Inactivation of von Hippel-Lindau Gene Induces Constitutive Phosphorylation of MET Protein in Clear Cell Renal Carcinoma

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Abstract
It is well known that inactivation of von Hippel-Lindau (VHL) gene predisposes for human clear cell renal carcinoma (CCRC). However, details about critical roles of VHL inactivation during tumorigenesis are still unknown. MET protein is a tyrosine kinase receptor for hepatocyte growth factor/scatter factor (HGF/SF), which regulates cell growth, cell morphology, and cell motility. We showed that MET protein overexpressed in CCRC cells was phosphorylated without HGF/SF. This constitutive phosphorylation of MET protein in CCRC cells was inhibited by the rescue of exogenous wild-type VHL gene without a decrease in expression level of MET protein. Interestingly, wild-type VHL gene suppressed the phosphorylation of MET protein only under high cell density conditions. Additionally, MET protein activated by the inactivation of VHL gene modified cell adherence, including N-cadherin and β-catenin. When activation of MET protein in CCRC cells was inhibited by the MET inhibitor K252a, the growth of CCRC cells in vitro and the tumorigenesis induced by CCRC cells in nude mice were suppressed. From these results, we concluded that inactivation of VHL gene induced constitutive phosphorylation of MET protein and modified intercellular adherence structure to trigger the cell growth released from contact inhibition, finally resulting in tumorigenesis. This is one of the mechanisms of CCRC oncogenesis, and MET protein has potential as a molecular target for novel CCRC therapies. (Cancer Res 2006; 66(7): 3699-705)

Introduction
Although patients who are found to have localized renal cell carcinoma have an excellent prognosis, those with advanced disease show significantly decreased survival (1). Immunotherapy with IFNs and interleukins is the standard therapy for metastatic renal carcinoma, but its response rate remains <20% (2). Recently, allogeneic hematopoietic stem cell transplantation and vaccination as immunotherapies of renal cell carcinoma were reported, but these are still experimental and still do not provide satisfactory outcomes (3, 4). To develop novel therapies for renal cell carcinoma, it is necessary to clarify details of the renal oncogenic mechanism and to identify targets for therapies.

Clear cell renal carcinoma (CCRC) constitutes >80% of human kidney cancers, and its oncogenic mechanism has been made apparent gradually by many investigators. von Hippel-Lindau (VHL) tumor suppressor gene was identified originally in families with VHL disease, a rare hereditary multitumor syndrome with CCRCs (5). Somatic mutations of VHL gene also occurred in most sporadic CCRCs (6), and the reintroduction of VHL gene into VHL-deficient CCRC cell line suppressed its tumorigenicity (7). These experiments indicated that CCRC was caused by inactivation of VHL gene. VHL gene product forms a stable complex with elongin C, elongin B, and Cul-2, called the VCB-Cul-2 complex (8, 9), which has ubiquitin ligase activities (10–12). One of the targets of the VCB-Cul-2 complex is hypoxia-inducible factor-1α (HIF-1α), the transcriptional factor responsible for the induction of hypoxia genes, including vascular endothelial growth factor (VEGF), platelet-derived growth factor-β, and Glut-1 (13–15). When VHL gene is inactivated, the accumulation of HIF-1α due to the decrease of ubiquitination produced high expression of VEGF and some other growth factors and eventually induced tumor angiogenesis. This is a recognized mechanism of CCRC tumorigenesis, but angiogenesis is a necessary factor for progression of tumor growth after onset of oncogenicity. The details about critical roles of VHL inactivation in the early stage of oncogenicity are still unknown. Recently, we reported the possibility that VHL mediated contact inhibition and suggested that loss of this inhibition may be the basis for tumorigenesis of CCRC (16). The normal origin of CCRC is thought to be the renal proximal tubule epithelial cells (RPTEC; refs. 17, 18). The tight monolayer structure of renal proximal tubule is maintained by intercellular adhesion of RPTEC. Cancer cells with inactivated VHL gene grow contrary to the restriction by intercellular adhesions to rupture the structure of renal proximal tubule.

