and Methods

Human Tumor Samples

Radical prostatectomies from patients with clinical stage B tumors were obtained from two institutions, ie, New York Department of Veterans Affairs Medical Center (NYVAMC) and Presbyterian Hospital. Tissue from NYVAMC was received fresh and normal and neoplastic tissues were divided and snap-frozen in liquid nitrogen. The remaining tissue was then formalin fixed and analyzed pathologically. The frozen tissues were analyzed by frozen section and tissue fragments containing a minimum of 50% carcinoma were used for DNA extraction by standard methods.[15] Control tissue containing no carcinoma on frozen section was also extracted. Tissue from Presbyterian Hospital was obtained from formalin-fixed, paraffin-embedded tissue from radical prostatectomies. Areas with 70% or greater tumor were selected by comparison to stained sections and DNA extracted from five to ten 10-μ sections from that area as described by Wright et al.[16] Control DNA was extracted from adjacent nonneoplastic tissue in a similar manner. Pathological data for the specimens from the two institutions is summarized in Table 1. Stage C and D prostate carcinoma specimens were paraffin-embedded biopsies from the site of local extension or metastasis. Staging was by the system of Whitmore and Jewett.

Single-Stranded Conformation Polymorphism (SSCP) Analysis

DNAs from areas of each tumor containing high percentages of carcinoma were analyzed for mutations in exons 5 to 8 essentially as described by Gaidano et al,[17] except for Presbyterian Hospital samples in which polyacrylamide gel electrophoresis was performed without glycerol for exons 5 and 7. Controls for detection of mutations were the T-ALL cell lines CEM (exons 5 and 7), Jurkitt (exon 6),[18] and the Burkitt's lymphoma cell line BL-60 (exon 8).[17] The majority of samples were analyzed at least twice.

Analysis for LOH

Two polymerase chain reaction (PCR)-based methods were used to detect LOH. All samples were analyzed for the dinucleotide repeat polymorphism p53CA by the method of Jones and Nakamura.[19] The method was validated using the BL-60 cell line and its normal control IARC277.[17] All NYVAMC samples were also analyzed for the codon 72 BSTU-1 restriction site polymorphism by a modification of the method of Meltzer et al.[5] Specifically, the outer primer set described by Meltzer et al[5] was used to conduct PCR reactions using 100 ng of genomic DNA, 20 pmol of each primer, 100 μM of all four deoxynucleotide triphosphates, 1.5 mM $MgCl_2$, manufacturer's reaction buffer (Perkin-Elmer, Norwalk, CT), and 2.5 U Taq polymerase (Perkin-Elmer) in a total volume of 50 μl for 35 cycles of 1 minute, 94 C for 1 minute, 55 C for 1 minute, 72 C. The resulting products were digested with 20 U of the restriction endonuclease BSTU-1 (New England Biolabs, Beverly, MA) for 4 hours at 60 C in the manufacturer's buffer. Digested products were analyzed by gel electrophoresis in 1% agarose/3% NuSieve (FMC Bioproducts, Rockland, ME) gels and stained with ethidium bromide.

Immunohistochemistry

All analysis was performed on representative paraffin-embedded sections containing carcinoma

as described by Greco et al[20] with minor modifications as described below. CM-1, a rabbit polyclonal serum (Novacastra, Newcastle upon Tyne, UK), was used at a dilution of 1:250 to 1:500 with overnight incubation. Aminoethylcarbazole was used for color development. All analyses were performed in parallel with p53-positive colon adenocarcinoma control tissue obtained from the same institution. Samples from the NYVAMC were also routinely treated with the antigen retrieval protocol of Shi et al[21] to enhance staining.

Southern Blotting

Prostate tissues from the NYVAMC containing high percentages of carcinoma were used for Southern blotting. Ten micrograms of genomic high molecular weight DNA was digested to completion with *Eco*RI then electrophoresed on a 1% agarose gel and transferred to nylon membranes. The Southern blot was hybridized with ^{32}P-labeled random-primed probes using standard techniques.[15] The MDM-2 probe consisted of the nucleotides 1–949 of the human MDM-2 cDNA.[10] Control probe was a 600-bp *Eco*RI fragment containing the first and a portion of the second immunoglobulin-like domains of the 3-immunoglobulin domain form of the fibroblast growth factor receptor-1 cDNA.[22] Blots were washed as described previously and autoradiography performed for 3 days at −70 C with intensifying screens.

