ITPR1 protects renal cancer cells against natural killer cells by inducing autophagy


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Clear cell renal cell carcinomas (RCC) frequently display inactivation of von Hippel-Lindau (VHL) gene leading to increased level of hypoxia inducible factors (HIFs). In this study, we investigated the potential role of HIF-2α 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 to the VHL-corrected cell line (WT7). This resistance was found to require HIF-2α stabilization. Based on global gene expression profiling and ChIP assay, we found ITPR1 (inositol 1,4,5-trisphosphate receptor, type 1) as a direct novel target of HIF-2α and targeting ITPR1 significantly increased susceptibility of 786-0 cells to NK-mediated lysis. Mechanistically, HIF-2α 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 insights into the link between HIF-2α, the ITPR1-related pathway and natural immunity and strongly suggest a role for the HIF-2α /ITPR1 axis in regulating RCC cell survival.
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 anti-cancer treatments. For many decades, immunotherapy based on interleukin-2 (IL-2) and interferon-alpha (IFN-α) 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 intra-tumoral 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 alloreactive NK cells has 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 towards NK cells. In this 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 HIF-1α and HIF-2α, for ubiquitin-mediated degradation via the proteasome (14). Consequently, VHL inactivation leads to constitutive stabilization of HIF’s and increased expression of target genes involved in the unfavorable tumor microenvironment (15). While the genes regulated by HIF-1 and HIF-2 often overlap (16), HIF-2 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 HIF-1α and tumor cell resistance to immune effectors (19-21), the specific role of VHL mutations and selective activation of HIF-2α in modulating RCC susceptibility to cytotoxic immune effector cells remains largely unknown.

Here, using a VHL-mutated RCC cell line that stabilizes only HIF-2α, we demonstrated that HIF-2α-dependent inositol 1,4,5-trisphosphate receptor, type 1 (ITPR1) expression regulates RCC cell susceptibility to NK-mediated cell lysis. Because HIF-2α 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 300U/ml recombinant human IL2 (rhIL2). The Renca murine renal carcinoma cell line was originally obtained from a tumor that arose spontaneously in the kidney of BALB/c mice (45). 786-0 cells were transfected with tomato-LC3 encoding vector by Lipofectamine 2000 according the manufacturer instructions (Invitrogen). Autophagy defective 786-0 cells were generated by transduction of Beclin1 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 (46). Primary antibodies (Abs) against HIF-2α, VHL, ITPR1 and Microtubule-associated protein 1A/1B-light chain 3 (LC3) were purchased respectively from Novus Biologicals, BD Phamrmingen 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 1000 target cells per well in round-bottom
96-well plates. After 4h co-culture, 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 RNA interference and shRNA transduction**

Gene silencing was performed using predesigned sequence-specific small interfering RNA purchased from Sigma (19). Briefly, 8.10^6 cells were electroporated twice over 48h in serum-free medium with 20 mM siRNA in an EasyJect Plus electroporation system (EquiBio; 260 V, 450µF). siRNA targeting luciferase was used as a negative control (59-GCAAGCUGACCCUGAAGUUCAU-39) (siRNA-CT). Predesigned lentiviruses (pLKO.1-puro) expressing shRNA against HIF-2α, ITPR-1 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 blot.

**SYBR-GREEN RT-qPCR**

Total RNA was extracted using TRIzol solution (Invitrogen) and mRNA levels were quantified by SYBR-GREEN qPCR method (Applied Biosystems) (19). Relative expression was calculated using the comparative Ct method (2^-ΔCt). Primer sequences are available upon request.

**ChIP assays**

ChIP (Chromatin immunoprecipitation) assays were performed as described (47).

Primers used were: VEGF-FWD 5’-TCAGTTCCCTGGCAACAT-3’; VEGF-REV 5’-ACCAAGTTTGGAGCGTCT-3’; ITPR1-FWD 5’-TCACATCTCACTAAGCCACT-3’; and ITPR1-REV 5’-GGGTCACTGCCTAACTCATTC-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 immunological 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 40X oil immersion objective.

