ITPR1 Protects Renal Cancer Cells against Natural Killer Cells by Inducing Autophagy

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Abstract

Clear cell renal cell carcinomas (RCC) frequently display inactivation of von Hippel-Lindau (VHL) gene leading to increased level of hypoxia-inducible factors (HIF). In this study, we investigated the potential role of HIF2α in regulating RCC susceptibility to natural killer (NK) cell–mediated killing. We demonstrated that the RCC cell line 786-0 with mutated VHL was resistant to NK-mediated lysis as compared with the VHL-corrected cell line (WT7). This resistance was found to require HIF2α stabilization. On the basis of global gene expression profiling and chromatin immunoprecipitation assay, we found ITPR1 (inositol 1,4,5-trisphosphate receptor, type 1) as a direct novel target of HIF2α and that targeting ITPR1 significantly increased susceptibility of 786-0 cells to NK-mediated lysis. Mechanistically, HIF2α in 786-0 cells lead to overexpression of ITPR1, which subsequently regulated the NK-mediated killing through the activation of autophagy in target cells by NK-derived signal. Interestingly, both ITPR1 and Beclin-1 silencing in 786-0 cells inhibited NK-induced autophagy and subsequently increased granzyme B activity in target cells. Finally, in vivo ITPR1 targeting significantly enhanced the NK-mediated tumor regression. Our data provide insight into the link between HIF2α, the ITPR1-related pathway, and natural immunity and strongly suggest a role for the HIF2α/ITPR1 axis in regulating RCC cell survival. Cancer Res; 74(23); 6820–32. ©2014 AACR.

Introduction

Clear cell renal cell carcinomas (RCC) account for approximately 3% of adult cancers (1). They are characterized by their hypervascularity and resistance to conventional anticancer treatments. For many decades, immunotherapy based on IL2 and IFNα has been the standard first-line treatment, although the response rate was typically less than 20% with multiple side effects (2). Thanks to improved understanding of RCC molecular pathogenesis, targeted therapies blocking angiogenesis or signal transduction pathways have been developed (3). While these therapies have undoubtedly improved clinical outcome, most patients eventually relapse and develop progressive disease (4).

Natural killer (NK) cells play an important role as first-line defenders in the host response to tumors and infections, in transplant rejection, and the development of tolerance (5). The presence of intratumoral NK in RCC correlates with improved patient survival (6). Recent studies suggest that immunotherapy may be an effective approach for patients with RCC (7), and emerging strategies are currently under investigation based on adoptive transfer of T or NK cells (8, 9). However, immune therapies based on adoptive transfer of in vitro activated autologous NK cells or on T-cell modulating agents such as antibodies against programmed death 1 and cytotoxic T lymphocyte–associated antigen-4 have not resulted in a significant clinical response (10). The activation status of NK cells has been reported as a reason for the lack of clinical response and evidence clearly indicates that NK cell activation is not sufficient to kill tumor cells due to complex interactions with the tumor and its inherent features. However, recent data from preclinical and clinical studies with donor-derived allogeneic NK cells have sparked interest in the possibility of exploiting the antitumor effect of NK cells (11). The major challenge for an effective NK-based immunotherapy is to overcome the mechanisms of tumor cell resistance toward NK cells.
HIF2α/ITPR1 Axis Regulates RCC Natural Killer–Mediated Lysis

regard, the molecular events involved in the susceptibility of RCC cells to natural cytotoxic effectors should be taken into consideration.

The majority of renal cancers presents clear cell carcinoma histology (12), which is usually associated with mutational or functional inactivation of the von Hippel-Lindau (VHL) gene (13). The VHL pathway targets the hypoxia-inducible factors (HIF) family of transcription factors, in particular HIF1α and HIF2α, for ubiquitin-mediated degradation via the proteasome (14). Consequently, VHL inactivation leads to the constitutive stabilization of HIFs and increased expression of target genes involved in the unfavorable tumor microenvironment (15). While the genes regulated by HIF1α and HIF2α often overlap (16), HIF2α is reported to regulate unique target genes and to be the critical factor in RCC development (17, 18). Although several reports have established a link between hypoxia-induced HIF1α and tumor cell resistance to immune effectors (19–21), the specific role of VHL mutations and selective activation of HIF2α in modulating RCC susceptibility to cytotoxic immune effector cells remains largely unknown.