Results

SSCP Analysis of p53 Exons 5 to 8

A total of 29 cases were analyzed by PCR-SSCP for the presence of mutations in exons 5 to 8 of the p53 gene. An example of the PCR-SSCP analysis of exon 8 for nine tumors is shown in Figure 1. The tumor samples are underexposed in this example because the DNAs are derived from paraffin-embedded tissues and amplify less readily than the control tissues, which were extracted from cell lines. Longer exposures revealed no bands with abnormal migration in the tumor samples. In 25 cases all four exons were examined, whereas in four others exon 5 was not examined due to difficulties in amplification using DNA from paraffin-embedded materials (Table 1). We found no evidence of mutation in any exon in the 112 exons tested. It is unlikely that our inability to detect mobility shifts was due to technical difficulties in the procedure because we routinely detected mobility shifts in controls with known mutations in all four exons. In addition, we were able to detect a mobility shift in exon 7 in one of four glioblastomas analyzed blindly, which is consistent with the known frequency of mutations in exons 4 to 8 in glioblastomas, ie, approximately 40%.[5]

Immunohistochemistry with Antibodies to p53 Protein

All 29 cases were analyzed by immunohistochemistry with the CM-1 rabbit polyclonal antibody to the p53 protein and were negative (Table 1). We have used as our criteria for positive staining 1% or more of the cancer cells showing nuclear and/or cytoplasmic staining. Although in some cases rare cells (<1%) showed nuclear and/or cytoplasmic staining with p53 antibodies, we have considered these cases as negative because we believe such low level staining is either artifactual or if real probably is not related to malignant transformation. This interpretation is supported by the presence of rare cells in the surrounding benign tis-

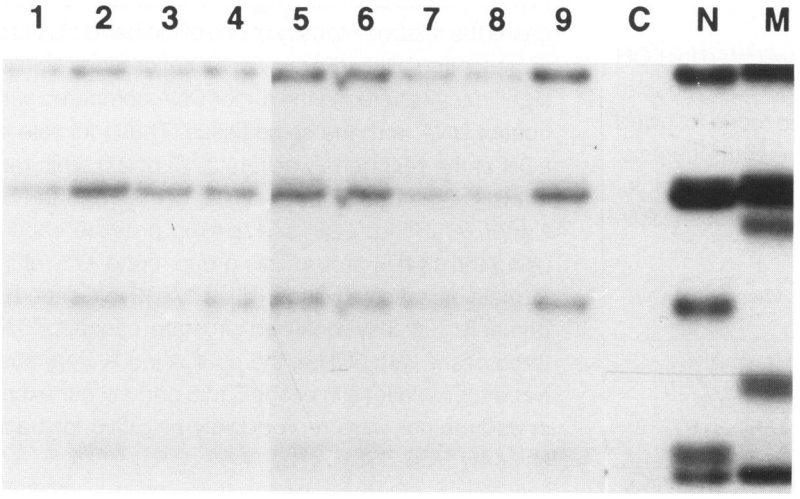

Figure 1. *SSCP analysis of p53 exon. DNA (5 to 50 ng) was amplified by PCR in the presence of ^{32}P-a-dCTP, denatured, electrophoresed on a nondenaturing 8% polyacrylamide gel containing 10% glycerol, and autoradiographed for 20 hours. Lanes 1 to 9 are from prostate cancer microdissected from formalin-fixed, paraffin-embedded tissue blocks. In lane C, H_2O was substituted for DNA in the PCR. Lane N is from the lymphoblastoid cell line NC-3, which contains two wild-type p53 alleles and lane M is from the tumor cell line BL-60, which has lost one p53 allele completely and contains a point mutation in exon 8 of the remaining allele. The fastest migrating band (seen best in lanes N and M) represents residual nondenatured PCR product. The presence of more than two additional bands indicates that one or both strands of the PCR product exhibit multiple conformations under these conditions.*