Treatment with perforin (PFN) and granzyme B (GzmB)

Native rat PFN was purified from RNK16 cells as described (48). Animal use was approved by the Animal Care and Use Committee of the Gustave Roussy Institute. Human GzmB was purified from the human NK cell line YT-Indy as described (49). Cells were washed and equilibrated 5 min in HBSS with 10 mM Hepes pH7.5, 4 mM CaCl₂, 0.4% BSA before adding sublytic PFN and/or GzmB at the indicated concentration, diluted in PFN buffer (HBSS, 10 mM Hepes pH7.5). Sublytic PFN concentration was determined independently for each experiment as the concentration required to induce 5-15% propidium iodide (PI) uptake (2 ug/mL) (Sigma) measured 20 min later by flow cytometry (BD Accuri™ C6 Flow Cytometer).

NK-delivered GzmB detection in target cells

The level of GzmB in target cells was assessed by co-culture of target cells with YT-INDY-NK cells as previously described (23). The presence of GzmB in target cells was evaluated by immunoblot using GzmB antibody (BD biosciences). Intracellular active GzmB in target cells was measured by using Prizm GzmB cell mediated cytotoxicity assay kit following manufacturer instructions (ORIGENE).

Immunohistochemistry (IHC)

IHC staining for HIF-2α and ITPR1 was performed as described earlier (50) using Tissue microarrays (TMA) from 235 RCC patients selected during the period 1993-2004 from the...
pathology department of Hôpital Kremlin 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 sub-cutaneously with 2.5 X 10^6 Renca cells.

**Calcium (Ca^{2+}) video imaging**

Measurements of the intracellular calcium concentration were performed at 37°C with a diaphot 300 microscope (Nikon, Melville, NY) and with MetaFluor software. The video acquisition was made for 10 min. Average Ca^{2+} 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 Figure 1A, transfection of VHL in WT7 cells resulted in the loss of HIF-2α expression, whereas parental 786-0 and PRC3 cells constitutively expressed HIF-2α (Figure 1A). Similarly, HIF-2α target genes: Vascular endothelial growth factor (VEGF), carbonic anhydrase-9 (CAIX) and Glucose transporter 1 (SLC2A1) were significantly down-regulated in WT7 (Figure 1B). As shown in Figure 1C and 1D, 786-0 and PRC3 cell lines were significantly more resistant to both NK92- and NKD-mediated lysis as compared to WT7 at all effector /target ratios. We observed similar results with another NK cell line (NKL) and two different NKD cells (Figure S1A, S1B and S1C). We also performed Cr51 cytotoxic assay using A498-pmock RCC VHL-deficient cell line (expressing exclusively HIF-2alpha) and A498 cell line stably transfected by a vector encoding the wild type VHL gene co-cultured with NKD 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 to 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 three RCC cell lines by confocal microscopy. 786-0, PRC3 and WT7 were able to form stable conjugates with NKL cells with the same efficiency (Figure 1E and 1F). Moreover, no difference was observed in the mean fluorescence intensity of the phospho-tyrosine staining when NK cells were co-cultured with 786-0, PRC3 and WT7 cell lines (Figure 1G). In addition, a panel of NK activating and inhibitory receptor ligands was analyzed. As shown in Figure 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 HIF-2α 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 HIF-2α, we knocked down HIF-2α gene expression (Figure 2A) and its transcriptional activity (Figure 2B) by two different siRNAs. Interestingly, HIF-2α silencing resulted in a significant increase of 786-0 target cell lysis by NK92 (Figure 2C) and NKD (Figure 2D). Similar results were also observed with another NKL and two different NKD (Figure S1D and S1E). In addition, we inhibited HIF-2 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 HIF-2 in HIF2 knocked down 786-0 cells restored their resistance to NK-mediated lysis, thus emphasizing the critical role of HIF-2 in the acquisition of 786-0 cells resistance to NK effectors (data not shown). Data depicted in Figure S4 demonstrate that knockdown of HIF-2α did not affect NK activating and inhibitory ligand expression. These data point to an essential role of HIF-2α in the acquisition of resistance to NK-mediated lysis in VHL-mutated RCC cells.

