

PDRG1, a novel tumor marker for multiple malignancies that is selectively regulated by genotoxic stress

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Key words: PDRG1, tumor marker, p53, genotoxic stress, DNA damage

Abbreviations: PDRG1, p53 and DNA damage-regulated gene 1; UV, ultraviolet; PDCD7, programmed cell death 7; MAP1S, microtubules-associated protein 1S; HEK293T, human embryonic kidney 293T cells

We have previously cloned and characterized a novel p53 and DNA damage-regulated gene named PDRG1. PDRG1 was found to be differentially regulated by ultraviolet (UV) radiation and p53. In this study, we further investigated stress regulation of PDRG1 and found it to be selectively regulated by agents that induce genotoxic stress (DNA damage). Using cancer profiling arrays, we also investigated PDRG1 expression in matching normal and tumor samples representing various malignancies and found its expression to be upregulated in multiple malignancies including cancers of the colon, rectum, ovary, lung, stomach, breast and uterus when compared to their respective matched normal tissues. Western blot and immunohistochemical analyses were also performed on select specimen sets of colon cancers and matching normal tissues and the results also indicated PDRG1 overexpression in tumors relative to normal tissues. To gain insight into the function of PDRG1, we performed PDRG1 knockdown in human colon cancer cells and found its depletion to result in marked slowdown of tumor cell growth. These results suggest that PDRG1 may be linked to cell growth regulation. Yeast two-hybrid screening also led to the identification of PDCD7, CIZ1 and MAP1S as PDRG1-interacting proteins that are involved in apoptosis and cell cycle regulation which further implicate PDRG1 in controlling cell growth regulation. Taken together, our results indicate that PDRG1 expression is increased in multiple human malignancies suggesting it to be a high-value novel tumor marker that could play a role in cancer development and/or progression.

Introduction

Tumorigenesis is a multistep process that involves abnormalities in various oncogenes, tumor suppressor genes, growth factors and their receptors.¹ Although significant progress has been made in recent years to better understand the molecular pathogenesis of human malignancies, the exact molecular mechanisms are still unclear. Identification of novel molecular markers, particularly those exhibiting altered expression during the progressive transition from normal to premalignant to malignant stages, is likely to reveal important insights into these mechanisms and facilitate the development of clinical assays capable of detecting the malignant potential of premalignant lesions.

We have previously reported the identification and initial characterization of a novel p53 and DNA damage-regulated gene 1 (PDRG1).² PDRG1 was found to be differentially regulated by ultraviolet (UV) radiation and p53. Interestingly, PDRG1 mRNA was upregulated by ultraviolet radiation (UV), but downregulated by tumor suppressor p53. We have also noted that p53-null cells constitutively express higher levels of PDRG1 and

that p53 downregulated PDRG1 gene promoter region suggesting that p53 negatively regulated PDRG1 at the transcriptional level.²

Signaling molecules involved in the DNA damage response are important candidates for tumor marker studies because DNA damage response and cancer development are closely linked and DNA, the carrier of genetic information, appears to be the most precious possession of a cell. However, DNA is vulnerable to damage caused by a variety of toxic insults including those mediated by intrinsic and extrinsic genotoxic agents. Cellular response to DNA damage (genotoxic stress) is complex and involves a variety of molecules.³ Defects in DNA response pathway could result in accumulation of mutations leading to genomic instability which may be one of the underlying causes of cancer development.

p53 is believed to play a key role in guarding the genome. To prevent the potential deleterious consequences of DNA damage, p53 serves as a signal-converging node for signals emanating from damage sensors and directing cells to either undergo growth arrest to repair the damaged-DNA or commit suicide when the

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Submitted: 10/12/10; Revised: 12/05/10; Accepted: 12/06/10
DOI: 10.4161/cbt.11.6.14412