Here, using a VHL-mutated RCC cell line that stabilizes only HIF2α, we demonstrated that HIF2α-dependent inositol 1,4,5-trisphosphate receptor, type 1 (ITPR1) expression regulates RCC susceptibility to NK-mediated cell lysis. Because HIF2α induced by VHL mutation or deletion is critical in RCC development, this study underscores the significance of its targeting in the future NK cell–based therapeutic strategies against RCC.

Materials and Methods

Cell culture and transfection

786-0, PRC3, and WT7 were obtained from Dr. William Kaelin, Jr. (Dana Farber Institute, Harvard Medical School, Boston, MA). WT7 and PRC3 cells were derived from 786-0 by stable transfection with pRC-HAVHL or the empty vector respectively (14). NKD cells were purified from healthy donor peripheral blood mononuclear cells using CD56-positive selection (STEMCELL technologies). NKD, NKL, and NK92 natural killer cell lines were maintained in RPMI supplemented with 20% FBS. Beclin-1 shRNA and lentivirus particles were purchased from Sigma and transduced according to the manufacturer’s instructions. NKD, NKL, and NK92 natural killer cell lines were transduced with tomato-LC3 encoding vector by transduction of Beclin-1 shRNA lentivirus particles (Santa Cruz Biotechnologies).

VHL-mutant constructions

The inducible VHL cell lines, 786-0-VHL WT (wild-type VHL), 786-0-VHL Y98H (VHL c.292T>C mutation associated with VHL disease type 2A), 786-0-VHL C162F (VHL c.485G>T mutation associated with VHL disease type 1), and 786-0 empty (empty vector), were created using the T-Rex lentiviral expression system (Invitrogen) and pLenti4/TO/V5-DEST-VHL according to the manufacturer’s protocol.

Western blotting

Western blotting was conducted as previously reported (23). Primary antibodies (Ab) against HIF2α, VHL, ITPR1, and microtubule-associated protein 1A/1B-light chain 3 (LC3) were purchased, respectively, from Novus Biologicals, BD Pharmingen, and Cell Signaling Technology.

Cytotoxicity assay

Four-hour chromium release assays were performed as described previously (19). Briefly, different effector:target (E:T) ratios were used on 1,000 target cells per well in round-bottom 96-well plates. After 4 hours of coculture, the supernatants were transferred to LumaPlate-96 wells (PerkinElmer), dried down, and counted on a Packard Instrument TopCount NXT. Percentage-specific cytotoxicity was calculated conventionally as described earlier (19).

Gene silencing by RNAi and shRNA transduction

Gene silencing was performed using predesigned sequence-specific siRNA purchased from Sigma (19). Briefly, 8 × 10⁴ cells were electroporated twice over 48 hours in serum-free medium with 20 mmol/L siRNA in an EasyJect Plus electroporation system (Equibio; 260 V, 450 μF). siRNA targeting luciferase was used as a negative control (5'-GCAAGUCUGACCUGAGUUCAU-3'; siRNA-CT). Predesigned lentiviruses (pLKO.1-puro) expressing siRNA against ITPR1, and shRNA control (CT) were purchased from Sigma and transduced according to the manufacturer’s instructions. Gene-specific targeting was evaluated by quantitative real-time PCR (RT-qPCR) and/or Western blotting.

SYBR Green RT-qPCR

Total RNA was extracted using TRizol solution (Invitrogen), and mRNA levels were quantified by SYBR Green qPCR method (Applied Biosystems; ref. 19). Relative expression was calculated using the comparative Ct method (2⁻ΔΔCt). Primer sequences are available upon request.

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) assays were performed as described (24). Primers used were: VEGF-FWD 5'-TCAAGCTTCCCTGGACACAT-3'; VEGF-REV 5'-ACCAAGTTTGGGAGCTGAG-3'; ITPR1-FWD 5'-TTCACATTCGAGACCCAGCACA-3'; and ITPR1-REV 5'-GGGTCACTGCTAATCATTCC-3'.