To gain insight into the mechanism by which HIF-2α 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 HIF-2α or control siRNAs. As shown in Figure 2E and 2F, 28 genes were differentially expressed with more than 2-fold change and an adjusted p-value of 0.05. We first focused on 10 down-regulated genes with more than 3-fold change namely: F3, PTHLH, SLC2A, ADM, ITPR1, GFRA2, VEDGFA, EDN1, ANGPTL4, and EPAS1. A strong positive correlation was found between microarray data and qRT-PCR results (Figure 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 (Figure S2A, S2B and S2C), a slight increase was observed following targeting of ADM (Figure S2D, S2E and S2F). Interestingly, siRNA-mediated silencing of ITPR1 (Figure 2H) resulted in a dramatic and significant increase of 786-0 susceptibility to both NK92- and NKD-mediated lysis (Figure 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 knock-down inhibited NK-mediated autophagy induction and increased GzmB activity in 786-0 cells.**

ITPR1 has been shown to be involved in the control of intracellular calcium signaling and the regulation of autophagy (22). To investigate how ITPR1 regulates 786-0 cells 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 (Figure 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 (Figure S3C). Moreover, 786-0 cells transfected with siRNA-HIF-2α or siRNA-ITPR1 neither induced nor inhibited autophagy after culture under serum starvation conditions or treatment with hydroxychloroquine (HCQ) (Figure 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 (Figure 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, co-cultured with NK cells and the formation of autophagosomes in target cells 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 (Figure 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 (Figure 3C and Supplementary movies S1 and S2).

We further analyzed the LC3-II levels by western blot using NK-92 cell line. Figure 3D showed a dramatic increase in LC3-II in 786-0 cells when co-cultured with NK cells (+) compared to 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 GzmB (23). We next evaluated whether targeting autophagy may affect the level and the activity of NK-derived GzmB in 786-0 cells. Strikingly, our results indicate that targeting Beclin1 significantly increased NK-derived GzmB level (Figure 3E) in 786-0 cells. Furthermore, no NK signal (NKG2D) was detected in CT or BECN1 siRNA cells after co-culture, thereby ruling out any contamination by NK cells. Our results (Figure 3F) demonstrate that 786-0 cells displayed a significant lower level of GzmB compared to WT7 cells.

Results depicted in Figure 3G further show that the serine protease activity of GzmB was significantly increased in ITPR1 and Beclin-1 siRNAs-treated 786-0 cells after they encountered NK cells. It is important to note that both ITPR1- and Beclin1-silenced cells formed the same number of immune conjugates as compared to control cells (Figure 3H).

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

**ITPR1 is a new direct target gene of HIF-2α in RCC cells**

We further asked whether ITPR1 could be a direct target of HIF-2α. 786-0 cells transfected with two different HIF-2α siRNAs resulted in a significant decrease in ITPR1 expression at mRNA (Figure 4A) and protein levels (Figure 4B). In addition, varying HIF-2α stabilization levels in four 786-0 derived cell lines with different VHL mutations (inducible wild type VHL, VHL-Y98H, VHL-C162F, empty vector) revealed that HIF-2α expression levels positively correlated with ITPR1 (Figure 4C and 4D). Similarly, in A498 cells transfected with a vector encoding wild type VHL (A498-pVHL), we also observed a decrease in HIF-2 and ITPR1 mRNA and protein expression as compared to A498 cells transfected with a control vector (A498-pmock) (Data not shown). These results further support our findings using 786-0, PRC3, and WT-7 cells.

To examine a putative direct transcriptional regulation of ITPR1 by HIF-2α, we analyzed the promoter region of ITPR1 gene using fuzznuc (EMBOSS) software and found 10 putative hypoxia-response elements (HRE) (A/GCGTG) suggesting a possible direct interaction between HIF-2α 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 HIF-2α antibody as compared to the WT7 cell line or the IgG antibody. Similar enrichment of VEGF promoter fragment containing HRE, a well known HIF-2α target gene (24), was also observed in PRC3 and 786-0, but not in WT7 cells (Figure 4E).