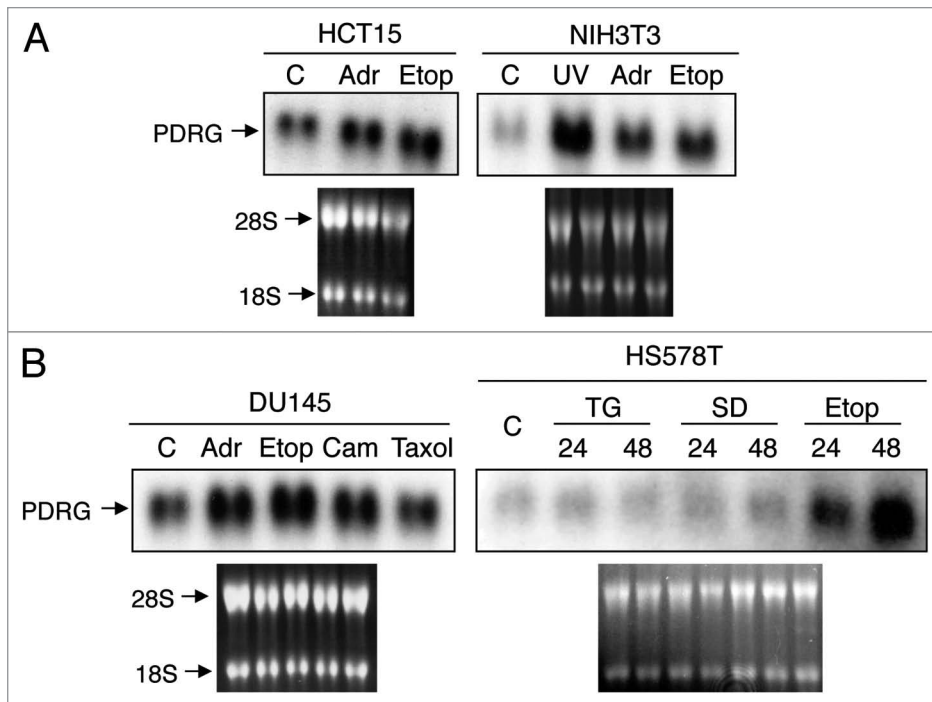


Figure 1. PDRG1 expression is selectively upregulated by genotoxic stress. (A) HCT15 and NIH3T3 cells were untreated as control (C) or treated with 2 μ M adriamycin (Adr), 30 μ M etoposide (Etop) or 20 J/m² ultraviolet radiation (UV) for 24 hours. Total RNA preparation and northern blot analyses were done as described in Material and Methods. A human PDRG1 cDNA probe was used for the blot containing RNA from HCT15 cells, and a mouse PDRG1 cDNA probe was used for the blot containing RNA from NIH3T3 cells. The integrity of total RNA and comparable loading are shown by ethidium bromide staining of the gel prior to transfer. (B) DU145 and HS578T cells were untreated as control (C) or treated with adriamycin (Adr), etoposide (Etop), camptothecin (Cam), Taxol, thapsigargin (TG) or sulindac sulfide (SD) and then harvested after 24 hours or at indicated time points. Total RNA was extracted and northern blot analyses of PDRG1 mRNA levels were done using human PDRG1 cDNA probe as described in Material and Methods. Ethidium bromide staining of the gel shows integrity of RNA and comparable loading.

damage is beyond repair.⁴ Loss of p53 function would leave the cells vulnerable to genetic damage and subsequently cancer development. Indeed, p53 inactivation is associated with a wide variety of human cancers.

The function of PDRG1 remains to be elucidated and because PDRG1 is regulated by UV and p53, it is possible that it could be linked to regulating cellular stress response and cancer development and progress. We undertook this study to gain further insight into the function of PDRG1 and our results indicate that it is selectively regulated by agents that induce genotoxic stress. Our results further indicate that PDRG1 is overexpressed in multiple human malignancies and that knockdown of PDRG1 in human colon cancer cells affects their growth. Yeast two-hybrid screening also led us to identify PDCD7, CIZ1 and MAP1S as PDRG1-interacting proteins that are involved in apoptosis and cell cycle regulation which further support the role of PDRG1 in controlling cell growth regulation. Collectively, our results indicate that PDRG1 expression is increased in multiple human malignancies suggesting it to be a high-value novel tumor marker that could play a role in cancer development and/or progression.

during progression from adenoma to carcinoma of the colon.¹³ Additionally, frequency of amplification in this region has been reported to further increase in the metastatic lesions of colorectal cancers.¹⁴ Thus, PDRG1 resides in the cytogenetic region that is frequently amplified in human colorectal cancers and perhaps in other types of malignancies. Furthermore, PDRG1 is transcriptionally downregulated by p53 and p53-null cells constitutively express higher levels of PDRG1.²

It is now well-established that p53 is frequently inactive in a variety of human malignancies including colorectal cancers. Therefore, we sought to investigate the expression of PDRG1 in primary human malignancies and to that end, analyzed the expression pattern of PDRG1 in primary tumors and matching normal tissues using commercially available cancer profiling arrays. As shown in **Figure 2**, PDRG1 expression was indeed upregulated in a variety of human cancer tissues when compared with matching normal tissues. The overall results are summarized in **Table 1** and indicate that PDRG1 expression is upregulated in tumors of the colon (82%), rectum (78%), ovary (71%), lung (70%), stomach (68%), breast (63%) and uterus (62%) when compared with their matching normal tissues.

