p53 Regulation and Function in Renal Cell Carcinoma

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Abstract
Loss of p53 function is a critical event in tumor evolution. This occurs through a range of molecular events, typically a missense p53 mutation followed by loss of heterozygosity. In many cancers, there is compelling evidence that cells that can compromise p53 function have a selective advantage. The situation in renal cell carcinoma is unclear. It has recently been suggested that p53 function is unusually compromised in renal carcinoma cells by a novel dominant, MDM2/p14ARF-independent mechanism. This is hard to reconcile with other recent studies that have identified p53 as an important prognostic indicator. Indeed, one of these latter studies found that the best predictor of poor outcome was the presence of high levels of both p53 (usually indicative of p53 mutation) and MDM2. Thus, it is important that we gain a clearer understanding of the regulation of p53 and the role of MDM2 in renal cell cancer. To address this, we have investigated the transcriptional activity of p53 in a panel of renal cell carcinoma cell lines and the contribution of MDM2 and p14ARF to p53 regulation. We have found that p53 is functional in p53 wild-type renal cell carcinoma cells and that this activity is significantly regulated by MDM2 and to a much lesser extent by p14ARF. Moreover, following induction of DNA damage with UV, the p53 response in these cells is intact. Thus, future studies of renal cell carcinoma that focus on p53 and MDM2 and their role in determining disease outcome will be required to create a better understanding of this notoriously difficult to manage disease.

Introduction
Renal cell carcinoma (RCC) is the most common form of kidney cancer in the United States/United Kingdom, accounting for some 17,800/3,200 deaths per annum, respectively. The unpredictable outcome of RCC following diagnosis is a major obstacle to the effective management of this disease. There are at least five subtypes of RCC currently recognized and the most common form is clear cell cancer, which accounts for roughly 70% to 80% of cases. Renal cell carcinoma is characterized by its lack of “warning” signs. The classic triad of pain, hematuria, and flank mass is seen in <10% of cases (1, 2). Most patients who present with symptoms have advanced disease and pain is usually an indication of invasion. Typically, it is seen that ~41% of patients present with pain, 38% with hematuria, 24% with mass, 36% have weight loss, fever is seen in 18%, hypertension in 22%, and hypercalcemia in 6%. Many (40-60%) are diagnosed as an incidental finding most frequently made during computer tomography and ultrasound scans for other complaints (1). The stage of disease, according to the Union Internationale Contre le Cancer tumor-node-metastasis classifications revised in 1997, is currently the most reliable prognostic factor for any patient group. The importance of p53 inactivation in RCC has been the subject of conflicting observations (3–8). The studies that describe a low frequency for p53 detection by immunohistochemistry are fairly limited in both number and scale. However, in a large study (n = 97), Haitel et al. (7) showed that p53 up-regulation, typically indicative of p53 mutation, is a frequent event in RCC occurring in roughly 36% of cases. A larger (n = 246) and more recent study obtained similar results with 29.5% of RCCs being p53 positive using 20% positive cells as a cutoff value (8). In both these studies, p53 was a significant prognostic indicator (P < 0.003 and P < 0.0005, respectively). In RCC cell lines, one of us (W.M.L.) previously described (9) loss of heterozygosity of the p53 locus in 14 of 29 (48%) lines. Mutations were found in 11 of these by single-strand conformational polymorphism, which was confirmed by sequencing. In conclusion, it seems likely that p53 mutation is not an uncommon event and more importantly is of prognostic significance in RCC. Nevertheless, it was recently (6) shown that p53 may be inactivated by a novel dominant mechanism in renal cells. Stress or drug treatment of these cells, while leading to increased p53 DNA binding, did not induce increased p53 transcription. The authors also showed that neither MDM2 nor p14ARF were responsible for the observed inactivation of p53 and thus postulated the existence of a novel dominant mechanism of inactivation of p53 in renal cells. They further proposed that this novel mechanism might explain the observation that p53 is rarely mutated in RCC. However, the idea that p53 is not mutated in RCC is not supported by the most recent and largest studies to date.

Given the previously described association between p53 and MDM2 expression and poor prognosis (7, 8), we set out to investigate the function of and regulation of p53 in RCC cells. We have found that not only is wild-type p53 functional in RCC cells, but it is transcriptionally active, responds normally to DNA damage induction by UV, and is negatively regulated by MDM2.