Immunohistochemistry staining was finally performed on tissue sections from 235 patients diagnosed with RCC to examine the relationship between ITPR1 and HIF-2α expression. Out 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 HIF-2α (87%). For
the ITPR1 positively stained patients (n=32), 20 were found to be stained by HIF-2α (62.5%). Statistical analysis showed a significant correlation between HIF-2α and ITPR1 staining in these patients (Table 1).

Taken together, these results clearly demonstrate that ITPR1 is a direct novel target gene of HIF-2α in RCC cell lines and point to the existence of a functional link between ITPR1 and HIF-2α in RCC.

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

In order to investigate the in vivo relevance of the HIF-2α /ITPR1 pathway, we used the Renca murine RCC with constitutively stabilized HIF-2α. When Renca cells were transfected with siRNA against HIF-2α, a significant decrease in ITPR1 was observed at both protein (Figure 5A) and mRNA levels (Figure 5B). Moreover, treatment of Renca cells with increasing doses of digoxin (an inhibitor of HIF-1α and HIF-2α) (25) resulted in a simultaneous decrease in HIF-2α and ITPR1 expression in a dose dependent manner (Figure 5C and 5D). 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 (Figure 5E), we demonstrated that ITPR1 inhibition resulted in a significant decrease in Renca tumor growth as compared to 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 to untreated mice. Importantly, the combination of ITPR1 inhibition and NK cell depletion resulted in more robust tumor growth than NK depletion alone (Figure 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 (26). 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 HIF-1α and HIF-2α, or in a selective stabilization of HIF-2α (27). Accumulating evidence indicates that HIF-2α is more important for tumor progression, while HIF-1α mostly behaves as a tumor suppressor (28). These antagonistic effects have been partially explained by the ability of HIF-2α to regulate genes involved in activation of proliferation (MYC, CyclinD), inhibition of apoptosis (p53) and promotion of metastasis (CXCR4). In contrast, HIF-1α regulates preferentially apoptotic genes (BNIP3, p53) and inhibits proliferation (MYC inhibition, p21, p27) (29). In the RCC cell line (786-0 cells) used in this study, VHL mutation selectively induces HIF-2α stabilization, thus representing an ideal model system to specifically examine the effect of HIF-2α on RCC susceptibility to NK-mediated lysis. Using this model, we showed that targeting HIF-2α in 786-0 cells attenuates their resistance to NK-mediated lysis, while HIF-2α overexpression in HIF2 knocked down 786-0 cells restored their resistance to NK-mediated lysis suggesting a critical role of HIF-2α in the acquisition of resistance to cytotoxicity associated with VHL mutations. Perier et al. 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 (30). These discrepancies from our
findings may be due to the fact that in our model, 786-0 cell line stabilizes only HIF-2α while the RCC4 and RCC6 cell lines stabilize both HIF-1α and HIF-2α. In this regard, it should be noted that hypoxia and HIF’s (HIF-1α and HIF-2α) 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 (31) while Siemens et al provided evidence that hypoxic stress promotes the shedding of MHC class I chain-related molecules MICA and MICB in prostate cancer cells, resulting in a decreased sensitivity to NK-mediated lysis (32).

DNA microarray-based global transcriptional profiling in HIF-2α targeted 786-0 cells and RT-qPCR revealed a set of genes related to their fold change. Among the differentially expressed genes, we selected three 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) (33-35). Under our experimental conditions, Ca^{2+} 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 Ca^{2+} in intracellular compartments (endoplasmic reticulum and mitochondria) (36) 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.

Since conflicting studies have suggested a role for HIF-2α and ITPR1 in the regulation of autophagy (22), 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 HCQ in the three RCC cell lines and in 786-0
transfected or not with siRNA HIF-2α and siRNA ITPR1 and no difference in autophagy markers was observed (Supplementary data 4). Menard et al. have reported that HIF-2α was a potent inhibitor of hypoxia-induced autophagy (37). Bohensky et al also showed that in maturing chondrocytes expressing both HIF-1α and HIF-2α, the latter acts as a brake on the autophagy-accelerator function of HIF-1α (38).