Results

We have previously reported that PDRG1 mRNA was upregulated by UV.² Because UV is a potent genotoxic stress inducing agent, next we sought to investigate PDRG1 regulation in cellular stress response to genotoxic and non-genotoxic agents. For this purpose, we used adriamycin (doxorubicin), etoposide, camptothecin and UV as genotoxic agents while paclitaxel (taxol), thapsigargin and sulindac sulfide as non-genotoxic agents. As shown in **Figure 1**, in multiple cell lines, genotoxic stress inducing drugs upregulated PDRG1 expression whereas non-genotoxic stress inducing agents did not. These results, therefore indicate that PDRG1 is selectively regulated in response to genotoxic stress.

We have found that PDRG1 resides at the long arm of chromosome 20 in the cytogenetic region 20q11.2. A large body of evidence indicates high-frequency chromosome gains of 20q in cancers of the breast, lung, liver, colon and rectum.⁵⁻⁹ Furthermore, colorectal cancer patients with 20q gains have a significantly short survival time.¹⁰ More recent evidence indicates that the cytogenetic region 20q11.2–20q13.2 is frequently amplified in human colorectal cancers.^{11,12} Evidence also suggests that this region is specifically amplified

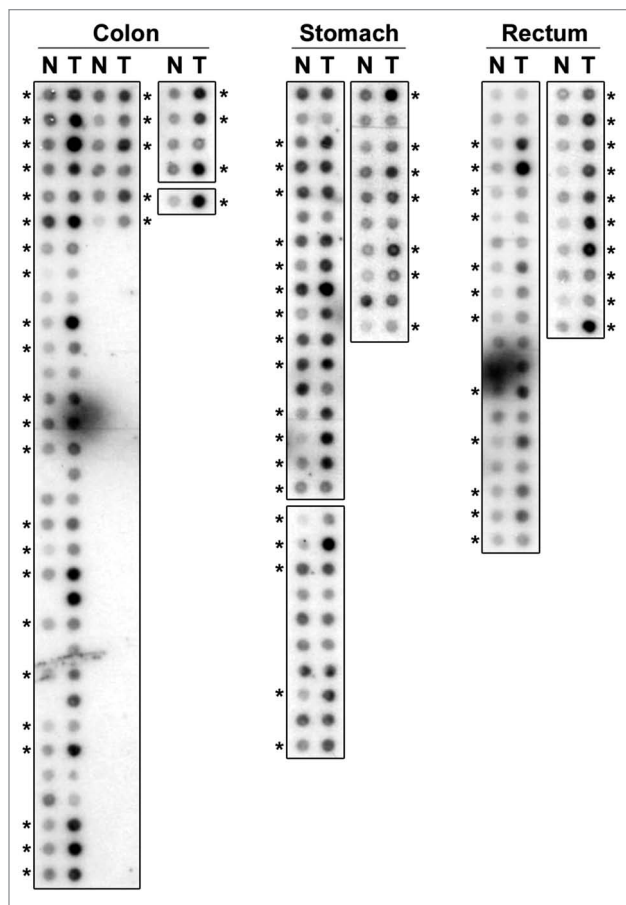


Figure 2. PDRG mRNA is overexpressed in human malignancies. Representative results showing PDRG expression in primary gastrointestinal tract tissue specimens. RNA samples representing matching normal (N) and tumor (T) specimens from colon, stomach and rectum were probed with PDRG cDNA probe as described in Material and Methods. Each pair indicates normal and tumor samples from a single patient. *Indicates higher PDRG mRNA level in tumor sample than that in the matching normal tissue sample.

Thus, PDRG1 expression was highly elevated in various human malignancies, a finding which implicated its role in cancer development and/or progression. The clinicopathologic features of the tumors exhibiting PDRG1 upregulation are presented in **Supplemental Tables 1–7**.

Our preceding results show that PDRG1 expression was increased in multiple human malignancies at the mRNA levels. Next, we sought to validate these results at the protein levels and to that end we selected human colon cancer as a representative malignancy.