Materials and Methods
Plasmids and antibodies. The human p53 (pCEP4-p53) expression plasmid and the β-galactosidase expression plasmid pβ-gal (SV40 promoter) were described previously (10). pCEP4 was obtained from Invitrogen (Carlsbad, CA). The p53 luciferase reporter construct p533-TA-Luc containing p53 binding sites (11, 12) driving luciferase expression from a minimal herpes simplex virus thymidine kinase promoter was obtained from BD Clontech (Mountain View, CA). The p21 (CDKNI)-promoter luciferase reporter (pWVPLuc) has been described previously (13). The 370-bp Bax promoter has also been described previously (14) and pGL3-Bax-Luc is a derivative of this that contains the whole of this
Bax promoter fragment cloned into pGL3-Basic (Promega, Madison, WI; ref. 15). Mouse monoclonal antibodies against human MDM2 (Ab-1), p53 (Ab-6), p21 (CDKN1A; Ab-1), β-galactosidase (Ab-1, used as a transfection efficiency control), and the sheep polyclonal antibody against p53 (Ab-7) were all purchased from Calbiochem (San Diego, CA). The anti-actin (C-2, used as a total protein loading control) and the anti-p21 (anti-CDKN1A; F-5) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). AffiniPure donkey anti-mouse FITC and anti-sheep Texas red conjugates were obtained from Jackson ImmunoResearch (West Grove, PA).

Cell culture and transfection. The cell lines A498 (primary RCC, p53 wild type), ACHN (primary RCC, p53 wild type), Caki-2 (primary clear cell RCC, p53 wild type), H1299 (non small cell lung carcinoma, p53 null), and U2-OS (osteosarcoma, p53 wild type) were obtained from the American Type Culture Collection (Manassas, VA) and 117 (p53 wild type) and 121 (p53 mutant) cells were previously generated by one of us (W.M.L.; ref. 9). A498, ACHN, 117, and 121 cells were maintained in Eagle's MEM supplemented with 2 mmol/L l-glutamine, 1.5 g/L sodium bicarbonate, 0.1 mmol/L nonessential amino acids, 1 mmol/L sodium pyruvate, and 10% fetal bovine serum (FBS). H1299 and U2-OS cells were maintained in DMEM supplemented with 2 mmol/L l-glutamine and 10% FBS. Caki-2 cells were maintained in McCoy's 5A supplemented with 1.5 mmol/L l-glutamine, 2.2 g/L sodium bicarbonate, and 10% FBS. Cells were transiently transfected using 3 μL Genejuice reagent (Novagen, San Diego, CA) per microgram of DNA, and empty vector was used to ensure equal DNA content in transfections. siRNA was delivered to cells by transfection with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. DNA, and empty vector was used to ensure equal DNA content in transfections. siRNA was delivered to cells by transfection with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. siRNA for p53 (5′-GGACGCAUCCUUCUUAAUU-3′; ref. 16), MDM2 (5′-GCCCAAAUUCGUAUAG-3′; ref. 17), and a negative control (5′-GGACGCAUCCUUAUAAU-3′; ref. 17) were synthesized by Dharmacon (LaJolla, CA). In some experiments, cells were subjected to 4 to 40 J/m2 whole body irradiation using 30-W UV lamp (Philips, New York, NY) calibrated using a Black-Ray Model J-225 shortwave UV measuring meter (UVP).

Western analysis. Cells were harvested and lysed using SLIP buffer [50 mmol/L HEPES (pH 7.5), 10% glycerol, 0.1% Triton X-100, 150 mmol/L NaCl] in the presence of the following protease inhibitors: aprotinin (2 μg/mL), leupeptin (0.5 μg/mL), pepstatin A (1 μg/mL), soybean trypsin inhibitor (100 μg/mL), and phenylmethylsulfonyl fluoride (1 mmol/L). After 10 minutes of incubation on ice, lysates were centrifuged at 20,000 × g and protein concentrations in the supernatant were determined using Bradford reagent (Bio-Rad, Hercules, CA). Typically, 50 μg samples of total protein in 1× protein sample buffer [50 mmol/L Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 0.25% β-mercaptoethanol, bromophenol blue (1 mg/mL)] were separated by SDS-PAGE and transferred to Hybond enhanced chemiluminescence nitrocellulose membrane (Amersham Pharmacia Biotech, Chalfont St Giles, United Kingdom). Membranes were blocked in PBS/Tween 20 (0.1% v/v) containing nonfat dry milk (Bio-Rad; 5% w/v) for 1 hour at room temperature before incubation with primary antibodies (each at 3 μg/mL, except anti-p53 at 1 μg/mL). Membranes were washed thrice for 15 minutes in PBS/Tween 20 before addition of horseradish peroxidase–conjugated anti-mouse (12,500) secondary antibody (Amersham Pharmacia Biotech) for 1 hour at room temperature. Membranes were washed as before and signal was detected by Western Lightning Chemiluminescence Reagent (Perkin-Elmer, Wellesley, MA).