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 (39). Several studies demonstrated that genetic or pharmacological inhibition of ITPR receptors can inhibit or stimulate autophagy (36). Inhibition of ITPR1 signaling pathway can suppress the autophagy indirectly by mechanisms involving endoplasmic reticulum and calcium remodeling (40). ITPR1 has been reported to directly inhibit starvation-induced autophagy through increased binding to Beclin1 (41). Recently, Baginska et al demonstrated that activation of autophagy under hypoxia resulted in the degradation of NK-derived GzmB, which compromises the ability of NK cells to eliminate tumor cells (23). 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 GzmB occurred in these cells. Our results strongly support that the increased expression of ITPR1 by HIF-2α leads to the activation of autophagy following contact with NK cells, which subsequently decreased the activity of NK-derived GzmB. Although the precise mechanism by which autophagy affects the level or serine protease activity of GzmB is not addressed here, we strongly believe that ITPR1-mediated NK-induced autophagy induction could be associated with the decrease in NK-derived GzmB 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 HIF-2α and that its expression was regulated by HIF-2α at both mRNA and protein levels. This was further confirmed by ChIP assay. Furthermore, using a large group (235) of RCC patients, we demonstrated the existence of a significant correlation between HIF-2α 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 (42), the direct involvement of HIFs in the regulation of ITPR1 has not been established. Nevertheless, since HIF-1α and HIF-2α are known to have many common target genes (15, 16), and given that the hypoxia response element (A/G CGTG) is common for HIF-1 and HIF-2 (43), we cannot exclude the possibility that HIF-1α could also regulate ITPR1 in cells stabilizing HIF-1α.

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 while ITPR3 inhibition increased cell death (44). 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 HIF-2α /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.

Since HIF-2α 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 HIF-2α 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 anti-tumor activity and their cross-talk with tumor microenvironment in the context of its stressor complexity and heterogeneity.
Further studies are needed to define whether HIF-2α 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.
Acknowledgments:

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References


Table 1. HIF-2α and ITPR1 staining correlates significantly in human RCC patients.

Association between HIF-2α and ITPR1 expression in RCC patients. Numbers in the “HIF-2α staining” and “ITPR1 staining” columns refer to the number of samples meeting each condition. Numbers in the “−” or “+” columns indicate the total number of samples with negative or positive staining respectively.

<table>
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<th>HIF-2α staining</th>
<th>−</th>
<th>%</th>
<th>+</th>
<th>%</th>
<th>n</th>
<th>p value=1.01x10(^{-10})</th>
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<tr>
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Figure legends

Figure 1. Differential susceptibility of 786-0, PRC3, and WT7 cell lines to NK-mediated lysis demonstrate comparable immune synapse formation.

(A) Western blot analysis of VHL and HIF-2α protein expression levels in 786-0, PRC3 and WT7 cells. (B) HIF-2α, VEGF, CAIX and SLC2A1 expression was evaluated by qRT-PCR in 786-0, PRC3 and WT7 cells. (C and D) Cr51 cytotoxicity assays were performed on 786-0, PRC3, and WT7 cells as targets at different E:T ratios. NK cell line (NK92) and N KD were used as effectors. Data represent 3 independent experiments with SD. Statistically significant differences (indicated by asterisks) are shown (*P < 0.05; **P < 0.005; and ***P < 0.0005).

(E) Conjugate formation between NK effector cells and target tumor cells was analyzed by confocal microscopy. Synapse formation, as defined by phosphotyrosine accumulation in the cell-to-cell contact area was assessed using anti-phosphotyrosine antibody (green fluorescence). Nuclei were stained with TO-PRO-3 iodide (blue fluorescence). Bars, 5μm. (F) Scoring of conjugate formation between NK and tumor cells by visual counting in 10 different randomly selected fields. (G) Quantification of phosphotyrosine staining by mean fluorescence intensity (MFI) was analyzed using the Region Measurement function of the ImageJ software. Data represent 3 independent experiments with SD.

