COP1, the Negative Regulator of p53, Is Overexpressed in Breast and Ovarian Adenocarcinomas

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Abstract

The tumor suppressor protein p53 plays a central role in protecting normal cells from undergoing transformation. Thus, it is fitting that cancer cells selectively dampen the p53 response to gain a selective growth advantage. In fact, the p53 gene is the most commonly mutated tumor suppressor gene in human cancers, and if the gene is not mutated, then other components of the p53 pathways are skewed to dampen the p53 response to stress. We recently identified COP1 as a novel and critical negative regulator of p53. COP1 is a RING finger-containing protein that targets p53 for degradation to the proteasome and is necessary for p53 turnover in normal and cancer cells. However, the association between COP1 and cancer remains to be determined. We performed expression analysis of COP1 in ovarian and breast cancer tissue microarrays. COP1 is significantly overexpressed in 81% (25 of 32) of breast and 44% (76 of 171) of ovarian adenocarcinoma as assessed by in situ hybridization and immunohistochemistry. Overexpression of COP1 correlated with a striking decrease in steady state p53 protein levels and attenuation of the downstream target gene, p21, in cancers that retain a wild-type p53 gene status. Overall, these results suggest that overexpression of COP1 contributes to the accelerated degradation of p53 protein in cancers and attenuates the tumor suppressor function of p53.

Introduction

The role of p53 as a classical tumor suppressor has been well established. Biochemically, p53 functions as a stress-activated sequence-specific transcription factor that activates transcription from promoters that harbor a p53 consensus-binding site (1). In addition, p53 also functions as a potent repressor of transcription, thereby adding an additional layer of gene regulation (2). As such, it protects cells from a variety of stress signals such as DNA damage, nucleotide depletion, and oncogene activation to name a few, by activating the transcription of a cadre of genes involved in cell cycle arrest, apoptosis, and DNA repair in addition to repressing genes involved in angiogenesis, antiapoptosis, and cell cycle progression. The physiologic consequence of p53 activation essentially leads to growth arrest, senescence, or apoptosis, thereby preventing cells from replicating a genetically compromised genome.

The high frequency of alterations in the p53 gene or deregulated components of the p53 pathway in human malignancies underscores the importance of p53 integrity to prevent carcinogenesis. For example, in breast tumors the estimated frequency of gene alteration is only 20%, whereas this frequency dramatically increases to >70% in cases of small cell lung carcinomas (3). Within malignancies where the p53 gene is not mutated, other mechanisms may exist to attenuate its function as a tumor suppressor.

p53 is rapidly turned over in unstressed cells by a proteasome-dependent pathway, and this is achieved by substrate recognition for the E3 ligases COP1(4), Pirh2 (5), and MDM2 (6, 7). The MDM2 gene has been shown to be up-regulated in tumors by gene amplification and overexpression. It has been suggested that overexpression of the negative regulator, MDM2, negates the requirement of cells to mutate p53 (8, 9). Surprisingly, the frequency of overexpression and/or amplification of MDM2 is relatively low in various cancers with a wild-type p53 gene status (9, 10), despite MDM2 harboring oncogenic properties (11). These data suggest that other mechanisms might also exist to dampen the p53 response independently of p53 gene mutation or MDM2 amplification or overexpression.

Recently we identified COP1 as a critical negative regulator of p53 (4). Therefore, it is important to examine the relationship between COP1 expression and p53 status in human cancer. Our analysis of both COP1 and p53 at the DNA, mRNA, and protein levels reveals that COP1 is overexpressed in breast and ovarian carcinomas suggesting that it may promote tumorigenesis by inactivating a p53-dependent pathway.

Materials and Methods

p53 Gene Sequencing, Real-Time PCR, and Western Blotting. Tumor samples were isolated and resuspended in lysis buffer [1% SDS, 20 mmol/L Tris (pH 7.5), 2 mmol/L EDTA, and 400 mmol/L NaCl] and were supplemented with 500 µg/mL Proteinase K (Sigma) and incubated overnight at 55°C. DNA was extracted with phenol-chloroform-isooamyl alcohol (25:24:1).