Table 1. *Summary of Stage B Prostate Cancers*

	New York VAMC (15 cases)	Presbyterian Hospital (14 cases)	Total (29 cases)
(1) Combined Gleason grade (mean)	6.7	6.0	6.4
(2) Seminal vesicle invasion (microscopic)	5/15 (33%)	2/14 (14%)	7/29 (24%)
(3) Gel shift on SSCP analysis			
Exon 5	0/15	0/10*	0/25
Exon 6	0/15	0/14	0/29
Exon 7	0/15	0/14	0/29
Exon 8	0/15	0/14	0/29
(4) Immunohistochemistry fraction staining positive with CM-1 antibody	0/15	0/14	0/29
(5) Loss of heterozygosity			
A. p53CA polymorphism			
Fraction informative	12/15 (80%)	11/13* (85%)	23/28 (82%)
Loss of heterozygosity	0/12 (0)	3/13* (28%)	3/25 (12%)
B. BSTU-1 polymorphism fraction heterozygous	7/15 (47%)	ND†	7/15 (47%)
Loss of heterozygosity	0/7	ND	0/7
C. Fraction with loss of heterozygosity by either method (of informative cases)	0/14	3/13	3/27 (11%)
(6) Amplification of MDM-2 gene	0/11	ND	0/11

* Some cases were not amplified by PCR reactions leading to a denominator less than the total number of cases.
† ND, not determined.

sues staining positively. In addition, such low level staining has been reported in a number of benign tumors[6] and other groups have seen such staining of occasional cells in malignant tumors with no evidence of mutations in the conserved exons 5 to 8.[4] It should be noted that in control colon adenocarcinomas approximately 60 to 80% of nuclei were stained using the CM-1 antibody. In contrast, when biopsies of locally advanced (stage C) or metastatic (stage D) prostate adenocarcinomas were stained with CM-1 antibody 33 to 66% stained positively (Table 2). Although the number of cases is small, this difference was statistically significant (stage B 0% positive *versus* stage C and D 45% positive, $P < 0.001$, Fisher exact test).

LOH at the p53 Locus

Of the 29 prostate cancers 28 were evaluated for LOH at the p53CA locus, a highly polymorphic dinucleotide repeat using PCR to amplify the locus followed by denaturing polyacrylamide gel electrophoresis. Twenty-three of 28 (82%) cases were informative, ie, two alleles could be resolved in normal tissue (Table 1). By mixing experiments we estimate that if LOH is present in 50% of more of the extracted cells we could detect it using this assay (Figure 2,A). In addition, all 15 NYVAMC samples were analyzed using the BSTU-1 polymorphism at codon 72 of the p53 gene using the method of Meltzer et al.[5] Seven of 15 (47%) cases were heterozygous for the BSTU-1 polymorphism (Table 1), very similar to the 52% reported by Meltzer et al.[5] By mixing known homozygotes in varying proportions we estimate that a LOH would be easily visible if 50% of more of extracted cells showed a LOH at this site (data not shown). Two NYVAMC cases that were not informative using the p53CA locus were heterozygous for the BSTU-1 polymorphism. Thus, a total of 27 cases were informative for LOH by one or both methods. We found three cases that showed LOH at the p53CA locus (Figure 2,B). In each of these cases the relative intensity of one of the band clusters, representing either the slow or fast migrating allele, is significantly altered in the tumor DNA compared with control DNA from the same tissue. Thus, our rate of LOH at the p53 gene was 11.1% (3 of 27). This rate of LOH at the p53 locus is similar to that reported by Carter et al[23] for LOH at 17p13 in prostate cancer using the 144D6 probe. This group found 17% of informative cases showing LOH at 17p13 in a series of primarily clinically localized prostate cancers.[23] All three cases with LOH at the p53CA locus were analyzed for mutations in exons 5 to 8 and all four exons in each tumor were reproducibly negative for band shifts by PCR-SSCP.

