Suppression of Hypoxia-Inducible Factor 2α Restores p53 Activity via Hdm2 and Reverses Chemoresistance of Renal Carcinoma Cells

Andrew M. Roberts,1 Ian R. Watson,1,3 Andrew J. Evans,1,4 David A. Foster,5 Meredith S. Irwin,1,2,3 and Michael Ohh1

1Department of Laboratory Medicine and Pathobiology, Faculty of Medicine, and 2Institute of Medical Sciences, University of Toronto; 3Department of Paediatrics, The Hospital for Sick Children; 4Department of Pathology, University Health Network, Princess Margaret Hospital, Toronto, Ontario, Canada; and 5Department of Biological Sciences, Hunter College of the City University of New York, New York, New York

Abstract

p53 mutations are rarely detected in clear cell renal cell carcinoma (CCRCC), but, paradoxically, these tumors remain highly resistant to chemotherapy and death receptor–induced death. Here, we show that the accumulation of hypoxia-inducible factor 2α (HIF2α), a critical oncogenic event in CCRCC following the loss of von Hippel-Lindau (VHL) tumor suppressor protein, leads to Hdm2-mediated suppression of p53. Primary CCRCC specimens exhibiting strong hypoxic signatures show increased levels of activated nuclear phospho-Hdm2(Ser166), which is concomitant with low p53 expression. The abrogation of Hdm2-p53 interaction using the small-molecule Hdm2 inhibitor nutlin-3 or the downregulation of HIF2α via HIF2α-specific short hairpin RNA or wild-type VHL reconstitution restores p53 function and reverses the resistance of CCRCC cells to Fas-mediated and chemotherapy-induced cell death. These findings unveil a mechanistic link between HIF2α and p53 and provide a rationale for combining Hdm2 antagonists with chemotherapy for the treatment of CCRCC. [Cancer Res 2009;69(23):9056–64]

Introduction

Renal cell carcinoma (RCC) accounts for approximately 3% of all malignancies, and patients with metastatic RCC have a median survival of 13 months. The most common form (75%) of kidney cancer is of the clear cell histology [clear cell renal cell carcinoma (CC RCC)], which is highly aggressive and unresponsive to radiation or chemotherapy (1). Surgery by radical or partial nephrectomy is the most effective treatment option for localized disease. However, in one third of patients, tumors recur postoperatively as distant metastases, and only 4% to 6% of these tumors respond to chemotherapy (1). Hence, the standard nonsurgical treatment for advanced CCRCC has been the administration of interleukin-2 (II-2). However, a high-dose IL-2 regimen has a response rate of only 21% and causes significant toxicities (1). Recently, clinical trials of receptor tyrosine kinase inhibitors, such as the vascular endothelial growth factor receptor-2 and platelet-derived growth factor (PDGF) receptor-β inhibitors sorafenib (Nexavar) and sunitinib (Sutent), have yielded promising results in clinical trials by prolonging progression-free survival in ~70% of patients with metastatic CCRCC. However, neither drug has had a significant effect on overall patient survival (1, 2).