Microarray analysis

DNA microarray analysis was performed as previously reported (19) using Agilent Human Whole Genome Microarray: 44,000 spots. Data mining was done using Rosetta Resolver software, IPA (Ingenuity Pathways Analysis).

Confocal microscopy and analysis of immunologic synapse formation

Effector (NKL) and tumor target cells were allowed to adhere on poly-lysine–coated coverslips (Sigma) at a 2:1 E:T ratio for 30 minutes and immunologic synapse formation was analyzed as described earlier (19). Efficiency of conjugate formation
between NK and RCC cells was calculated by determining the ratio of effector cells forming conjugates with target cells to total target cells × 100. The formation of autophagosomes in tomato-LC3-expressing 786-0 cells was monitored by laser scanning confocal microscopy (LSM)-510-Meta (Carl Zeiss) using 40× oil immersion objective.

Treatment with perforin and granzyme B

Native rat perforin was purified from RNK16 cells as described (25). Animal use was approved by the Animal Care and Use Committee of the Gustave Roussy Institute (Villejuif, France). Human granzyme B was purified from the human NK cell line YT-Indy as described (26). Cells were washed and equilibrated 5 minutes in HBSS with 10 mmol/L HEPES, pH 7.5, 4 mmol/L CaCl₂, 0.4% BSA before adding sublytic perforin and/or granzyme B at the indicated concentration, diluted in granzyme B buffer (HBSS, 10 mmol/L HEPES, pH 7.5). Sublytic perforin concentration was determined independently for each experiment as the concentration required to induce 5% to 15% propidium iodide (PI) uptake (2 μg/mL; Sigma) measured 20 minutes later by flow cytometry (BD Accuri C6 Flow Cytometer).

NK-delivered granzyme B detection in target cells

The level of granzyme B in target cells was assessed by coculture of target cells with YT-INDY-NK cells as previously described (27). The presence of granzyme B in target cells was evaluated by immunoblot using granzyme B antibody (BD biosciences). Intracellular active granzyme B in target cells was measured by using Prizm granzyme B cell-mediated cytotoxicity assay kit following manufacturer’s instructions (Origene).

Immunohistochemistry

Immunohistochemical (IHC) staining for HIF2α and ITPR1 was performed as described earlier (28) using tissue microarrays (TMA) from 235 patients with RCC selected during the period 1993 to 2004 from the pathology department of Hôpital Kremlin Bicêtre (Bicêtre, France). The criterion for immunopositivity was a minimum of 10% positive cells evaluated using a binary qualitative score (0, negative; 1, positive).

In vivo tumorigenic assay

To avoid tumor rejection (human 786-0 cells) by the immune system of mice, we used murine tumor cells. Six- to 7-week-old mice Balb/c mice (Harlan) were used. Mice were housed at the Institut Gustave Roussy animal facility and treated in accordance with institutional animal guidelines. Mice (n = 10 per group) were inoculated subcutaneously with 2.5 × 10⁵ Renca cells.

Calcium video imaging

Measurements of the intracellular calcium concentration were performed at 37°C with a diaphot 300 microscope (Nikon) and with MetaFluor software. The video acquisition was made for 10 minutes. Average calcium responses were calculated for all tumor cells with MetaFluor Analyst software, and statistical analysis was made with GraphPad Prism software.

Results

VHL mutation decreased RCC cell susceptibility to NK-mediated lysis independently of the alteration of synapse formation and NK ligand expression

To investigate the influence of VHL mutations on the regulation of RCC susceptibility to NK effector cells, we used the VHL-mutated 786-0 and PRC3 cell lines as well as WT7 cell lines stably transfected with wild-type VHL (14). As shown in Fig. 1A, transfection of VHL in WT7 cells resulted in the loss of HIF2α expression, whereas parental 786-0 and PRC3 cells constitutively expressed HIF2α. Similarly, HIF2α target genes, such as VEGF, carbonic anhydrase-9 (CAIX), and glucose transporter 1 (SLC2A1), were significantly downregulated in WT7 (Fig. 1B). As shown in Fig. 1C and D, 786-0 and PRC3 cell lines were significantly more resistant to both NK92- and NKD-mediated lysis than to WT7 at all E:T ratios. We observed similar results with another NK cell line (NKL) and 2 different NKD cells (Supplementary Fig. S1A–S1C). We also performed Cr⁵¹ cytotoxic assay using A498-pmock RCC VHL-deficient cell line (expressing exclusively HIF2α) and A498 cell line stably transfected by a vector encoding the wild-type VHL gene cocultured with NKL and NKL effector cells. Our data indicate that reintroduction of a wild-type pVHL in A498 cells resulted in a significant increase in NK-mediated lysis as compared with A498-pmock cells (data not shown). These data indicate that VHL mutations play a critical role in the acquisition of RCC cell resistance to NK-mediated lysis.