First, we used seven matched pairs of normal and colon cancer tissues and performed Western blot analysis to investigate PDRG1 expression pattern at the protein level. Consistent with our results at the mRNA levels, PDRG1 protein levels were significantly higher in all seven cancer samples when compared to the matching normal tissues (**Fig. 3**). We also performed immunohistochemical analysis to investigate the level and distribution of PDRG1 in tumors and normal tissues using specimen sections of matched colorectal normal and tumor samples. As shown in

Table 1. PDRG expression in primary tumors

Primary site	Total # of patients	PDRG mRNA levels in tumors
Colon	39	Increased, 32/39 = 82%
Rectum	28	Increased, 22/28 = 78%
Ovary	24	Increased, 17/24 = 71%
Lung	27	Increased, 19/27 = 70%
Stomach	37	Increased, 25/37 = 68%
Breast	56	Increased, 35/56 = 63%
Uterus	47	Increased, 29/47 = 62%

Figure 4, PDRG1 was predominantly expressed by epithelial cells and the tumor sample exhibited a significant increase in PDRG-specific staining compared to the matching normal tissue.

Our results thus far indicated PDRG1 to be overexpressed in human malignancies, a finding which could link PDRG1 to cancer development and/or progression. To gain some insight into this issue, we also investigated the effect of PDRG1 depletion on human cancer cell growth. To that end, shRNA approach was employed to knockdown the expression of PDRG1 in RKO human colon cancer cells and cell proliferation was analyzed by MTT assays. Results in **Figure 5** indicate that two different PDRG1 shRNAs which targeted different regions of the PDRG mRNAs efficiently depleted the expression of PDRG. Next, we analyzed the growth pattern of PDRG1-proficient and -deficient cells and as shown in **Figure 5**, PDRG1 depletion significantly affected cell growth, a finding that could link PDRG1 to modulating cell growth regulation.

Our preceding results indicated that PDRG1 was overexpressed in human malignancies, and that depletion of PDRG1 slowed down tumor cell growth. To gain further insight into the potential mechanisms by which PDRG1 could play such a role, we performed a yeast two-hybrid screening to identify the interaction partners of PDRG1. This screening led us to identify three proteins including PDCD7 (programmed cell death 7), CIZ1 (Cip1 interacting zinc finger) and MAP1S (microtubules-associated protein 1S). PDCD7 has been implicated in modulating apoptosis and also reported as a component of U12-type spliceosome.¹⁵ CIZ1, via its interaction with p21, is believed to modulate cell cycle progression by shuttling p21 and itself between nucleus and cytoplasm.¹⁶ It has also been reported that CIZ1 promotes mammalian DNA replication and thus, plays a role in S-phase progression of the cell cycle.¹⁷ MAP1S, on other hand, is a microtubule-binding protein that has been shown to interact with RASSF1A tumor suppressor protein to modulate cell cycle progression.^{18,19}

Using PDCD7 as a representative protein, we also performed co-immunoprecipitation assay to investigate its interactions with PDRG1 in mammalian cells. For that purpose, HEK-293T cells were transiently co-transfected with HA-tagged PDRG1 expression vector along with myc-tagged PDCD7 or tag-alone control vector and cell lysates were then analyzed by protein co-immunoprecipitation assays. As shown in **Figure 6**, myc-tagged PDCD7, but not the myc-tag alone, co-immunoprecipitated with HA-tagged PDRG1 indicating that PDRG1 specifically interacted with PDCD7 in 293T cells.

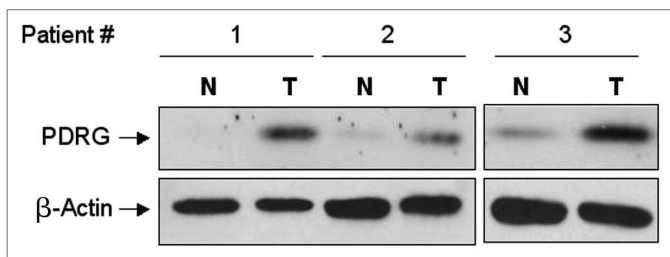


Figure 3. PDRG1 protein is overexpressed in human colon cancer. Representative western blots showing PDRG expression in matching normal and tumor tissues. Protein samples prepared from primary matching normal (N) and tumor (T) tissues were subjected to western blotting using anti-PDRG antibody. Same blots were subsequently probed with anti-β-actin antibody to show loading in each lane.

Discussion

In the current study, we have found that PDRG1 is selectively regulated in response to genotoxic stress. It is interesting that genotoxic stress upregulates while p53 downregulates PDRG1 expression. Because genotoxic stress regulation of PDRG1 occurs

in both p53-positive and -negative cells, it is likely that genotoxic stress-mediated upregulation of PDRG1 occurs in a p53-independent manner. Furthermore, PDRG1 gene promoter harbors Oct1-binding sites and Oct1 strongly upregulates PDRG1 expression.² Oct1 is positively regulated in response to genotoxic stress and therefore, it is possible that, following genotoxic stress, strong positive regulation by Oct1 could override the negative effect of p53 on PDRG1 in p53 wild-type background. We have also found that PDRG1 is overexpressed in multiple human malignancies and that knockdown of PDRG1 in human colon cancer cells affects their growth.