The efficacy of most chemotherapies is dependent on a successful execution of p53-mediated apoptosis to override the survival signals acquired by cancer cells (3, 4). Consequently, tumors harboring p53 mutations are associated with chemoresistance and, in general, predict a considerably worse patient prognosis in comparison with malignancies with wild-type p53 (5). Intriguingly, p53 mutations are infrequently detected in CCRCC (6, 7), but nevertheless, these tumors are very resistant to chemotherapy. Although there is no general consensus, several models have been proposed to explain the resistance of CCRCC to apoptosis, which may contribute to chemoresistance. For example, CCRCC cells devoid of von Hippel-Lindau (VHL) are resistant to death receptor tumor necrosis factor receptor–mediated cell death due, at least in part, to the increased activity of nuclear factor κB (NFκB) and downstream NFκB-mediated expression of antiapoptotic proteins (8). Yang and colleagues showed that VHL acts as an adaptor molecule that binds and promotes the inhibitory phosphorylation of the NFκB agonist Card9 by casein kinase 2 in a hypoxia-inducible factor (HIF)–independent manner. Downregulation of Card9 in VHL−/− CCRCC normalized NFκB activity and sensitivity to cytokine-induced cell death and attenuated the tumorigenic potential of CCRCC cells (9). The effect of the other major death receptor Fas-mediated signaling in CCRCC is unknown. There are limited and conflicting reports about the significance of p53 in CCRCC. In particular, Gurova and colleagues suggest that p53 is inactive via unknown dominant-negative mechanisms independent of Hdm2 (7, 10), whereas Warburton and colleagues showed that p53 in several CCRCC cell lines can respond to UV radiation and is negatively regulated by Hdm2 (10, 11). Furthermore, Hdm2 positivity was found significantly more frequently in CCRCC tumors of higher grade (12). The presence of a specific single nucleotide polymorphism in the Hdm2 promoter (SNP309), which results in elevated Hdm2 transcription and expression (13), has also been identified to be predictive of poor prognosis and survival in RCC (14). These findings suggest a possible oncogenic involvement of Hdm2 in CCRCC.

Approximately 80% of sporadic CCRCC arise due to the biallelic inactivation of the VHL tumor suppressor protein. In addition, individuals who inherit one faulty copy of VHL develop a rare multisystemic VHL cancer syndrome characterized by the development of retinal and cerebellar hemangioblastoma and pheochromocytoma, as well as CCRCC, on loss of the remaining wild-type VHL.
allele in a susceptible cell. VHL is the substrate-specifying component of the multiprotein E3 ubiquitin ligase ECV (elongins B and C/cullin 2/VHL) that catalyzes the polyubiquitylation of prolyl-hydroxylated HIFα for subsequent destruction via the 26S proteasome. HIFα is hydroxylated on conserved proline residues by prolyl-hydroxylase domain–containing enzymes in an oxygen-dependent manner. Under hypoxia, the unhydroxylated HIFα escapes recognition by VHL and thereby escapes ECV-mediated degradation. Stabilized HIFα associates with the constitutively stable partner HIFβ to form an active heterodimeric HIF transcription factor, which binds to hypoxia-responsive elements located in the promoter/enhancer regions of numerous hypoxia-inducible genes to initiate the various adaptive responses to hypoxia, such as anaerobic metabolism, erythropoiesis, and angiogenesis (15, 16).

Several lines of evidence have strengthened the notion that HIF2α stabilization is critical for CCRCC progression. For example, the inhibition of HIF2α, but not of HIF1α, in CCRCC cells was sufficient to abolish the tumorigenic potential of CCRCC cells in a mouse xenograft assay (17, 18). Conversely, the stable expression of nondegradable HIF2α in VHL-reconstituted CCRCC cells overcame the tumor-suppressive role of VHL (17). Moreover, a subset of CCRCC is caused by an inactivation of TSC1/2 tumor suppressor complex. The loss-of-function mutations in TSC1/2 result in
increased translation of HIFα via mammalian target of rapamycin-dependent and –independent mechanisms (19). In the Eker rat renal tumor model, HIF2α was shown to be upregulated in RCC with a loss of TSC2 (20). Recently, VHL-null CCRCC exclusively expressing HIF2α showed elevated c-Myc activity associated with enhanced proliferation and resistance to replication stress in comparison with CCRCC overexpressing both HIF1α and HIF2α (21). These observations suggest that whereas HIF1α antagonizes c-Myc, HIF2α promotes c-Myc activity associated with increased disease aggressiveness (21). Although these findings further support a critical role of HIF2α in the progression of CCRCC, the role of HIF2α in chemoresistance is unknown.