We next examined conjugate formation between NKL and the 3 RCC cell lines by confocal microscopy. 786-0, PRC3, and WT7 were able to form stable conjugates with NKL cells with the same efficiency (Fig. 1E and F). Moreover, no difference was observed in the mean fluorescence intensity of the phospho-tyrosine staining when NKL cells were cocultured with 786-0, PRC3, and WT7 cell lines (Fig. 1G). In addition, a panel of NK-activating and -inhibitory receptor ligands was analyzed. As shown in Supplementary Fig. S3, no significant difference in the surface expression of NK ligands between 786-0, PRC3, and WT7 cell lines was observed. This indicates that the differential RCC cell line susceptibility to NK-mediated lysis associated with VHL mutations was not due to a differential NK ligand expression or an alteration of immune synapse formation.

Silencing HIF2α attenuated 786-0 cell resistance to NK-mediated lysis via ITPR1

To determine whether resistance of VHL-mutated 786-0 cells to NK-mediated lysis involved the constitutive expression of HIF2α, we knocked down HIF2α gene expression (Fig. 2A) and its transcriptional activity (Fig. 2B) by 2 different siRNAs. Interestingly, HIF2α silencing resulted in a significant increase of 786-0 target cell lysis by NK92 (Fig. 2C) and NKL (Fig. 2D). Similar results were also observed with another NKL and 2 different NKD (Supplementary Fig. S1D and S1E). In addition, we inhibited HIF2 expression by siRNA in 786-0 cells and then transfected these cells with a plasmid encoding pcDNA3-HIF-2. The results showed that overexpression of HIF2 in HIF2-knocked down 786-0 cells restored their resistance to NK-mediated lysis, thus emphasizing the critical role of HIF2 in the acquisition of 786-0 cells resistance to NK effectors (data not shown).
Data depicted in Supplementary Fig. S4 demonstrate that knockdown of HIF2α did not affect NK-activating and inhibitory ligand expression. These data point to an essential role of HIF2α in the acquisition of resistance to NK-mediated lysis in VHL-mutated RCC cells.

To gain insight into the mechanism by which HIF2α regulated RCC susceptibility to NK-mediated lysis in VHL-mutated cells, we performed a global gene expression analysis using DNA microarray. We used 786-0 cells transfected with HIF2α or control siRNAs. As shown in Fig. 2E and F, 28 genes were differentially expressed with more than 2-fold change and an adjusted P value of 0.05. We first focused on 10 downregulated genes with more than 3-fold change namely: F3, PTHLH, SLC2A4, ADAM, ITPR1, GFRα2, VEGFα, EDN1, ANGPTL4, and EPAS1.
strong positive correlation was found between microarray data and qRT-PCR results (Fig. 2G). Three candidate genes (ANGPTL4, ADM, and ITPR1) were then selected on the basis of their fold change and their involvement in cell death and survival. While silencing of ANGPTL4 had no effect on 786-0 tumor cell susceptibility to NK-mediated lysis (Supplementary Fig. S2A–S2C), a slight increase was observed following targeting of ADM (Supplementary Fig. S2D–S2F). Interestingly, siRNA-mediated silencing of ITPR1 (Fig. 2H) resulted in a dramatic and significant increase of 786-0 susceptibility to
both NK92- and NKD-mediated lysis (Fig. 2I and J). These results clearly demonstrate a significant role of ITPR1 in the resistance of VHL-mutated RCC cells to lysis by NK effectors.