Yeast two-hybrid screening also led us to identify PDCD7, CIZ1 and MAP1S as PDRG1-interacting proteins that are involved in apoptosis and cell cycle regulation, which further support the role of PDRG1 in controlling cell growth regulation. Together these results suggest PDRG1 to be a high-value novel tumor marker that could play a role in cancer development and/or progression. Our finding that knockdown of PDRG1 in human colon cancer cells affects their growth highlights its potential role in modulating cancer cell growth. Previously, we had found that overexpression of PDRG1 in HCT116 human colon cancer cells decreases clonogenic survival in response to UV irradiation.² Such an outcome

could occur due to overexpression, which may not be perceived as a very physiologically-relevant approach to evaluate PDRG's role in cellular sensitivity to DNA damage. Further in-depth studies are, therefore, needed to explore how PDRG1 depletion would affect cellular sensitivity to genotoxic stress.

In a recent study that used mass spectrometry analysis, PDRG1 was identified as a subunit of a prefoldin complex.²⁰ Prefoldin is a heterohexameric molecular chaperone complex that binds to nascent unfolded polypeptides and then delivers them to a group II chaperonin for proper folding.²⁰ Tubulin and actin are its best known substrates.²¹ MAP1S, the PDRG1 binding partner identified in our yeast two-hybrid screening, is also a microtubule-associated protein.^{18,22} Together, this information would suggest that PDRG1 might be involved in the regulation of microtubules. Microtubule dynamics are essential to a number of cellular functions including cell cycle progression. Studies on the potential involvement of PDRG1 in modulating microtubule dynamics would likely shed more light on PDRG's cellular functions and its role in cell growth control.