Here, we show that HIF2α suppresses p53 expression and function via Hdm2. In patient CCRCC samples, we show an increased accumulation of nuclear phospho-Hdm2(Ser166) and, correspondingly, negligible levels of p53, and provide evidence that HIF2α-dependent Hdm2-mediated suppression of p53 contributes to the resistance of CCRCC cells to Fas-mediated or chemotherapy-induced cell death. Importantly, CCRCC cells can be rendered highly sensitive to apoptotic stimuli by restoring p53 function via pharmacologic Hdm2 inhibitors. These findings provide the first mechanistic link between HIF2α and p53-dependent resistance to apoptosis in CCRCC, and support the notion that CCRCC can be successfully sensitized to conventional chemotherapy if combined with modalities designed to reactivate p53.

Materials and Methods

Cells. HEK293A embryonic kidney cells, MCF7 breast carcinoma cells, and A498 (VHL−/−, HIF1α−/−) and 786-O (VHL−/−, HIF1α−/−) CCRCC cells were obtained from the American Type Culture Collection and maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich) at 37°C in a humidified 5% CO2 atmosphere. 786-VHL (WT), 786-VHL(C162F), and 786-VHL(L188V) were previously described (22, 23). 786-VHL(WT) isogenic subclone harboring mutant p53(R249W) was described previously (24). RCC4-VHL(WT) and RCC4-MOCK have also been previously described (25). 786-O subclones stably expressing pRetro-SUPER-empty (786-RetroEMPT) or pRetro-SUPER-HIF2α (786-RetroshHIF2α) short hairpin RNA (shRNA) were previously described (26) and generously provided by Dr. William G. Kaelin (Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA).

Antibodies. Monoclonal anti-HA antibody (12CA5) was obtained from Roche Molecular Biochemicals. Polyclonal anti-HA (Y-11) and anti-Hdm2 (SMP14) antibodies were obtained from Santa Cruz Biotechnology. Anti-cleaved poly(ADP-ribose) polymerase (PARP), anti-PUMA, anti–phospho-Hdm2(Ser166), and anti–phospho-Hdm2(Ser202) antibodies were obtained from Cell Signaling Technology. Anti-HIF2α and anti–carbonic anhydrase IX (CAIX) antibodies were obtained from Novus Biologicals. Anti-HIF1α antibody was obtained from BD Biosciences. Anti-Hdm2 (Ab-1), anti-p53 (DO-1), anti-p73, and anti-Noxa antibodies were obtained from Calbiochem. Anti–v-inhibitor and anti–Fas (CH-11) antibodies were obtained from Upstate. Anti-α-tubulin and anti–α-tubulin antibodies were obtained from Sigma-Aldrich. Polyclonal anti-p53, anti–GLUT-1, anti–cleaved caspase-3, and anti–hnRNP C1-C2 antibodies were obtained from Abcam. Anti-Cul2 antibody was obtained from Zymed.

Chemicals. LY294002 and nutlin-3 were obtained from Upstate and Chemicals.

Materials and Methods

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Chemicals. LY294002 and nutlin-3 were obtained from Upstate and Cedarlane Laboratories, respectively. Doxorubicin, etoposide, cisplatin, Taxol, 5-fluorouracil, desferrioxamine, and neocarzinostatin were obtained from Sigma-Aldrich.

Immunoprecipitation and immunoblotting. Immunoprecipitation and Western blotting were done as described previously (27).

Small interfering RNA–mediated knockdown. A small interfering RNA (siRNA) duplex targeted to p53 (5′-GUGAGGCCGCUUGGAAUGUUU-3′; Dharmacon) and a nontargeting scrambled siRNA duplex (5′-CCAUUCGAUCCUAGUCCG-3′; Dharmacon), as well as ON-TARGETplus SMARTpool siRNA targeted to HIF2α (Dharmacon) and siGENOME RISC-Free Control siRNA (Dharmacon), were transfected into the indicated cell lines according to the instructions of the manufacturer.

Subcellular fractionation. Subcellular fractionation was done as previously described (28).

In vitro binding assay. In vitro binding assay was done as previously described (29).