Luciferase reporter assay. For reporter assays, cells were cotransfected using Genejuice with 0.05 to 0.1 μg plasmid DNA per cm² of culture dish as described in the figure legend. Reporter constructs were either the p53-responsive luciferase reporters p53-3A-luc, pGL3-Bax-Luc, or pWWP-Luc as indicated.

Cells were lysed and luciferase activity measured 4 seconds after addition of sample to substrate using the Luciferase Assay Kit (Stratagene, La Jolla, CA) essentially according to the manufacturer's instructions with an integration period of 60 seconds in a TD 20/20 luminometer (Turner Design, Sunnyvale, CA). We have loaded equal amounts of total protein and only used data from experiments in which transfection efficiencies were comparable, to avoid generating artifacts which might occur for example when normalizing to another reporter construct (e.g., Renilla luciferase) which can be affected by many variables within the cell such as oncogene activity or DNA damage (18, 19).

Immunofluorescence analysis. Cells were transfected and seeded 8 hours later onto glass chamber slides (Nunc, Rochester, NY). Twenty-four hours later, cells were washed in PBS, fixed with 4% paraformaldehyde in PBS, and then permeabilized with 0.1% Triton X-100. All subsequent steps were carried out in solutions containing 0.1% Triton X-100 in PBS. Cells were washed thrice and were then blocked in 1% normal donkey serum (NDS) for 1 hour. Primary antibody was added at 1:500 for anti-p53 (Ab-7) or 1:100 for anti-p21 (a 1:1 mixture of Ab-1 plus F5) in 1% NDS.Slides were washed thrice and secondary antibodies were added at 1:100 in 1% NDS. Following three further washes, cells were mounted in DAKO fluorescent mounting medium before analysis. Dual labeling was done as above for p53 first followed by p21 and no cross-reaction between secondary antibodies was detected.

Cell cycle analysis. Cells were harvested and analyzed by fluorescence-activated cell sorting (FACS) essentially as described previously (10). Cells were harvested 24 hours after addition of siRNA and washed in Dulbecco's PBS containing 1% bovine serum albumin. Cells were then fixed in ethanol, stained in propidium iodide and analyzed using a Beckman-Coulter EPICS ALTRA flow cytometer.

Results

A recent study regarding the function and regulation of p53 in renal carcinoma cells suggested that p53 function is compromised by a novel dominant mechanism (6). We wanted to address three critical questions raised by that work. First, is exogenous p53 active in renal cells regardless of endogenous p53 status? Second, is endogenous p53 active in p53 wild-type renal cells and is it regulated by MDM2 and/or p14ARF? Finally, is the p53-mediated DNA damage response (i.e., up-regulation of p53 transcriptional activation) intact in p53 wild-type renal cells? Figure 1 shows the result of transfection of a wild-type p53 expression construct into a panel of renal cell carcinoma cells lines. In Fig. 1A, the results of p53 luciferase reporter assays are shown. In Fig. 1B and C, the corresponding raw data and the western blot from the luciferase assay, respectively, are shown for comparison. As expected, transfection of wild-type p53 induces a significant increase in p53 transcriptional activity in all of these cells regardless of their p53 status. We have also done these studies with the p21 (pWWP-Luc) and Bax (pGL3-Bax-Luc) promoter luciferase reporter constructs and have obtained similar results (data not shown). Note that in 121 cells (p53 mutant) the steady-state level of p53 following transfection is only slightly increased. Nevertheless, there is a >200-fold increase in p53 transcriptional activity. We have found that we routinely achieve a plasmid transfection efficiency of between 0.1% and 1% in these RCC cell lines. Therefore, to detect an effect of p53 transfection upon an endogenous target gene, we used immunofluorescence to monitor endogenous p21 (CDKN1A) levels. Figure 1D shows that cells transfected with an expression construct for p53 and expressing the highest levels of p53 also express higher levels of p21. Thus, p53 can function to up-regulate endogenous target genes in RCC cells.