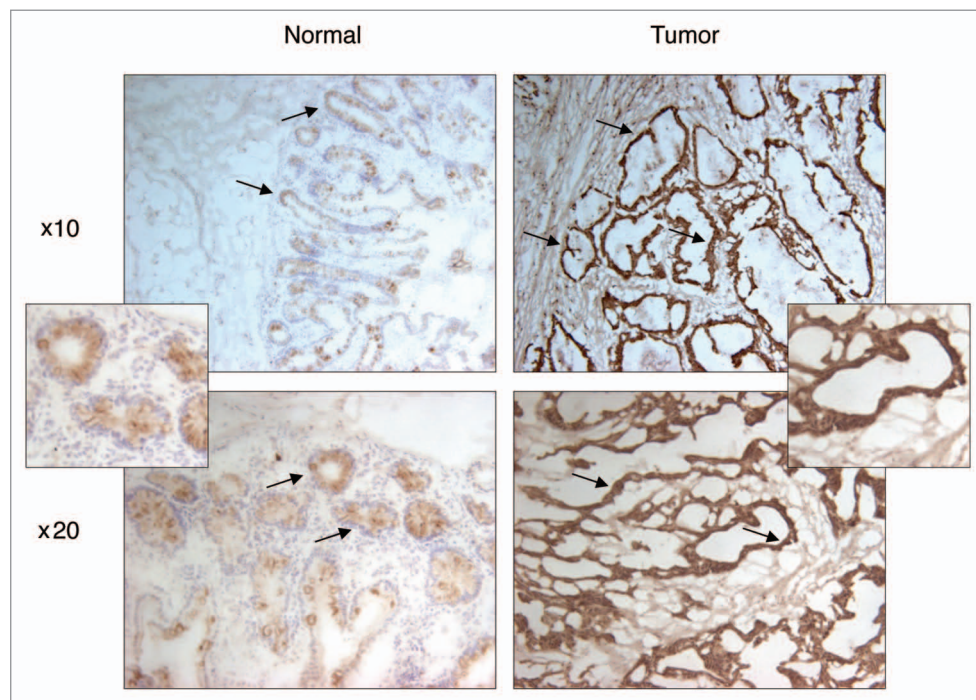


Figure 4. Immunohistochemical staining of PDRG in matched normal and cancer tissues from colon cancer patients. Frozen primary colon normal and tumor tissues from same patient were sectioned (6 μm) then fixed with 4% paraformaldehyde for 30 min. Endogenous peroxidase was quenched with hydrogen peroxide and the sections were then blocked in PBS with 10% goat sera for 20 min and incubated sequentially with anti-PDRG antibody for 1 hour, biotinylated anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA) for 45 min and ABC reagent (Vector laboratories) for 30 min. After several washes, the sections were then incubated with peroxidase substrate DAB solution (Vector laboratories) and counterstained with hematoxylin. Brown color indicates the PDRG-specific signal. Photomicrographs from different regions captured at x10 and x20 magnifications are shown. Please note that PDRG1 is predominantly expressed by the epithelial cells as indicated by arrows and the tumor sample exhibits very strong PDRG-specific staining than the matching normal tissue. Insets show sections of enlarged images from x20 photomicrographs.

The molecular basis for PDRG1 overexpression in human malignancies remains to be elucidated. We have found that PDRG1 resides at the long arm of chromosome 20 in the cytogenetic region 20q11.2. This region is frequently amplified in human colorectal cancers and perhaps in other types of malignancies.⁵⁻⁹ It is possible that increased expression could result due to PDRG1 gene amplification. We have previously reported that p53-null cells constitutively express higher levels of PDRG1.² Because p53 negatively regulates PDRG1 expression and the fact that p53 is inactive in a large number of various human tumors, it is also possible that PDRG1 expression is increased as a consequence of p53 inactivation due to either p53 mutation or p53 gene deletion.

As shown in current study, PDRG1 is highly expressed in multiple human malignancies highlighting its value as a novel tumor marker. Our current findings have laid the groundwork for more in-depth studies to further investigate the exact function of PDRG1 and firmly establish its potential as a high-value tumor marker.

Material and Method

Cell lines and culture conditions. Human cancer cell lines HCT15 and RKO (colon), DU145 (prostate), HS578T (breast) as well as HEK-293T (human embryonic kidney cells), were regularly maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gemini Bioproducts Inc., Calabasas, CA). Mouse fibroblast NIH3T3 cells were maintained in DMEM (Cellgro, Mediatech, Herndon, VA) supplemented with 10% calf serum (Invitrogen, Grand Island, NY).

Plasmids. For yeast two-hybrid screening, we generated PDRG1 and Gal4 DNA binding domain (DNA-BD) fusion protein, the open reading frame (ORF) of PDRG1 was first amplified using primers: 5'-GGA ATT CCA TAT GCT ATC ACC CGA GGC AGA-3' and 5'-CGC GGA TCC TCA TCC TTT CAA GAT GAC CTT GAG A-3', then digested with Nde I and BamH I and subcloned in frame at carboxal terminal of Gal4 DNA binding domain (DNA-BD) in pGBKT7 vector (a kind gift from Dr. Friedman, SUNY Upstate Medical University). The resulting plasmid was named pBD-PDRG1 and was sequenced to confirm the in-frame fusion of PDRG1 and DNA-BD. HA-tagged PDRG1 expression vector (pCEP-PDRG) was generated by subcloning of PDRG1 whole ORF into the mammalian expression vector pCEP4 as described previously in reference 2. The full-length PDCD7 ORF was generated using two ESTs (BU855083 and BC016992, ATCC, Manassas, VA) that contain overlapped partial PDCD7 ORF. 5' part of PDCD7 ORF was amplified by primers: 5'-CCG CTC GAG CCA CCA TGG CCC TGC CAC CAT TCT T-3' and 5'-AGG GCT CTC TCT TCA GCT TC-3' using EST BU855083 as template. 3' part of PDCD7 ORF was amplified by primers: 5'-TGT CCT CCA GCC TCA GCA GA-3' and 5'-CCG GAA TTC AAT GCA GCT TAA CAG CAG TTG-3' using EST BC016992 as template. The amplified 5' part of PDCD7 ORF was digested with Xho I and BsmA I, and the