Figure 2. Targeting HIF-2α attenuated 786-0 cell resistance to NK-mediated lysis by a mechanism involving ITPR1.

(A) 786-0 tumor cells were transfected with two different sequences of HIF-2α siRNA or control siRNA (siRNA-CT). Western blot analysis was performed with antibodies as indicated. Actin was used as a loading control. (B) Inhibition of HIF-2α target genes following siRNA-mediated HIF-2α targeting was evaluated by qRT-PCR. (C and D) Cr51 cytotoxicity assays were performed on 786-0 transfected with either siRNA against HIF-2α or siRNA-CT as targets at different E:T ratios. NK cell line (NK92) and N KD were used as effectors. Data
represent 3 independent experiments with SD. Statistically significant difference (indicated by asterisks) are shown (*P < 0.05; **P < 0.005; and ***P < 0.0005).

(E) Volcano plot of gene expression (Log² fold change) and adjusted p-values for 786-0 cells transfected with siRNA-HIF2α. (F) Heat map of HIF-2α-regulated genes in 786-0 cells. Red signifies up-regulation, green signifies down-regulation. (G) Validation by qRT-PCR of HIF-2α-regulated genes with more than 3-fold change. (H) 786-0 tumor cells were transfected with 2 different siRNA targeting ITPR1, or control siRNA and gene silencing was confirmed by qRT-PCR. (I and J) Cr⁵¹ cytotoxicity assays were performed on 786-0 transfected with siRNA against ITPR1, or control siRNA as targets at different E:T ratios. NK 92 and NKD cells were used as effectors. Data represent 3 independent experiments with SD. Statistically significant difference (indicated by asterisks) are shown (*P < 0.05; **P < 0.005; and ***P < 0.0005).

Figure 3. ITPR1 regulates NK-induced autophagy and NK-derived GzmB in 786-0 cells

(A) The formation of autophagosomes was assessed by confocal microscopy in 786-0 cells transfected with siRNA-CT or siRNA-ITPR1 overexpressing tomato-LC3 cultured without NK cells. (B) The formation of autophagosomes was assessed by confocal microscopy in 786-0 cells transfected with siRNA-CT or siRNA-ITPR1 overexpressing tomato-LC3 cultured with PKH-67 stained NK92 cells (middle panel) at 5/1 E:T ratio. The formation of red dot structures corresponding to autophagosomes in target cells was assessed by confocal microscopy (upper panel). Lower panel corresponds to merge images of upper and middle panels. Scale bar: 10 µm. (C) 786-0 cells described in Figure 3B were recorded by time-lapse video microscopy (presented as Movies S1 and S2). At the indicated times, representative images were extracted from the movies. After 81 min of co-culture, NK cells were able to kill ITPR1-silenced cells more efficiently. (Scale bar: 10 µm). (D) Analysis of LC3-II level in 786-0 cells. Control (+) or ITPR1-defective (-) 786-0 cells were cultured alone (-) or in the presence of NK-92 cells. After separation of NK cells from targets by using beads
coated with monoclonal CD56 antibodies, target cell lysates were subjected to Western blot for LC3-I and -II expression. (E) 786-0 cells stably transfected with shRNA-CT and shRNA-beclin1 (BECN1) (autophagy defective cells) and co-presented to NK cells at 5/1 E:T ratio for 30 min. Following separation from NK cells, lysates of tumor cells were subjected to immunoblot analysis to evaluate the GzmB intracellular content. NK cells lysate (NK) was used as control for GzmB detection. (F) 786-0 cells expressing (+) or not (-) BECN1 as well as WT7 were co-culture with NK-92 cells for 1h. NK cells were separated from targets by using beads coated with monoclonal CD56 antibodies. Target cell lysates were subjected to Western blot for analysis of Granzyme B content. (G) 786-0 cells were transfected with siRNA-CT or siRNA-BECN1 or siRNA-ITPR1 and co-cultured with NK cells at 3/1 E:T ratio for 60 min. Shown is the percentage of target cells with intracellular active Granzyme B. (H) 786-0 cells were transfected with siRNA-CT or siRNA-BECN1 or siRNA-ITPR1 and co-cultured with NK cells at 3/1 E:T ratio for 60 min. Shown is the percentage of conjugate formation between effector and target cells assessed by flow cytometry. Data represent 3 independent experiments with SD. Statistically significant difference (indicated by asterisks) are shown (*P < 0.05; **, P < 0.005, and ***, P < 0.0005). (I) 786-0 cells were transfected with siRNA-CT or siRNA-ITPR1, incubated with sublytic PFN and GzmB and western blot was performed. Actin was used as loading control.