Immunohistochemistry and tissue microarray. Formalin-fixed, paraffin-embedded sections from 10 nephrectomy CCRCC specimens and matched normal tissue and tissue microarray consisting of quadruplicate representative 10-mm needle-biopsy cores from 56 CCRCC were obtained from the files of the Department of Pathology and Laboratory Medicine at The University Health Network (Toronto, Ontario, Canada). These samples were used and processed in accordance with a University Health Network Research Ethics Board–approved protocol on gene expression in RCC and analyzed as previously described (31).

Results

The acquisition of mechanisms promoting resistance to apoptosis is a common feature of all transformed cells. The significance and deregulation of the Fas-mediated apoptotic pathway has been observed in several cancer types including ovarian (32) and prostate (33) cancers, as well as T-cell leukemia (34). However, the status of Fas-mediated apoptosis in CCRCC is unknown.

Activation of HIF2α is associated with resistance to Fas-mediated apoptosis. 786-O (VHL−/−, HIF1α−/−) CCRCC cells ectopically expressing empty plasmid (786-MOCK) or HA-VHL(WT) (786-VHL) were treated with the agonistic anti–Fas receptor antibody CH-11. 786-VHL(WT) cells were sensitive to CH-11 and showed high levels of cleaved caspase-3 and 86-kDa PARP cleavage product, as measures of apoptosis (Fig. L4; Supplementary Fig. S1). In contrast, 786-MOCK cells exhibited very low levels of cleaved PARP and caspase-3 (Fig. L4; Supplementary Fig. S1). As expected, PARP cleavage inversely correlated with cell viability as measured by trypan blue exclusion assay (Fig. L4, graph). Similar results were obtained using another CCRCC cell line system (RCC4-MOCK and RCC4-VHL; Supplementary Fig. S2).

VHL promotes the destruction of HIFα via the ubiquitin-proteasome pathway. Thus, we asked whether the resistance to Fas-mediated apoptosis in CCRCC is HIF dependent. 786-O cells stably reconstituted with wild-type VHL or disease-causing VHL mutants possessing varying abilities to degrade HIF2α were treated with CH-11 and monitored for cleaved PARP. Similar to 786-MOCK, 786-VHL(WT) cells (Fig. 1B) showed negligible PARP cleavage on CH-11 treatment (Fig. 1B). However, in response to CH-11 treatment, 786-VHL(L188V) (low HIF2α) cells showed significantly higher levels of cleaved PARP, comparable to levels observed in 786-VHL(WT) cells (Fig. 1B). In addition, CH-11 treatment of 786-O cells with stable integration of retrovirus encoding HIF2α-specific shRNA (786-RetroshHIF2α) showed significantly higher levels of cleaved PARP in otherwise CH-11–resistant parental 786-O or 786-RetroEMPT cells (Fig. 1C; data not shown). Similarly, siRNA-mediated knockdown of HIF2α markedly increased the sensitivity of CCRCC cell lines A498 (VHL−/−, HIF1α−/−) and RCC4-MOCK (VHL−/−), the latter of which overexpresses both HIF1α and HIF2α, to CH-11–induced apoptosis (Fig. 1D). These results suggest that the stabilization of HIF2α contributes to the resistance of CCRCC to Fas-mediated apoptosis irrespective of HIF1α status. Consistent with this notion,
ectopic expression of HIF2α likewise increased the resistance of MCF7 breast carcinoma cells to Fas-mediated apoptosis (Supplementary Fig. S3).

**Resistance to Fas-mediated apoptosis is p53 dependent.** p53 influences Fas-mediated apoptosis (35, 36). We asked whether the HIF2α-dependent sensitivity of CCRCC cells to Fas-mediated apoptosis is dependent on p53 by first determining the levels of p53 and p53 target gene products in 786-MOCK and 786-VHL cells. 786-MOCK cells possess significantly reduced levels of p53 and p53-regulated gene products PUMA and Noxa in comparison with 786-VHL cells reconstituted with VHL (Fig. 2A), suggesting that the loss of VHL leads to the suppression of p53 expression and activity. Importantly, siRNA-mediated knockdown of endogenous wild-type p53 in 786-VHL(WT) cells resulted in the diminution of PARP cleavage on CH-11 treatment (Fig. 2B). In addition, the 786-VHL isogenic subclone harboring a sporadic inactivating p53(R248W) mutation (24) exhibited considerable resistance to CH-11–induced apoptosis similar to 786-MOCK cells (Fig. 2C). These results suggest that VHL-mediated restoration of p53 activity is required for Fas-induced apoptosis.