The second question we wanted to address was whether the endogenous p53 in RCC cells harboring wild-type p53 was transcriptionally active. We found, as shown in Fig. 2, that p53 is indeed active in these cells and using siRNA for p53, we have confirmed that the luciferase signal is p53-dependent (note that in contrast to the poor plasmid transfection efficiency seen in Fig. 1, we are able, as Fig. 2 shows, to efficiently deliver siRNAs to these cells). In addition, down-regulation of MDM2 with siRNA results in up-regulation of
p53 transcriptional activity. This is reflected by changes in the steady-state levels of both MDM2 and p53 as shown in Fig. 2C. In 117s, A498s, and ACHNs the effect of MDM2 knockdown is an ~1.5-fold increase in p53 transcriptional activity. In Caki-2s, the effect is 2-fold. We have previously seen similar, 2-fold, effects using this same siRNA for MDM2 on MCF-7 cells (17), and indeed, this degree of p53 up-regulation from knockdown of MDM2 is typical of this sort of experiment (20). In addition, increased p53 activity resulting from knockdown of MDM2 was associated with an increase in endogenous p21 steady-state levels as shown in Fig. 2C. Because p21 is not exclusively regulated by p53 (21), it was not surprising that knockdown of p53 did not always (compare ACHN with A498, Caki-2, and 117s) lead to a reduction in p21 levels. We also examined the effect of knockdown of p14ARF with siRNA and these results

Figure 1. Transient transfection and expression of p53 results in functional p53 activity in RCC cell lines regardless of endogenous p53 status. Renal cell lines were transfected with 1 μg of plasmids expressing either p53 (pCEP4-p53) or an empty vector control (pCEP4) as indicated together with 1 μg of β-galactosidase plasmid (pβ-gal). A, luciferase assay of cell lysates. B, raw data from (A). C, Western blot analysis of the same lysates shown in (A), probed for the indicated proteins. D, immunofluorescent detection of p53 and endogenous p21 in cells transfected with 1 μg pCEP4-p53. Experiments have been repeated thrice and the results shown are typical and from a single experiment. Columns, means (n = 3); bars, ± SE.
suggest that whereas p14\textsuperscript{ARF} plays no role in 117s and ACHNs, it apparently contributes, albeit weakly, towards p53 regulation in Caki-2s and also very weakly in A498s. One caveat is that we have not been able to detect the endogenous p14\textsuperscript{ARF} protein in these cells by western blot, but the siRNA clearly works well in those where we can detect endogenous p14\textsuperscript{ARF} such as 117s and 121s.

We then looked at the consequences of induction of p53 activity on the cell cycle by knockdown of MDM2. All of the RCC cell lines

\begin{figure}[h]
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\caption{Wild-type p53 in renal cell carcinoma cell lines is transcriptionally active and is regulated by MDM2 and to a lesser extent by p14\textsuperscript{ARF}. Renal cell lines were transfected with the indicated siRNA and 8 hours later were transfected with 1 \(\mu\)g of a p53-luciferase reporter construct (pp53-TA-Luc) and 1 \(\mu\)g of a \(\beta\)-galactosidase expression plasmid (\(\beta\)-gal). Cells were harvested a further 24 hours later. A. luciferase assay of cell lysates. B. raw data from (A). C. Western blot analysis of the same lysates shown in (A), probed for the indicated proteins. D. FACS analysis of Caki-2 cells transfected with the indicated siRNA. Cells were transfected and harvested 36 hours later, fixed in ethanol, and stained with propidium iodide. E. Western blot analysis of the Caki-2 cells analyzed in (D). Experiments have been repeated thrice and the results shown are typical and from a single experiment. Columns, means (\(n = 3\)); bars, \(\pm\) SE.}
\end{figure}
we have used in this study are very difficult to transfect (compared with many other tumor cell lines) with the possible exception of Caki-2s; therefore, we examined the cell cycle profile of these cells following treatment with MDM2 siRNA. As shown in Fig. 2D and E, reducing the level of MDM2 with siRNA increases the steady-state level of p53 in these cells with a resulting increase in the G1 population. Thus, we conclude that p53 is transcriptionally and functionally active in wild-type p53 RCC cell lines and is substantially regulated by MDM2. Moreover, the degree of regulation of p53 by MDM2 is typical of many of the tumor cell lines that we and others have studied derived from a range of tissues (17, 19).

The final question we addressed was whether there was any defect in the p53 response to DNA damage. RCC cells are notoriously resistant to chemotherapeutics (22). Therefore, to induce DNA damage we exposed cells to shortwave UV radiation (UV-C). As shown in Fig. 3, there is a significant dose-dependent induction of p53 transcriptional activity in RCC cells that ranges from 1.4-fold in ACHNs through 2-fold in Caki-2s to 5-fold in A498s. U2-OS cells in comparison express unusually high levels of wild-type p53 but even so display only a 4.7-fold induction of p53. Under similar conditions values in the literature typically range from 2- to 4-fold (19, 23). These results are reflected by induction of endogenous p21 steady-state levels in these cells. There is no induction in the p53 mutant 121 cell line, although there is a reduction in transcription in these cells (typical of the generalised reduction in transcription observed in cells exposed to high levels of UV radiation; ref. 19). Note that in Fig. 2 we had shown that the comparatively low levels of luciferase produced in these cells were not the result of p53 transcriptional activity. In conclusion, we find that the UV-induced p53-mediated DNA damage response is essentially intact in p53 wild-type RCC cells.