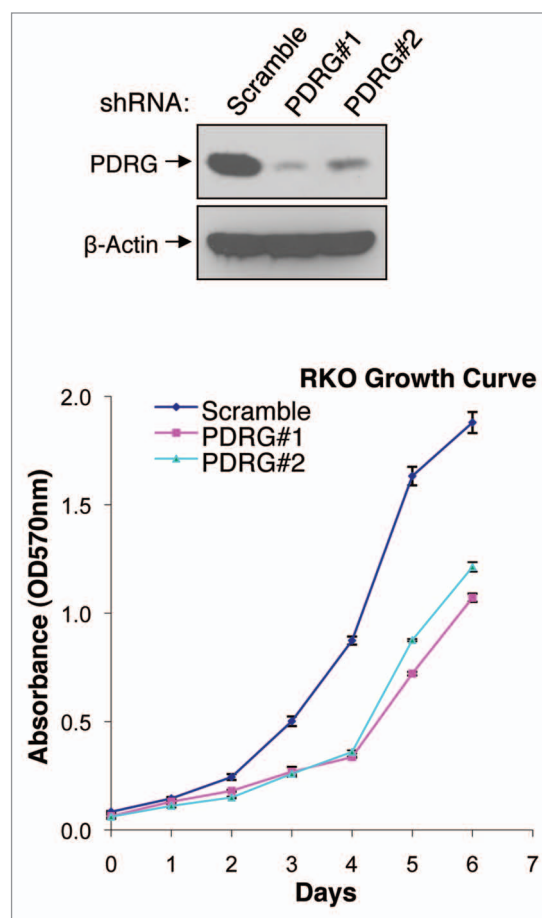


Figure 5. Depletion of PDRG suppresses tumor cell growth. Top, western blot analyses using anti-PDRG antibody show depletion of endogenous PDRG expression by lentiviral-based knockdown in the RKO cells; bottom, graphic representation of MTT assay depicting decreased cell proliferation due to PDRG knockdown in RKO cells. Cells were seeded at equal density and infected with lentiviral scrambled shRNA or PDRG-specific shRNAs. Nine days after infections, equal numbers of cells were reseeded onto 24-well plates (1×10^4 per well). Cell viability was determined by methyl thiazole tetrazolium (MTT) assay each day over a 7 day period. Points, mean of three independent experiments; bars, SE.

amplified 3' part of PDCD7 ORF was digested with EcoR I and BsmA I. Both digested fragments were mixed and ligated with Xho I and EcoR I digested pDsRed N1 vector. The resulting plasmid was sequenced to confirm that it contains correct the full-length PDCD7 ORF sequence. The mammalian myc-tagged PDCD7 expression vector was constructed by subcloning the full-length PDCD7 ORF in pDsRed N1 vector into pCEP4 vector using primers: 5'-CGG GGT ACC AAG CTT ACC ATG GAA CAA AAA CTC ATC TCA GAA GAG GAT CTG ATG GCC CTG CCA CCA TTC TT-3' and 5'-CCG CTC GAG CTC GAG TTA CTA ATG CAG CTT AAC AG-3'. The resulting plasmid was named pCEP-PDCD7 and was sequenced to confirm that it contains the correct myc-tagged PDCD7 sequence.

Lentivirus-mediated shRNA silencing. Scramble shRNA construct were purchased from Addgene. All other shRNA constructs were from Open Biosystems (Huntsville, AL). The 21-bp

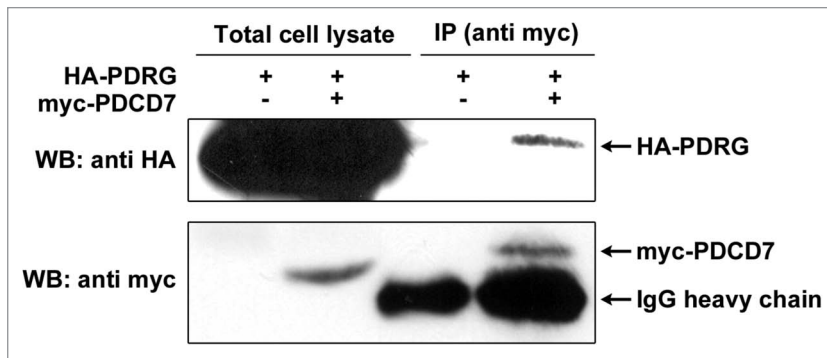


Figure 6. PDRG interacts with PDCD7 in mammalian cells. HEK 293T cells were transiently transfected with expression vector pCEP-PDRG1 and pCEP-PDCD7 or pCEP-PDRG1 and pCEP4 vector without PDCD7 insert. After 24 hours, cells were harvested and lysed. About 500 total lysates were precipitated with anti-myc antibody (9E10) and protein G-bead. The precipitated proteins were resolved by SDS-PAGE along with 150 μ g total cell lysates. HA-tagged PDRG was detected by western blotting with rat monoclonal anti-HA antibody and myc-tagged PDCD7 was detected using mouse monoclonal anti-myc antibody (9E10).

nucleotide sequences targeting human PDRG1 used in this study were

#1, 5'-GAT GGT TTG CTT CGG GAA CAT-3'.