**ITPR1 knockdown inhibited NK-mediated autophagy induction and increased granzyme B activity in 786-0 cells**

ITPR1 has been shown to be involved in the control of intracellular calcium signaling and the regulation of autophagy (29). To investigate how ITPR1 regulates 786-0 cell resistance to NK-mediated lysis, we first assessed intracellular calcium accumulation in either 786-0 cells alone or 786-0 cells transfected with siRNA-ITPR1 with or without NK stimulation. Surprisingly, no significant difference in intracellular calcium accumulation was observed (Supplementary Fig. S3A and S3B). Similarly, we did not observe any difference in autophagy induction in VHL-mutated (786-0 and PRC3) and VHL-corrected RCC (WT7) cells in the absence of NK (Supplementary Fig. S3C). Moreover, 786-0 cells transfected with siRNA-HIF2α or siRNA-ITPR1 neither induced nor inhibited autophagy after culture under serum starvation conditions or treatment with hydroxychloroquine (Supplementary Fig. S3D and S3E). Finally, 786-0 cells were transfected with tomato-LC3 vector, and the formation of autophagosomes was assessed by confocal microscopy. We did not detect any difference in the level of autophagosomes in 786-0 cells transfected with siRNA-control or siRNA-ITPR1 in the absence of NK cells (Fig. 3A).

We then examined whether NK cells can induce autophagy in target cells. For this purpose, 786-0 cells were transfected with tomato-LC3 vector and siRNA-CT or siRNA-ITPR1, cocultured with NK cells and the formation of autophagosomes was assessed by confocal microscopy. Interestingly, we show that NK cells were able to induce autophagy process only in 786-0 cells transfected with siRNA-CT but not in ITPR1 knocked down cells (Fig. 3B). The induction of autophagy in siRNA-CT cells seems to be responsible for the resistance of 786-0 cells to NK-mediated lysis. Time lapse video microscopy performed on control and ITPR1-defective cells provided strong evidence that control cells displaying several autophagosomes survived NK-mediated killing; however, ITPR1-defective cells displaying no autophagosomes underwent NK-mediated cell killing (Fig. 3C and Supplementary Movies S1 and S2).

We further analyzed the LC3-II levels by Western blotting using NK92 cell line. Figure 3D showed a dramatic increase in LC3-II in 786-0 cells when cocultured with NK cells (+) compared with cells cultured without NK cells (−). This result indicates that autophagy in target cells is induced by a signal derived from NK cells. Interestingly, targeting ITPR1 in target cells blocks the ability of NK cells to activate autophagy in target cells, indicating that ITPR1 positively controls autophagy in target cells.

We have recently shown that selective autophagy impairs innate tumor immune response by degrading NK-derived granzyme B (27). We next evaluated whether targeting autophagy may affect the level and the activity of NK-derived granzyme B in 786-0 cells. Strikingly, our results indicate that targeting Beclin-1 significantly increased NK-derived granzyme B level (Fig. 3E) in 786-0 cells. Furthermore, no NK signal (NKG2D) was detected in CT or BECN1 siRNA cells after coculture, thereby ruling out any contamination by NK cells. Our results (Fig. 3F) demonstrate that 786-0 cells displayed a significant lower level of granzyme B than WT7 cells.

Results depicted in Fig. 3G further show that the serine protease activity of granzyme B was significantly increased in ITPR1 and Beclin-1 siRNA-treated 786-0 cells after they encountered NK cells. It is important to note that both ITPR1- and Beclin-1–silenced cells formed the same number of immune conjugates as compared with control cells (Fig. 3H).

To validate these results, we measured activation of key mediators of the granzyme B signaling pathway in 786-0 cells transfected with either siRNA-control or siRNA-ITPR1 and incubated with sublytic perforin and human granzyme B. We observed that Bid (noncleaved form of Bid, 22 kDa), pro-caspase-9, and pro-caspase-3 cleavages (Fig. 3I) were significantly increased after perforin/granzyme B loading of ITPR1-silenced cells as compared with control cells. Taken together, these results clearly demonstrate that ITPR1 knockdown inhibits NK-induced autophagy and subsequently increased NK-delivered granzyme B activity leading to an increased susceptibility to granzyme B and NK-mediated cell death.