For the paraffin-embedded tissue microarray samples, DNA was extracted by incubating microdissected tumor tissue in 30 µL of PicoPure Proteinase K extraction buffer (Artucus, Mountain View, CA) for 48 hours at 65°C. The digest was heat inactivated at 95°C for 10 minutes and added directly to PCR reactions. Isolated genomic DNA was subject to PCR with the following primers for exons 5 to 8 of the p53 gene incorporating M13-specific sequences: Exon 5R (CAGAAAGACCTATGACGCCCGCTCTGCTCTTCA), Exon 5F (TGTAAACACGCGCGCCATTTACAGTTCTTCCCTTC), Exon 6R (CAGAAAGGCTATGACCTTACCCCTTCCCTCAGA), Exon 6F (TGTAAACACGCGCGCCATTTACAGTTCTTCCCTTC), Exon 7R (CAGAAAGGCTATGACCTTACCCCTTCCCTCAGA), Exon 7F (TGTAAACACGCGCGCCATTTACAGTTCTTCCCTTC), Exon 8R (CAGAAAGGCTATGACCTTACCCCTTCCCTCAGA), Exon 8F (TGTAAACACGCGCGCCATTTACAGTTCTTCCCTTC).

PCR products were sequenced with M13F and M13R sequencing primers.

Real-time PCR was carried out with specific probes for cop1, p21, and rpl19 as described previously (4) from total RNA isolated from normal and tumor samples.

Tissues were harvested in tissue lysis buffer [0.5% NP40, 20 mmol/L Tris (pH 7.5), 5 mmol/L EDTA, and protease inhibitor mix; Roche, Mannheim, Germany] followed by homogenization. Nitrocellulose membranes were probed with antibodies to COP1, p53 (DO-1 and 1801, Santa Cruz Biotechnology, Santa Cruz, CA), and actin (ICN, Irvine, CA).

In situ Hybridization, Northern Blots, and Immunohistochemistry. Iso- topic in situ hybridization was performed on sections of paraffin-embedded...
tissues and tissue microarrays as described previously (12) using a COP-1–specific 688-bp 32P-labeled antisense riboprobe. Mouse Multiple Tissue Northern Blots (Clontech, Palo Alto, CA) were carried out according to the manufacturer’s recommendations with a 32P-labeled murine COP1 cDNA.

For p53 and COP1 staining, Dako Target Retrieval (Dako, Carpenteria, CA; S1700) or High pH target Retrieval (Dako S3308) solution were used according to the manufacturer’s recommendations. Five micrograms per milliliter of anti-p53 (Novus Biologicals, Littleton, CO; NB200-104) or 1 µg/mL anti-COP1 (clone ID5) were used to obtain optimal staining. Slides were then counterstained with Mayer’s hematoxylin, dehydrated, mounted, and coverslipped for bright field viewing.

Results and Discussion

COP1 is well conserved across species. In Arabidopsis, COP1 is essential for photomorphogenesis (13). However, the role of COP1 in mammals is now only beginning to be elucidated (4, 14, 15). To facilitate our understanding of the role of COP1 in mammalian biology, we first performed expression analysis in normal murine tissues by in situ hybridization (Fig. 1A), reverse transcription-PCR (RT-PCR; Fig. 1B), and Northern blot (Fig. 1C). In situ hybridization analyses demonstrated that cop1 was expressed in normal murine skeletal muscle, intestines, and testes. In the testes, prominent expression was evident in the Leydig cells (Fig. 1A, arrows), although moderate signal was also present in the germ cells (Fig. 1A, arrowheads). The Northern blot panel confirmed that cop1 was expressed in the testes as well as the heart, liver, and kidneys (Fig. 1C). The cDNA panel RT-PCR also demonstrated that cop1 was expressed in brain, stomach, small intestine, pancreas, adrenal gland, uterus, and prostate. Notably, the RT-PCR and Northern blot panels were in good agreement for the tissue distribution of the full-length cop1 mRNA expression. In addition to murine tissues, we evaluated human tissue by in situ hybridization and identified cop1 expression in normal human tonsil, spleen, testes, pancreas, and colon (data not shown). This is consistent with previous findings showing prominent cop1 expression by Northern blot in normal human testes, thymus, colon, heart, prostate, spleen, intestine, and liver (14).

With our recent discovery that COP1 is a critical negative regulator of p53 in normal and cancer cell lines (4), we wished to determine whether the COP1 gene expression was altered in cancers. Therefore, total RNA was harvested from various normal and tumor samples, and relative cop1 expression was determined by real-time PCR and normalizing to rpl19 mRNA. Notably, there was a significant increase of 2- to 8-fold in cop1 mRNA over normal controls (Fig. 2A). Therefore, we carried out a larger-scale study using a cop1-specific probe for in situ hybridization on an ovarian tissue microarray composed of 0.6-mm cores of 67 ovarian adenocarcinoma cases in triplicate. Thirty percent (8 of 27) of the cases of serous adenocarcinoma displayed a positive signal only within the malignant cells, whereas no signal was observed within the stromal compartment (Fig. 2B) or normal ovarian tissues (data not shown). Moreover, 16% (3 of 19) cases of endometrioid adenocarcinoma and 27% (3 of 11) cases of clear cell adenocarcinoma displayed a positive signal for mRNA encoding cop1. None of the 10 cases of mucinous adenocarcinoma were positive for cop1 mRNA.