**Figure 4. ITPR1 is a direct novel target of HIF-2α.**

(A) 786-0 tumor cells were transfected with three different sequences of HIF-2α siRNA or control siRNA (siRNA-CT). Inhibition of ITPR1 mRNA expression following siRNA-mediated HIF-2α targeting was evaluated by qRT-PCR. (B) Western blot analysis and densitometry was performed. Actin was used as a loading control. (C and D) 786-0 cells were transfected with empty vector, a vector encoding wild type VHL and 2 vectors encoding two different VHL mutations, C162F and Y98H, respectively associated with high and low levels of HIF-2α. ITPR1 expression was analyzed by Western blot (C) and qRT-PCR (D). SLC2A1 expression
reflects HIF-2α transcriptional activity. (E) ChIP was performed in 786-0, PRC3 and WT7 cells using anti-HIF-2 or control non-specific antibodies. Samples were analyzed by real-time PCR using HRE-specific primers. VEGF was used as a positive control. Data represent 3 independent experiments with SD. Statistically significant difference (indicated by asterisks) are shown (*P < 0.05; **, P < 0.005, and ***, P < 0.0005). (F) Representative image of H and E staining and immunohistochemical staining of HIF-2α and ITPR1 expression in normal renal tissue and in tissue from RCC patients. Boxed regions are displayed at high magnification in insets (x20).

**Figure 5. ITPR1 silencing impairs Renca tumor growth in Balb/c mice.**

(A) Renca cells were transfected with two different sequences of HIF-2α siRNA or control siRNA (siRNA-CT). Western blot analysis was performed with antibodies as indicated. Actin was used as a loading control. (B) Inhibition of HIF-2α target genes following siRNA-mediated HIF-2α targeting was evaluated by qRT-PCR. (C and D) Renca cells were treated with increasing doses of Digoxin for 16hrs. (C) HIF-2α and ITPR1 expression was analyzed by Western blot. (D) Digoxin inhibition of HIF-2 target genes VEGF and SLC2A1 and of ITPR1 was analyzed by qRT-PCR. (E) qRT-PCR validation of ITPR1 silencing efficiency in Renca cells transduced stably with Lenti-shRNA-ITPR1-1 and Lenti-shRNA-ITPR1-2. (F) Renca cells stably expressing Lenti-shRNA-ITPR1-1 or Lenti-shRNA-CT were implanted sub-cutaneously in BALB/c mice (n=10). Mice were depleted for NK cells using anti-Asialo-GM1 antibody. Tumors were measured every 2 days. Data represent 3 independent experiments with SEM. Statistically significant difference (indicated by asterisks) are shown (*, P < 0.05; **, P < 0.005, and ***, P < 0.0005).
Figure 1

A

B

C

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E

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G

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Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
Figure 2

A

B

C

D

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H

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J

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
Figure 4

A

![Graph showing relative expression of ITPR1](image)

B

![Bar graph showing relative expression of HIF-2α](image)

C

![Image of HIF-2α, ITPR1, and ACTIN](image)

D

![Bar graph showing relative expression of SLC2A1 and ITPR1](image)

E

![Comparison of recruitment in WT, PRIC3, and 786-O](image)

F

![Images of H&E, HIF-2α, and ITPR1 staining in normal kidney, Patient 235499, and Patient 255648](image)
ITPR1 protects renal cancer cells against natural killer cells by inducing autophagy

Yosra Messai, Muhammad Zaeem Noman, Meriem Hasmim, et al.

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