**HIF2α suppresses p53 via Akt-mediated activation of Hdm2.** Akt-mediated phosphorylation of the E3 ubiquitin ligase Hdm2 on Ser166 or Ser186 leads to nuclear localization of Hdm2 and enhanced degradation of p53 (37, 38). The activation of Akt is triggered on Ser473 phosphorylation, which represents a well-established survival signaling event downstream of growth factor receptors, many of which, including epidermal growth factor receptor and PDGF receptor, have been shown to be hyperstimulated in response to HIF2α-mediated production of secreted growth factors (39–41). Thus, the overexpression of HIF2α on loss or inactivation of VHL could lead to downregulation of p53 activity via the Akt/Hdm2 pathway (see Fig. 3, left). Consistent with this model, 786-MOCK cells that overexpress HIF2α showed elevated levels of phospho-Akt(Ser473) and phospho-Hdm2(Ser166) accompanied by a significant reduction in p53 levels in comparison with 786-VHL(WT) cells (Fig. 3A, right). In keeping with the notion that Ser166 phosphorylation promotes Hdm2 nuclear localization, 786-MOCK cells showed greater levels of nuclear Hdm2 than 786-VHL cells (Supplementary Fig. S4). To directly assess the contribution of Hdm2 in regulating p53 expression and function, 786-MOCK cells were treated with nutlin-3, a small-molecule inhibitor of Hdm2-p53 interaction. Nutlin-3 treatment resulted in a marked increase in p53 levels and p53 transcriptional activity as detected by upregulation of the p53 target gene product PUMA (Fig. 3B). Nutlin-3 treatment likewise increased p53 expression in A498 cells (Supplementary Fig. S5). Furthermore, treatment of 786-MOCK cells with LY294002, a small-molecule negative regulator of Akt (42), resulted in decreased level of phospho-Akt(Ser473), concomitantly with attenuation of phospho-Hdm2(Ser166) level, predictively resulting in increased p53 and PUMA levels (Fig. 3C, left). Notably, total Hdm2 levels also decreased in the presence of LY294002 (Fig. 3C), which is consistent with the notion that Akt-mediated phosphorylation of Hdm2 leads to increased Hdm2 stability (43). To determine whether hypoxia- or HIF2α-dependent production of growth factors was responsible, at least in part, for the activation of Akt as evidenced by increased levels of phospho-Akt (Ser473) in 786-MOCK cells (see Fig. 3A, right), we maintained 786-VHL(WT) cells in various conditioned media and subsequently measured phospho-Akt(Ser473) levels (Fig. 3C, right). The treatment of 786-VHL(WT) cells with 786-MOCK (i.e., high HIF2α–conditioned media as well as with conditioned medium from 786-VHL(WT) cells grown under hypoxia (i.e., high HIF2α) resulted in increased levels of phospho-Akt(Ser473) (Fig. 3C, right). In contrast, treatment with conditioned medium from 786-VHL(WT) cells grown under normoxia (i.e., low HIF2α) had a comparably negligible effect on phospho-Akt(Ser473) levels (Fig. 3C, right). These results support the notion that the activation of Akt in CCRCC cells devoid of VHL is driven, at least in part, by receptor-mediated signaling initiated by HIF2α-dependent secretion of growth factors. In addition, 786-RetroHIF2α cells infected with retrovirus-driving shRNA specifically targeting HIF2α showed a significant reduction in nuclear Hdm2 levels, indicative of reduced phospho-Hdm2(Ser166) levels (Supplementary Fig. S6), and restoration of p53 expression in comparison with 786-RetroEMPTY cells (Fig. 3D). Similarly, siRNA-mediated HIF2α knockdown in A498 cells increased p53 levels (Supplementary Fig. S6). These results support a novel link between HIF2α and p53, in which the stabilization of HIF2α in CCRCC leads to the suppression of p53 activity via the Akt/Hdm2 pathway.