**ITPR1 is a new direct target gene of HIF2α in RCC cells**

We further asked whether ITPR1 could be a direct target of HIF2α. 786-0 cells transfected with 2 different HIF2α siRNAs resulted in a significant decrease in ITPR1 expression at mRNA (Fig. 4A) and protein levels (Fig. 4B). In addition, varying HIF2α stabilization levels in 4 786-0–derived cell lines with different VHL mutations (inducible wild-type VHL, VHL-Y98H, VHL-C162F, empty vector) revealed that HIF2α expression levels positively correlated with ITPR1 (Fig. 4C and D). Similarly, in A498 cells transfected with a vector encoding wild-type VHL (A498-pVHL), we also observed a decrease in HIF2 and ITPR1 mRNA and protein expression as compared with A498 cells transfected with a control vector (A498-pmock; data not shown). These results further support our findings using 786-0, PRC3, and WT7 cells.

To examine a putative direct transcriptional regulation of ITPR1 by HIF2α, we analyzed the promoter region of ITPR1 gene using fuzznuc (EMBOSS) software and found 10 putative hypoxia-response elements (HRE; 5′GCGTGG 3′) suggesting a possible direct interaction between HIF2α and the ITPR1 promoter. Immunoprecipitation of the chromatin complexes formed in 786-0 and PRC3 cells showed significant enrichment of the ITPR1 promoter fragment containing HRE-7 with the specific HIF2α antibody as compared with the WT7 cell line or the IgG antibody. Similar enrichment of VEGF promoter fragment containing HRE, a well-known HIF2α target gene (30), was also observed in PRC3 and 786-0, but not in WT7 cells (Fig. 4E).

IHC staining was finally performed on tissue sections from 235 patients diagnosed with RCC to examine the relationship between ITPR1 and HIF2α expression. Of 235 patients, 203 were negative for ITPR1 staining and 32 were positively stained. Among the ITPR1 negatively stained patients (n = 203), 178 were also negative for HIF2α (87%). For the ITPR1 positively stained patients (n = 32), 20 were found to be stained...
by HIF2α (62.5%). Statistical analysis showed a significant correlation between HIF2α and ITPR1 staining in these patients (Table 1).

Taken together, these results clearly demonstrate that ITPR1 is a direct novel target gene of HIF2α in RCC cell lines and point to the existence of a functional link between ITPR1 and HIF2α in RCC.

In vivo targeting of ITPR1 in Renca cells promoted tumor regression in mice

To investigate the in vivo relevance of the HIF2α–ITPR1 pathway, we used the Renca murine RCC with constitutively stabilized HIF2α. When Renca cells were transfected with siRNA against HIF2α, a significant decrease in ITPR1 was observed at both protein (Fig. 5A) and mRNA levels (Fig. 5B). Moreover, treatment of Renca cells with increasing doses of digoxin (an inhibitor of HIF1α and HIF2α; ref. 31) resulted in a simultaneous decrease in HIF2α and ITPR1 expression in a dose-dependent manner (Fig. 5C and D). Furthermore, using stably transduced Renca cells with 2 different sequences of shRNA targeting ITPR1 (Lenti-shRNA–ITPR1–1 and Lenti-shRNA–ITPR1–2) or control shRNA (Fig. 5E), we demonstrated that ITPR1 inhibition resulted in a significant decrease in Renca tumor growth as compared with control tumors. When NK cells were depleted in Balb/c mice using anti-Asialo-GM1 antibodies, a more significant tumor growth was observed as compared with untreated mice. Importantly, the combination of ITPR1 inhibition and NK cell depletion resulted in more robust tumor growth than NK depletion alone (Fig. 5F). These data are consistent with a role of ITPR1 in tumor progression, at least in part, by a mechanism involving the regulation of tumor cell susceptibility to NK-mediated cell lysis.