Table 2. *p53 Immunohistochemistry of Advanced Stage Prostate Cancers*

Stage	IHC$^+$/Total	% Positive
C	1/3	33
D1	2/4	50
D2	4/6	66

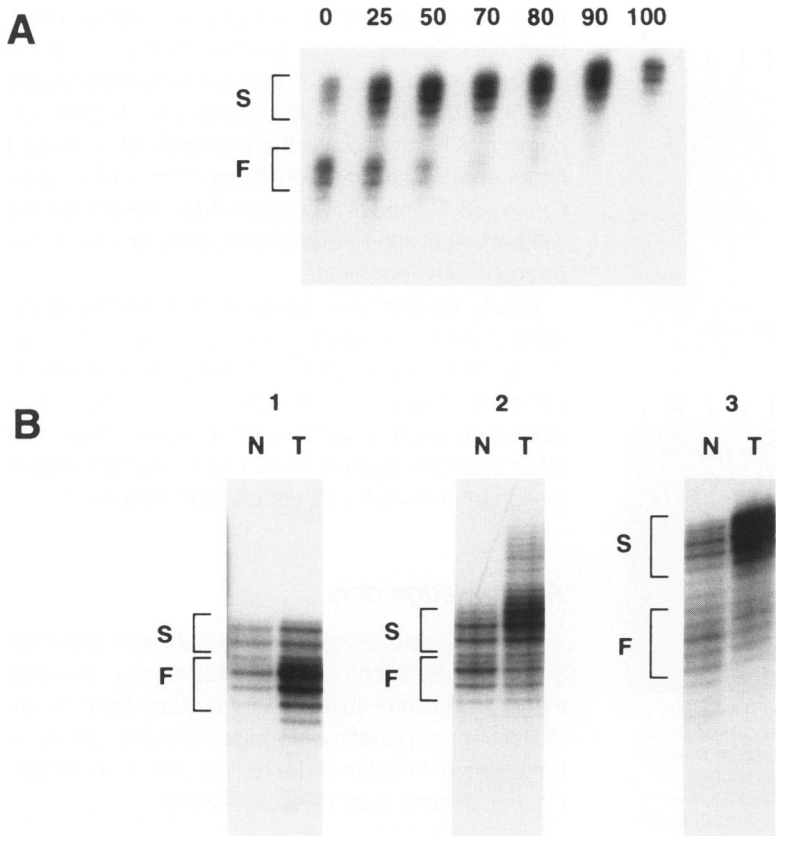

Figure 2. *LOH at the p53 locus. Matched sets of normal and tumor DNA (5 to 50 ng) were amplified by the PCR in the presence of ^{32}P-a-dCTP, denatured, electrophoresed on a denaturing 6% polyacrylamide gel, and autoradiographed for 16 hours.* **A:** *Reconstruction experiment to demonstrate detection sensitivity. DNA from the tumor cell line BL-60 (which contains a single p53 allele) and from a normal lymphoblastoid cell line (IARC277) derived from the same patient were mixed together before the amplification step. The lanes are numbered to indicate the percentage of tumor DNA contained in each sample. Alleles, represented by clusters of bands, are labeled S (for slow migrating) and F (for fast migrating).* **B:** *p53 allele loss in three prostate cancers. Three matched pairs (corresponding to tumors 1 to 3 in Figure 1) of normal (N) and tumor (T) DNA obtained by microdissection from formalin-fixed, paraffin-embedded tissue blocks are shown. In each case two clusters of bands are identified in the normal DNA sample, as per* **A**. *One of these clusters is significantly reduced in relative intensity in each tumor sample.*

Lack of Amplification of the MDM-2 Gene

It has previously been shown that approximately 35% of soft tissue and osteogenic sarcomas show 5- to 50-fold amplification of the MDM-2 gene.[10] This gene appears to negatively regulate the function of the p53 gene[8] so that amplification and subsequent overexpression would result in down-regulation of p53 function. We analyzed 11 stage B prostate carcinomas by Southern blotting of *Eco*RI-digested DNA followed by hybridization with the MDM-2 probe (Figure 3). No evidence of amplification was seen relative to non-neoplastic tissue or control gene fragments of similar size (fibroblast growth factor receptor-1).

Discussion

The data reported here indicate that alterations of p53 function are uncommon in clinically localized prostate cancers. We found no evidence of mutation in exons 5 to 8 by PCR-SSCP. The lack of mutations in exons 5 to 8 is in agreement with our finding of no accumulation of p53 protein by immunohistochemistry because there is a strong correlation between the presence of missense mutations in exons 5 to 8 and p53 protein accumulation.[2] Our results are lower but within the range of the rates of p53 staining in clinically localized prostate cancers in the two largest series reported previously in which similar stage cases can be identified. Bookstein et al[13] report 2 of 36 (6%) stage II tumors showing accumulation of p53 protein and Soini et al[1] found accumulation in only 2 of 34 (6%) prostatic carcinomas removed by radical prostatectomy (presumably for clinically localized disease). Visakorpi et al[11] report p53 accumulation in 4 of 51 (7.8%) T1 and T2 tumors, which would be clinically localized disease assuming no metastasis was present in this subset of tumors. However, the same group has shown that 26% of carcinomas from patients with more advanced local disease (T4) have accumulation of p53 protein, whereas Bookstein et al[13] found p53 accumulation in 22% of stage III and 24% of stage IV carcinomas. This agrees with our finding that a high percentage of locally advanced or metastatic prostatic adenocarcinomas show p53 overexpression on immunohistochemistry. Thus, although it appears that only a relatively small fraction of clinically localized prostate cancers show alterations of p53 function, there is a substantially higher percentage in advanced disease. Whether mutation