**Primary CCRCC samples exhibit increased phospho-Hdm2 (Ser166) expression and low p53 expression.** High levels of intense membranous staining and positive cytoplasmic staining of a well-established HIF target, CAIX, were observed in CCRCC tumor cells from all 10 nephrectomy specimens, whereas normal renal cortex showed an absence of CAIX staining in the glomerulus and a moderate cytoplasmic staining in the adjacent proximal...
convoluted tubules (Fig. 4A). We next assessed phospho-Hdm2 (Ser166) levels and showed that the normal renal cortex displayed negative nuclear staining of phospho-Hdm2(Ser166) in proximal convoluted tubular epithelium along with positive nuclear staining in scattered glomerular epithelial cells and mesangial cells (Fig. 4B, left). However, tumor cells in 9 of 10 CCRCC nephrectomy samples exhibited intense nuclear phospho-Hdm2(Ser166) staining patterns (Fig. 4B, right), showing for the first time an increased nuclear staining of the activated phospho-Hdm2(Ser166) in CCRCC. Normal renal cortex showed weak nuclear p53 staining in glomerular cells and tubular epithelium (Fig. 4C, left). Tumor cells in all 10 CCRCC nephrectomy samples showed negative nuclear p53 staining (Fig. 4C, top right; data not shown). A breast carcinoma specimen served as a positive control for anti-p53(DO-1) antibody (Fig. 4C, bottom right).

Previous studies have shown that VHL status invariably correlates with HIF2α expression (21, 44). For example, Turner and colleagues showed that 94% of VHL-negative tumors overexpress HIF2α (21, 44), whereas Gordan and colleagues showed that 100% of VHL-negative tumors overexpress HIF2α (21, 44). Thus, VHL negativity implies HIF2α overexpression. Therefore, we examined phospho-Hdm2 and p53 status in the context of VHL status on tissue microarray consisting of 56 quadruplicate representatives of needle-core CCRCC biopsies (Fig. 4D). Consistent with the above nephrectomy specimens, the majority (82%) of CCRCCs exhibited negative p53 staining and the majority (73%) of p53-negative tumors with known VHL status, as determined by immunohistochemistry and direct DNA sequencing because subtle mutations could give "false-positive" VHL immunostaining, was determined to be VHL negative (Fig. 4D). Furthermore, the majority of phospho-Hdm2(Ser166)–positive samples showed negative VHL (69%) and negative p53 (63%) profiles (Fig. 4D). These results collectively support the notion that p53 expression is influenced by VHL/HIF2α via Hdm2.

Suppression of HIF2α restores p53 activity and reverses chemoresistance of CCRCC. We asked whether the restoration

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**Figure 3.** HIF2α suppresses p53 via Akt-mediated activation of Hdm2. A, model of HIF2α-dependent regulation of p53 via Hdm2 (left). Equal amounts of total cell lysates from 786-MOCK and 786-VHL(WT) cells were immunoblotted with the indicated antibodies (right). B, 786-MOCK cells treated with 5 μmol/L nutlin-3 were immunoblotted with the indicated antibodies. C, 786-MOCK cells treated with 5 μmol/L LY294002 were immunoblotted with the indicated antibodies (left). 786-VHL (WT) cells were treated for 16 h with conditioned media collected from 786-MOCK or 786-VHL(WT) maintained under normoxia or 786-VHL(WT) cells maintained under hypoxia. Total cell lysates were prepared and immunoblotted with the indicated antibodies (right). D, total cell lysates from 786-RetroEMPTY or 786-RetroshHIF2α cells were immunoprecipitated with anti-Hdm2 antibody and immunoblotted with anti-phospho-Hdm2(Ser166) antibody, which was subsequently stripped and reimmunoblotted with anti-Hdm2 antibody (bottom). IP, immunoprecipitation.
of p53 expression or activity could sensitize 786-MOCK cells to CH-11–induced apoptosis. 786-MOCK cells, which are resistant to CH-11–induced apoptosis (Fig. 5A, lane 2; see Figs. 1 and 2), were sensitive to CH-11 treatment in combination with nutlin-3 as evidenced by a dramatic induction of cleaved PARP (Fig. 5A, lane 4). Notably, nutlin-3 treatment alone was not sufficient to cause death in CCRCC cells, as the apoptotic stimulus provided by CH-11 was required to execute the apoptotic process in the presence of restored p53 expression (Fig. 5A, compare lanes 3 and 4). These results show that the restoration of p53 activity can sensitize CCRCC devoid of VHL to apoptotic signals.