#2, 5'-GCT TCG GGA ACA TGT TTA TCA-3'.

Viruses were produced in HEK-293T cells using lipofectamine 2000 (Invitrogen) according to Addgene's protocol. Lentivirus particle was added to the cells in DMEM containing \sim 8 μ g/ml polybrene. Cells were cultured with 2 μ g/ml puromycin (Sigma) for 7 days prior to analyses.

MTT assay. MTT proliferation assays were done as we have previously described in reference 23. Briefly, cells were incubated with 1 mg/ml MTT (Sigma) for 3 hours, then the MTT precipitate was dissolved in isopropanol with 0.04 M HCl. Absorbance was read with a Bio-Rad SmartSpec 3100 at 570 nm with background subtraction read at 690 nm.

Northern blot analyses. RNA extraction and Northern blot analyses were done as we have previously described in reference 2. Briefly, total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) and about 20 μ g of total RNA for each sample were fractionated on 1.2% agarose gel and transferred to Nytran SuperCharge membranes (Schleicher & Schuell, Keene, NH). Human PDRG1 probes were labeled with 32 P and prehybridizations and hybridizations were done in QuikHyb Solution (Stratagene, La Jolla, CA) at 65°C. To detect PDRG1 expression in paired human normal and cancer samples, Cancer Profiling Array I and Cancer Profiling Array II were purchased from Clontech, BD Biosciences, and probed with PDRG1 cDNA probe as described above.

Yeast two-hybrid screening. Yeast two-hybrid screening was done using MATCHMAKER GAL4 Two-Hybrid System 3 (Clontech Laboratories Inc., Palo Alto, CA) per the company's manual. In brief, pBD-PDRG1 vector was co-transformed with pACT2 vector based human normal testis MATCHMAKER cDNA library (Clontech) into AH109 yeast cells. The transformed yeast cells were spread on minimal synthetic

dropout medium (SD medium) agar plates without Adenine (Ade), Histidine (His), Leucine (Leu) and Tryptophan (Trp), but containing X- α -Gal. After 3 or 7 days incubation at 30°C, blue clones grown on SD medium agar plate were selected, then pACT2 plasmids in the yeast were rescued and sequenced to identify the inserts using Blast online comparison.

Immunoprecipitation and western blot analyses. Immunoprecipitation were done as we have previously described in reference 24. Briefly, HEK-293T cells were transiently co-transfected with pCEP-PDRG1 and pCEP-PDCD7 or pCEP4 vectors using Lipofectamine 2000 (Invitrogen) according to the instructions of the manufacturer. Cells were harvested and lysed in lysis buffer (50 mmol/L Tris-HCl (pH 7.5), 100 mmol/L NaCl, 1% chaps, 10% glycerol, 0.5 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 μ g/mL leupeptin, 1 mmol/L NaF and 1.5 μ g/mL aprotinin). About 0.5 mg of 1 mg/ml total cell lysate was mixed with 2 μ g of anti-myc monoclonal antibody 9E10 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and rotated for about 2 hours at 25 rpm in 4°C cold room. Then 100 μ l of protein G-bead slur was washed with lysis buffer and added to total cell lysate. After 3–4 hours rotation, protein G-bead was pelleted by brief centrifuge and washed with lysis buffer three times. Bound protein was washed off by adding 100 μ l 2x SDS-PAGE gel loading buffer and resolved by SDS-PAGE. Western blot analyses was performed as previously described in reference 25. HA-tagged PDRG1 was detected using a rat monoclonal anti-HA antibody 3F10 (Roche, Indianapolis, IN). Myc-tagged protein was detected using mouse monoclonal anti-myc antibody 9E10.

Immunohistochemical staining. Frozen primary colon normal and tumor tissues from same patient were sectioned (6 μ m) then fixed with 4% paraformaldehyde for 30 min. Endogenous peroxidase was quenched with hydrogen peroxide and the sections were then blocked in PBS with 10% goat sera for 20 min and incubated sequentially with anti-PDRG1 antibody for \sim 1 hour, biotinylated anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA) for 45 min and ABC reagent (Vector laboratories) for 30 min. After several washes, the sections were then incubated with peroxidase substrate DAB solution (Vector laboratories) and counterstained with hematoxylin.

Acknowledgements

This work was supported in part by NIH grants ES005633-02, CA121850 and CA128096 to M.S.S. and Y.H. L.J. is supported by a pre-doctoral fellowship from the US Department of Defense (Grant BC073479).

Note

Supplemental materials can be found at: <http://www.landesbioscience.com/journals/cbt/article/14412>

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