To confirm that COP1 is overexpressed in these tumors at the protein level, immunohistochemistry analysis using a specific antibody to COP1 was done on the same ovarian tissue microarray described above as well as the ovarian samples used for RT-PCR in Fig. 2A. The tissue microarray showed 47% (11 of 27) of serous adenocarcinomas, 63% (12 of 19) of endometrioid adenocarcinomas, 36% (4 of 11) of clear cell adenocarcinomas, and 50% (5 of 10) of mucinous adenocarcinomas displayed a robust COP1 immunoreactivity within the nuclear and cytoplasmic compartments of the malignant cells; however, no signal was detected within the stroma (Fig. 2C). In addition, normal tissues were negative for any reactivity with the COP1 antibody (data not shown). Collectively, these data indicate that COP1 mRNA and protein are specifically overexpressed in the malignant cells. There was relatively good agreement with the in situ hybridization and immunohistochemistry data (data not shown); however, endometrioid and mucinous adenocarcinomas displayed a higher percentage of COP1-positive samples by immunohistochemistry relative to the in situ hybridization data, suggesting post-transcriptional regulation of COP1.

Next we wished to determine whether any of the tumor samples that overexpress COP1 harbored a defect in a p53-dependent response. To
address this question we carried out real-time PCR on the ovarian tumors that overexpressed cop1 (Fig. 2A) using specific probes to the p53 target gene and cyclin-dependent kinase inhibitor, p21/WAF1, with fold-change in mRNA assessed by comparing to normal control samples. Interestingly, the majority of the samples that overexpressed cop1 showed a significant decrease in p21 mRNA over matched normal controls (Fig. 2D). These results are consistent with COP1 negatively regulating the ability of p53 to activate transcription of p21 (4). Given that p53 can be mutated in ovarian cancers, it is important to determine the p53 gene status in these samples, because specific mutants of p53 lose their inherent ability to transactivate from the p21 promoter. For example, if p53 is mutated in the samples where COP1 is overexpressed then the down-regulation of p21 mRNA observed might be independent of p53. Therefore, we sequenced exons 5 to 8, as well as the intron-exon boundaries, where p53 mutation is frequently identified, and discovered that 7 of 8 of these samples were wild-type, and 1 of 8 had a R249S mutation, which is located in the p53 DNA binding region and has been shown to immortalize normal mammary epithelial cells (16). Moreover, the R249S mutant ovarian sample (number 8) had normal levels of p21 mRNA (Fig. 2D), consistent with the hypothesis that COP1 is repressing wild-type p53-dependent transcription.

We additionally expanded our studies to breast tumors. Using the COP1-specific antibody, immunohistochemistry was carried out on a tissue microarray featuring 32 cases of breast adenocarcinomas arrayed in 1-mm cores. Strikingly, 81% (25 of 32) of cases displayed strong immunoreactivity with the COP1 antibody (Fig. 3, A and B) exclusively in the malignant cells but not within the stroma or normal epithelial cells. Immunohistochemistry analysis using the 1801 p53-specific antibody, which is not affected by protein phosphorylation (17), revealed only 3 of 32 cases with a positive signal (Fig. 3, E and F). In contrast, most cases that are positive for COP1 are negative for p53 (Fig. 3, C and D). Given that p53 is difficult to detect in tissues by immunohistochemistry unless stabilized by DNA-damaging agents or gene mutation, the p53 gene from the breast tissue microarray was sequenced to confirm that these particular samples were indeed mutant p53. The mutations identified in each of the three cases resulted in amino acid substitutions as follows: C242F, P278S, and R175H.