We therefore asked whether the HIF2α-dependent suppression of p53 contributes to the chemoresistance of CCRCC because the efficacy of most chemotherapies is highly dependent on p53-mediated...
apoptosis to override the survival signals acquired by cancer cells (3, 4). CCRCC is one of most resistant tumors to conventional chemotherapy (1). Consistent with this view, 786-MOCK cells compared with their wild-type VHL-reconstituted counterparts displayed considerable resistance to doxorubicin (Fig. 5B, left), a DNA-damaging agent currently used in the clinic for the treatment of various tumors such as breast and ovarian cancers (45, 46). If the chemoresistance of CCRCC is indeed attributed to the impairment of p53 function via HIF2α overexpression, one prediction is that the suppression of HIF2α, or restoring the activity of p53 activity via nutlin-3 treatment, would reverse the chemoresistance of 786-MOCK cells to doxorubicin-induced death. Suppression of HIF2α via HIF2α-specific shRNA caused derepression of p53 expression via the inactivation of Hdm2 (see Fig. 3D), and as predicted, 786-RetroshHIF2α cells showed

Figure 5. Suppression of HIF2α restores p53 activity and reverses chemoresistance of CCRCC. A, 786-MOCK cells treated with (+) or without (−) 30 μmol/L nutlin-3 and/or 50 ng/mL CH-11 were immunoblotted with the indicated antibodies. B, 786-MOCK and 786-VHL(WT) cells treated with doxorubicin (Dox) were immunoblotted with the indicated antibodies (left). 786-RetroEMPTY and 786-RetroshHIF2α cells treated with (+) or without (−) 10 ng/mL doxorubicin were immunoblotted with the indicated antibodies (right). C, 786-MOCK cells treated with (+) or without (−) 30 μmol/L nutlin-3 and/or 10 ng/mL doxorubicin were immunoblotted with the indicated antibodies (top left) or stained with Annexin V-FITC and propidium iodide and analyzed by flow cytometry (top right). 786-MOCK and A498 cells treated with (+) or without (−) 30 μmol/L nutlin-3 and/or 200 nmol/L etoposide were immunoblotted with the indicated antibodies (bottom). D, 786-MOCK cells treated with 640 ng/mL neocarzinostatin (NCS) were immunoblotted with the indicated antibodies. Arrow, Hdm2; *, nonspecific protein band (left). 786-MOCK cells treated with (+) or without (−) 50 ng/mL CH-11 or 640 ng/mL neocarzinostatin were immunoblotted with the indicated antibodies (right).
increased sensitivity to doxorubicin-induced cell death (Fig. 5B, right). In accord, HIF2α knockdown enhanced p53 expression in A498 cells (Supplementary Fig. S7) and led to enhanced sensitivity to etoposide-induced apoptosis (Supplementary Fig. S8). 786-MOCK and A498 cells treated with nutlin-3 increased the levels of p53 and were dramatically sensitized to doxorubicin-induced, as well as eto-
poside-induced, cell death (Fig. 5C). Notably, such a treatment strategy has been adopted in preclinical trials against a variety of cancers including leukemias, lung cancer, and neuroblastoma (47–49). These results show that a death-inducing stimulus (i.e., DNA-damaging agent or death receptor activation) is required in addition to the restoration of p53 activity, via nutlin-3 or HIF2α suppression, to promote the death of CCRCC cells.