Discussion

VHL mutations play a significant role in regulating the development, invasiveness, and survival characteristics of RCC (32). However, the role of VHL mutations in modulating RCC susceptibility to cytotoxic immune effector cells remains largely unknown. In this study, we show that the restoration of VHL function in 786-0 and A498 cells significantly increases their sensitivity to NK-mediated lysis, suggesting a role for VHL mutations in attenuating RCC resistance to lysis. We further show that VHL operates by a mechanism independent of synapse formation and NK ligand expression, thereby excluding an alteration of target recognition by NK cells. It is well established that VHL mutations in RCC may result in stabilization of both HIF1α and HIF2α or in a selective stabilization of HIF2α (33). Accumulating evidence indicates that HIF2α is more important for tumor progression, whereas HIF1α mostly behaves as a tumor suppressor (34). These antagonistic effects have been partially explained by the ability of HIF2α to regulate genes involved in activation of proliferation (MYC, CyclinD), inhibition of apoptosis (p53), and promotion of metastasis (CXCR4). In contrast, HIF1α regulates preferentially apoptotic genes (BNIP3, p53) and inhibits proliferation (MYC inhibition, p21, p27; ref. 35). In the RCC cell line (786-0 cells) used in this study, VHL mutation selectively induces HIF2α stabilization, thus representing an ideal model system to specifically examine the effect of HIF2α on RCC susceptibility to NK-mediated lysis. Using this model, we showed that targeting HIF2α in 786-0 cells attenuates their resistance to NK-mediated lysis, whereas HIF2α overexpression in HIF2α knocked down 786-0 cells restored their resistance to NK-mediated lysis, suggesting a critical role of HIF2α in the acquisition of resistance to cytotoxicity associated with VHL mutations. Perier and colleagues have previously reported that the restoration of VHL function in RCC4 and RCC6 cell lines resulted in a decreased susceptibility to NK-mediated lysis by a mechanism involving modulation of HLA-1 (HLA-A and HLA-G) expression (36). These discrepancies from our findings may be due to the fact that in our model, 786-0 cell line stabilizes only HIF2α whereas the RCC4 and RCC6 cell lines stabilize both HIF1α and HIF2α. In this regard, it should be noted that hypoxia and HIF (HIF1α and HIF2α) effects on NK ligand expression have been reported to be cell-type-specific. Very recently, it was shown that hypoxia did not alter the surface expression of NK cell ligand in multiple myeloma (37), whereas Siemens and colleagues provided evidence that hypoxic stress promotes the shedding of MHC I chain–related molecules MICA and MICB in prostate...
cancer cells, resulting in a decreased sensitivity to NK-mediated lysis (38).

DNA microarray–based global transcriptional profiling in HIF2α–targeted 786-0 cells and RT-qPCR revealed a set of genes related to their fold change. Among the differentially expressed genes, we selected 3 genes, ANGPTL4, ADM, and ITPR1, on the basis of their fold change and involvement in cell death and survival. Very interestingly, ITPR1 was found to be the most prominent gene involved in the regulation of RCC resistance to NK-mediated lysis.

It is well established that ITPR1, a member of IP3 receptor family, is a ligand-gated ion channel that mediates calcium release from the intracellular stores specially the endoplasmic reticulum (ER; refs. 39–41). Under our experimental conditions, calcium accumulation was unchanged in cells regardless of whether they expressed or not ITPR1 in response to NK effectors, ruling out a role for ITPR1 in global calcium accumulation. However, it should be noted that ITPR1 overexpression could lead to a differential distribution of calcium in intracellular compartments (endoplasmic reticulum and mitochondria; ref. 42), which could modify the cell response to apoptotic stimuli. In addition, compensatory effects by other ITPR family members including ITPR2 and ITPR3 members could also occur.
Because conflicting studies have suggested a role for HIF2α and ITPR1 in the regulation of autophagy (29), we checked in our model whether autophagy was involved in the acquisition of resistance to NK by the 786-0 cells. We therefore examined the effect of autophagy modulators including serum starvation and hydroxychloroquine in the 3 RCC cell lines and in 786-0 transfected or not with siRNA-HIF2α and siRNA-ITPR1 and no difference in autophagy markers was observed (Supplementary Data S4). Menard and colleagues have reported that HIF2α was a potent inhibitor of hypoxia-induced autophagy (43). Bohensky and colleagues also showed that in maturing chondrocytes expressing both HIF1α and HIF2α,
the latter acts as a brake on the autophagy accelerator function of HIF1α (44).