To compare the steady state protein levels of p53 and COP1 in breast cancer samples, we performed Western blot analysis of tumor lysates (Fig. 4). Immunoblotting with anti-COP1 revealed a very weak signal in the normal breast tissue (sample N), but a significant increase in COP1 was detected in 67% (10 of 15) of cases, confirming the previous observations with the breast tissue microarray (Fig. 3, A and B) that COP1 is indeed overexpressed in breast adenocarcinomas. The p53 levels were reduced in 53% (8 of 15) but significantly increased in 20% (3 of 15) of the cases when compared with normal breast tissue. To additionally elucidate the consequence of COP1 overexpression on p53, it was necessary to determine the p53 gene status within these samples, because overexpression of COP1 is more likely to have an effect on wild-type p53 function and stability rather than mutant p53. Therefore, exons 5 to 8 were sequenced and analyzed for mutations at the intron-exon boundaries as well as that of the exons. Twenty seven percent (4 of 16) of cases harbored a mutation within the exon 8 (Fig. 4B); sample T6 and T12 contained the R290H mutation, whereas samples T7 and T10 harbored the R273H mutation. Of particular interest in these data were the steady-state protein levels of p53 when COP1 was overexpressed in the breast tumors; in 75% (6 of 8) of the cases where wild-type p53 levels were significantly reduced, COP1 was overexpressed. Only 25% (2 of 8) of cases displayed a reduction in p53 levels, presumably through a COP1-independent mechanism. Where COP1 was overexpressed and no concomitant decrease in p53 levels were observed, the p53 gene status was in fact mutant thereby indicating that COP1 can negatively regulate wild-type p53.

Fig. 2. COP1 overexpression in ovarian adenocarcinomas. A, real-time PCR analysis of cop1 mRNA from ovarian tumors. RNA was prepared from tumor samples with matched normal controls and subject to real-time PCR Taqman analysis. Data are represented as fold increase over matched normal cop1 mRNA and were normalized to RPL19 mRNA. B, overexpression of cop1 mRNA by in situ hybridization. Bright-field (left) and dark-field illumination (right) shows cop1 expression in neoplastic epithelial cells but not associated stroma; normal ovarian tissue was negative. Inset, ×400 magnification demonstrates silver grains over neoplastic epithelial cells. C, overexpression of COP1 at the protein level in ovarian adenocarcinomas. The same case of ovarian adenocarcinoma as in B demonstrates COP1 immunoreactivity in the cytoplasm and nucleus of neoplastic epithelial cells (inset, ×400 magnification) but not associated stroma. D, p53 gene status in ovarian tumors and correlation of COP1 overexpression with decrease in p21 mRNA. DNA was extracted from the samples in A and subject to PCR of exons 5 to 8 of the p53 gene with the products being analyzed by DNA sequencing and designated as wild-type (wt) or mutant (mu). The graph below the p53 gene status shows the same samples from A that overexpressed COP1 also had decreased p21 mRNA. The relative levels of p21 mRNA were determined by real-time PCR as in A and normalized to RPL19 mRNA. Data are represented as fold decrease in p21 message; bars, ±SD.

4 D. Dorman and T. Hupp, unpublished observations.
The proto-oncogene and negative regulator of p53, MDM2, is amplified in cancers such as breast carcinomas (18), astrocytomas, glioblastomas (19), and fibrosarcomas, albeit at a low frequency. It has been suggested that MDM2 can substitute the need for p53 gene mutation (8); however, there exists other negative regulators of p53 in cancers, because there have been many cases harboring a wild-type p53 gene without any evidence of MDM2 amplification (8). Furthermore, MDM2 is very rarely amplified in ovarian cancers, and many ovarian tumors carry a wild-type p53 gene (20).

The results herein demonstrate that at least 45% of ovarian adenocarcinomas and 80% of breast adenocarcinomas analyzed in this study had robust overexpression of COP1. Importantly, a defect in p53 function or steady-state protein level was evident in samples that contained a wild-type p53 gene and overexpressed COP1 but not in samples that contained mutant p53 and overexpressed COP1 (Figs. 2 and 4). Overexpression of COP1 was detected predominantly but not exclusively in wild-type p53-containing cancers indicating that one of the major roles of COP1 is to repress p53-dependent tumor suppression. In the few cases where COP1 is overexpressed and p53 is mutated, it is feasible that COP1 may have p53-independent functions or that COP1 overexpression alone at the early stages of cancer was not sufficient to drive malignancy in those particular cancers and, hence, selected for mutation of p53.

In summary, we have shown that COP1 is overexpressed in breast and ovarian cancers, and this is concomitant with a decrease in wild-type p53 steady-state protein levels or p53-dependent transcrip-
Our studies strongly suggest that COP1 may function as an oncogene and promote tumorigenesis, especially for tumors in which p53 is not mutated.

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References

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