**Discussion**

Our results support the conclusion that p53 function is essentially normal in RCC cells that possess wild-type p53. It is probably more accurate to say that p53 function in RCC cells is not evidently unusual when compared with tumor cell lines derived from other tissues. There have been a number of studies of p53 in renal cancer and these have come to a range of conclusions. Several relatively small studies have suggested that p53 mutation, inferred from immunohistochemical detection in clinical samples, is relatively rare (25%, n = 36; 16%, n = 31; and 2%, n = 53; refs. 3–5). However, a more recent and larger study (n = 97) has concluded that p53 expression is present in 36% of cases (7). When combined with MDM2 up-regulation, these authors found that p53 expression was an independent prognostic indicator (P = 0.00179). This latter suggests that inferred mutations of p53 and up-regulation of MDM2 occur in RCC in much the same way that they do in other cancers such as those of the bladder (24). In another even larger study of 246 RCC primary and metastatic samples, p53 overexpression was detected in 29.5% of cases (8). This study also found a statistically significant difference in metastasis-free survival between p53-positive and p53-negative tumors for RCC (P = 0.0005) and that p53 expression was an independent prognostic indicator (P = 0.01). When combined, these latter two studies suggest that, as with other cancers, one effective way that tumors can lose p53 function is through mutation. This also suggests that there is a clonal advantage, associated with loss of p53 function, conferred on tumor cells in the kidney. In studies of cell lines derived from renal cell carcinomas loss of heterozygosity of 17p has been documented at a higher frequency (48%) than has been inferred from the above clinical studies. This presumably reflects the selection for loss

![Figure 3](cancerres.aacrjournals.org)
of p53 function in cells that are better able to adapt to growth ex vivo (9).

It has recently been proposed that p53 was rarely mutated in renal cancer because it was already activated in a novel dominant manner that was neither MDM2 nor p14 ARF dependent (6). We therefore set out to investigate the regulation and function of p53 in RCC cell lines. We have used a panel of cells that overlap with those used by Gurova et al. (ACHN, H1299, and U2-OS) plus renal cell lines obtained either from the American Type Culture Collection (Caki-2 and A498) or that we have generated previously (117 and 121; ref. 9) to address the question of p53 functional status in renal cell carcinomas. We have found that ectopically expressed wild-type p53 is transcriptionally active, independent of the p53 status of the cells. In RCC cells that possess wild-type p53, the endogenous p53 is functional, responds to DNA damage and is regulated by MDM2 to a comparable degree with cells from other types of cancer (e.g., in MCF-7s derived from breast adenocarcinoma or U2-OS cells derived from an osteosarcoma). We are left with an unsatisfactory discordance between our results and those of Gurova et al. to some extent, we have used different cell lines, but in both studies, the ACHN cell line was used. Our experience with these cells suggests that they are even more refractory to transfection than any of the other RCC lines that we have used. This can lead to problems with siRNA which in our hands is much more efficiently delivered than a plasmid (see, e.g., the weak effect of siRNA on ACHN cells in Fig. 2C). Nevertheless, comparing Fig. 2A and B with Fig. 2C, it is clear that siRNA is delivered to a substantial number of those ACHN cells successfully transfected with the reporter construct. In Figs. 2 and 3, we have normalized our signal to the level detected in the scrambled siRNA ("control" lane) rather than plot absolute RLUs. Thus, the fold differences shown for RCC cells are comparable with those we and others have detected in similar assays (17, 19). It would of course be misleading to compare the fold differences in the p53 wild-type RCC cells with the p53 null H1299 cell line. Note also that although the histograms for the p53 mutant RCC line 121 are included on these normalized scales, the absolute values detected are much lower than for any of the p53 wild-type lines (as shown in Figs. 1B, 2B, and 3B).

Two important recent articles strongly suggest that loss of p53 function is a critical event in the evolution of renal cell carcinoma (7, 8). Our study shows that p53 function is essentially "normal" and is regulated by MDM2 to a "normal" degree and is to a lesser extent regulated by p14 ARF. Based upon what is generally understood about the regulation of p53 in a range of cell types, and in light of the more recent immunohistochemical studies implicating p53 in RCC, these results are perhaps not surprising. The important challenge now becomes to use this information to develop appropriate diagnostics and therapeutics to manage this notoriously difficult disease.

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**References**

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