We next evaluated whether NK cells can induce autophagy in 786-0 cells. Interestingly, we showed that NK cells were able to induce autophagy only in 786-0 cells transfected with siRNA-CT but not in ITPR1 knocked down cells, suggesting an involvement of ITPR1 in NK-induced autophagy in VHL-mutated tumor target cells. This result is in agreement with a recent report indicating that NK cells are able to induce autophagy promoting tumor cell survival and treatment resistance (45). Several studies demonstrated that genetic or pharmacologic inhibition of ITPR receptors can inhibit or stimulate autophagy (42). Inhibition of ITPR1 signaling pathway can suppress the autophagy indirectly by mechanisms involving endoplasmic reticulum and calcium remodeling (46). ITPR1 has been reported to directly inhibit starvation-induced autophagy through increased binding to Beclin-1 (47). Recently, Baginska and colleagues demonstrated that activation of autophagy under hypoxia resulted in the degradation of NK-derived granulysin B, which compromises the ability of NK cells to eliminate tumor cells (27). As ITPR1-regulated autophagy was also involved in the impairment of NK-mediated lysis of 786-0 cells, we asked whether a subsequent degradation or deactivation of granulysin B occurred in these cells. Our results strongly support that the increased expression of ITPR1 by HIF2α leads to the activation of autophagy following contact with NK cells, which subsequently decreased the activity of NK-derived granulysin B. Although the precise mechanism by which autophagy affects the level or serine protease activity of granulysin B is not addressed here, we strongly believe that ITPR1-mediated NK-induced autophagy induction could be associated with the decrease in NK-derived granulysin B activity in target cells making them less sensitive to NK-mediated killing.

Here, we obtained experimental evidence indicating that ITPR1 is a novel target of HIF2α and that its expression was regulated by HIF2α at both mRNA and protein levels. This was further confirmed by ChIP assay. Furthermore, using a large group (235) of patients with RCC, we demonstrated the existence of a significant correlation between HIF2α and ITPR1 expression. Although the existence of a link between ITPR1 and hypoxia in neuronal cells, mouse kidney, and human embryonic kidney 293 cells has been reported (48), the direct involvement of HIFs in the regulation of ITPR1 has not been established. Nevertheless, as HIF1α and HIF2α are known to have many common target genes (15, 16), and given that the hypoxia response element (A/G CGTG) is common for HIF1 and HIF2 (49), we cannot exclude the possibility that HIF1α could also regulate ITPR1 in cells stabilizing HIF1α.

The in vivo data presented here reveal that ITPR1 targeting combined with NK depletion significantly enhanced tumor growth, supporting the involvement of ITPR1 in regulating the in vivo susceptibility of Renca cells to NK activity. This is in agreement with previous reports indicating that overexpression of ITPR3 in colorectal carcinoma cells reduced apoptosis, whereas ITPR3 inhibition increased cell death (50). However, we cannot exclude the role of other immune cells in regulating Renca tumor growth. Taken together, these data support a role of the inositol triphosphate receptor family in tumor progression. Thus, the HIF2α/ITPR1 axis, triggered by VHL mutations in RCC, may play a critical role in controlling the switch from antitumor immunity to tumor cell survival and growth.

Because HIF2α has been reported to be a critical factor in tumor progression in RCC (18), it is tempting to speculate that ITPR1 could be one of the mechanisms by which HIF2α drives tumor growth in RCC. In this context, future protocols of NK cell–based immunotherapy should integrate the intrinsic features of tumor cells (i.e., VHL mutations and subsequent hypoxia status) to improve NK cell–mediated antitumor activity and their cross-talk with tumor microenvironment in the context of its stressor complexity and heterogeneity.

Further studies are needed to define whether HIF2α or ITPR1 may be considered as a potential target in future therapeutic protocols that aim to improve NK cell responses in patients with RCC and other solid malignancies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Acknowledgments
The authors thank Dr. William Kaelin, Jr. for providing RCC cell lines used in this study and for his helpful suggestions; Denis Martinvalet for the human tumor xenografts; Y. Laplanche for her assistance in statistical analysis.

Grant Support
This work was supported by the ARTuR (Association pour la Recherche sur les Tumeurs du Rein), ARC (Association pour la Recherche sur le Cancer) 2012-2013; N° SPF201212050624, and a grant from CRP-Santé (LHCE-2013 11 05).

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Received February 5, 2014; revised August 19, 2014; accepted September 22, 2014; published OnlineFirst October 8, 2014.


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