Neocarzinostatin is a DNA-damaging radiomimetic drug known to induce sequence-specific single-strand and double-strand breaks, as well as phosphoinositide-3-kinase–dependent Hdm2 autoubiquitylation that results in Hdm2 destabilization and consequent accumulation of p53 (50, 51). One prediction is that, unlike conventional DNA-damaging chemotherapeutic agents including cisplatin, Taxol, and 5-fluorouracil, which are unable to destabilize Hdm2 or induce apoptosis in 786-MOCK cells (Supplementary Fig. S9), neocarzinostatin treatment alone would induce 786-MOCK cell death because neocarzinostatin induces both DNA damage and p53 expression by destabilizing Hdm2. Notably, similar low doses of cisplatin, Taxol, and 5-fluorouracil sufficiently killed several other tumor cell lines, including H1299 lung carcinom-
a, SW-480 colorectal adenocarcinoma, HCT-116 colorectal, and SCC-9 head and neck squamous cell carcinoma cell lines (data not shown). As expected, 786-MOCK cells treated with neocarzi-

neostatin rapidly decreased Hdm2 levels as early as 1 hour post-
treatment and sustained this effect up to 24 hours (Fig. 5D; left; Supplementary Fig. S10). Importantly, neocarzinostatin treatment significantly increased p53 protein levels and PARP cleavage (Fig. 5D, right, lane 3), whereas CH-11 treatment alone resulted in an insignificant induction of PARP cleavage (Fig. 5D, right, lane 2; see also Figs. 1, 2, and 5A). These results support the notion that CCRCC cells could be rendered highly sensitive to chemotherapy-induced cell death by first restoring p53 function.

Discussion

Among urologic malignancies, CCRCC is the most lethal, with more than one third of the patients succumbing to this disease. Approximately one quarter of the patients present with metastatic disease and one third of patients who have been treated for surgery for clinically localized CCRCC eventually develop metastases. Patients with metastatic CCRCC have a median survival of 13 months with a chemotherapy response rate of only 4% to 6% (1).

The majority of human cancers have compromised p53-induced apoptotic pathways due to mutations in p53, which is tightly cor-
related with poor treatment response (5). CCRCC is arguably one of the most chemoresistant tumors despite rarely harboring the p53 gene mutations (6, 7). This paradox suggests that p53 may be inactivated via nonmutational mechanisms. In support of this notion, Gurova and colleagues showed that despite normal p53 status, p53-dependent transactivation in CCRCC cells is repressed via unknown mechanisms (10). Galban and colleagues showed that the reintroduction of VHL in CCRCC cells results in p53 accumulation, in part by enhanced binding of the RNA-stabilizing protein HIF2α (see Fig. 3D), which argues that VHL-dependent upregulation of p53 is indirect via HIF2α. Moreover, we did not observe any appreciable binding between VHL and p53 (Supplementary Fig. S11).

Accumulation of HIF2α is regarded as a critical oncogenic event in CCRCC (17). Here, we show that HIF2α induces Akt-mediated phosphorylation of Hdm2(Ser166), which activates and promotes the nuclear localization of Hdm2, resulting in the downregulation of p53. In support, multiple patient-derived CCRCC tumor samples almost invariably exhibited both strong nuclear-phospho-Hdm2 (Ser166) and membranous expression of CAIX, a classic marker of hypoxia and a reliable indicator of HIF activity. Consistent with increased nuclear-phospho-Hdm2(Ser166) expression, the level of nuclear p53 was negligible in virtually all CCRCC tumor samples tested, providing in vivo support for the link between aberrant HIF2 activity and inactivation of p53 in kidney cancer. These results provide a mechanistic explanation of why CCRCC cells are resistant to apoptosis triggered via Fas death receptor or DNA damage, and support a rationale for combining conventional chemotherapy drugs, such as doxorubicin or etoposide, with modalities that disarm Hdm2 such as nutlin-3 for the treatment of CCRCC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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