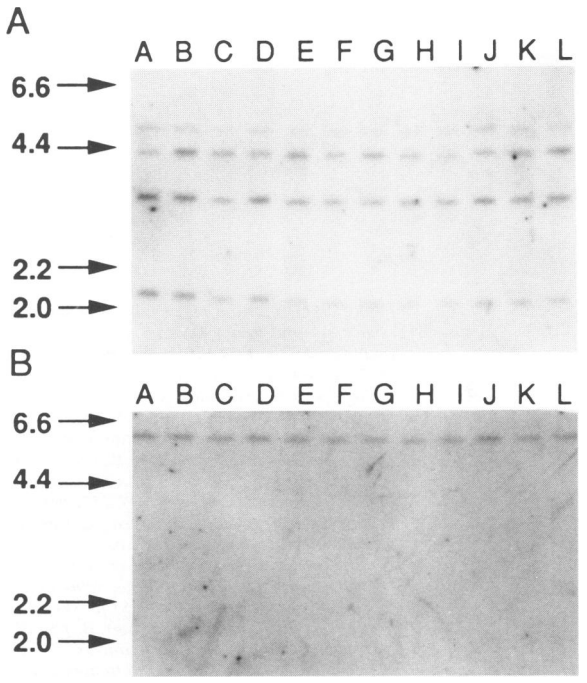

Figure 3. *Lack of amplification of the MDM-2 gene in clinically localized prostate cancers.* A: *The 10 μg of DNA from normal prostate (A) or prostate carcinomas (B-L) was digested with* EcoRI *then electrophoresed, transferred to nylon membranes, and hybridized with a* ^{32}P*-labeled MDM-2 cDNA probe corresponding with nucleotides 1 to 949 (10). After washing autoradiography was performed.* B: *The same filter as in (A) was stripped and rehybridized with a* ^{32}P*-labeled fibroblast growth factor receptor-1 cDNA probe (22) then washed and autoradiography performed.*

in the p53 gene is present initially in such cancers and results in a more aggressive clinical course or is acquired later in the progression of the disease by tumors that do not have p53 mutations initially is not known.

The presence of LOH at the p53 locus without evidence of mutations in the conserved exons is of interest. A similar discordance between the presence of LOH on 17p and accumulation of p53 protein and/or mutations in the conserved exons has been observed in several other malignant neoplasms including carcinomas of the lung,[3] breast,[4] malignant gliomas,[9] and medulloblastomas.[24] There are three possible explanations for the discordance between the 11% rate of LOH at the p53 locus and the lack on any mutations in exons 5 to 8 or accumulation of the p53 protein. First, it is possible that in the cases showing LOH that mutations are present in exons 2 to 4 or 9 to 11, which were not analyzed. Such mutations are often nonsense or splice site mutations and lead to loss of protein expression[2] so that they would not show p53 accumulation by immunohistochemistry. However, such mutations are much less frequent than mutations in exons 5 to 8[7] and it seems unlikely that such mutations would be the only type seen in the p53 gene. The second possible explanation is that a second tumor suppressor gene is present at 17p near the p53 gene so that the p53 gene is deleted secondary to LOH at this locus. Recent studies in soft tissue sarcoma cell lines indicate the presence of a second tumor suppressor gene on chromosome 17 in addition to p53.[25] Third, it is possible that loss of one allele without inactivation of the second allele is sufficient to promote transformation.[8]

Finally, we find no evidence of amplification of the MDM-2 gene in prostate cancers. Oliner et al,[10] although finding a high rate of MDM-2 amplification in sarcomas, found no evidence of amplification in 74 colon and gastric carcinomas. It seems likely that MDM-2 amplification is restricted to distinct types of malignant tumors for as yet unclear reasons.

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