MET protein is one of the candidates that may have an important role in the oncogenesis of CCRC. MET protein is a tyrosine kinase receptor activated by stimulation of its ligand, hepatocyte growth factor/scatter factor (HGF/SF; refs. 19, 20). Binding of HGF/SF to the extracellular portion triggers autophosphorylation of critical tyrosines located in the intracellular portion. Phosphorylated tyrosines located in COOH terminus in MET protein bind to a variety of second messengers, including GAB-1, Grb-2, c-Src, phosphatidylinositol 3-kinase, and phospholipase Cγ, to activate these signal pathways and regulate cell growth, cell morphology, and cell motility (21). MET protein is expressed in a variety of organs and plays important roles in organ development, organ reconstruction, and oncogenicity (22). In normal kidney, MET protein is expressed in RPTEC (23). We reported previously that mutations in the tyrosine kinase domain of the MET gene produced constitutive phosphorylation of MET protein and predisposed to both hereditary and sporadic papillary renal carcinomas, which make up ~5% to 15% of human kidney cancer (24–26). The mutations of MET gene have not been reported in CCRC. However, it is speculated that MET protein may play an important role during oncogenesis of CCRC because MET protein

References
2. American Association for Cancer Research. doi:10.1158/0008-5472.CAN-05-0617
and its ligand, HGF/SF, were overexpressed in CCRC (23, 27, 28). Additionally, Pennacchi et al. reported that HIF, the target molecule of the VCB-Cul-2 complex, activated the transcription of met proto-oncogene (29).

Another important molecule associated with oncogenesis of CCRC is cadherin. Cadherin is a transmembrane protein mediating calcium-dependent homophilic intercellular adhesion in a variety of cell types. Its COOH terminus binds to β-catenin, which in turn interacts with α-catenin that bridges the complex to the actin-based cytoskeleton (30–32). Cadherin has a variety of subclasses, and N-cadherin is expressed in RPTEC and CCRC. N-cadherin expressed in RPTEC was thought to maintain the structure of renal proximal tubule, and it was suggested that N-cadherin was associated with oncogenesis and progression of CCRC (33–36).

In this report, we monitored the phosphorylation of MET protein in CCRC cells to understand the role of MET protein in oncogenicity of CCRC and showed the potential of MET protein as a molecular target for novel human CCRC therapies.

**Materials and Methods**

**Cell culture.** Human normal RPTEC was purchased from Clonetics (Walkersville, MD) and maintained in the recommended medium, REGM (Clonetics). CCRC cell lines lacking wild-type VHL and stable transfectants expressing wild-type VHL were gifts. 786-O and 786-O stable transfectant expressing wild-type pVHL were from Dr. William G. Kaelin (Howard Hughes Medical Institute, Dana-Farber Cancer Institute, Brigham and Women's Hospital, Harvard Medical School, Boston, MA). UMRC-6 and UMRC-6 stable transfectant expressing wild-type pVHL were from Dr. Igor Kuzmin (Basic Research Program, SAIC-Frederick, Inc., Frederick, MD). Caki-1 and UMRC-3 were purchased from American Type Culture Collection (Manassas, VA). These cell lines were maintained in DMEM with or without 10% FCS at 37°C before analysis under specific conditions.

**Adenovirus vector.** Adenovirus vectors for human pVHL were generated using cosmid vector pA × C Adv (37). Mutant VHL R167W cDNA was generated by introducing a point mutation to human VHL cDNA as described elsewhere (38). 786-O cells were infected with Ax-W1 (empty vector), Ax-VHL, or Ax-VHL R167W at multiplicity of infection of 20, a condition sufficient for ~100% infection of the cells.

**Immunoprecipitation and Western blotting.** Cells were harvested under appropriate conditions. Cells were washed twice with ice-cold PBS, lysed in ice-cold buffer [20 mmol/L Tris (pH 8.0), 137 mmol/L NaCl, 10% glycerol, 0.1% SDS, 0.5% NPNP, 100 mmol/L sodium fluoride, 200 mmol/L sodium orthovanadate, 1 mmol/L EGTA, 2 mmol/L phenylmethylsulfonyl fluoride, 1 µg/mL leupeptin, 3 µg/mL aprotinin], and centrifuged (30 minutes, 4°C, 14,500 × g). Following quantitation, each cell lysate (400 µg) was pre cleared with protein A-Sepharose and then incubated with antibody of interest and protein A-Sepharose for 3 hours at 4°C with rotation. The samples were then washed thrice with ice-cold buffer [20 mmol/L Tris (pH 7.4), 100 mmol/L NaCl, 0.5% NPNP, 1 mmol/L EDTA]. The samples were subjected to SDS-PAGE on 8% gel and electrotransferred to Immobilon-P (Millipore, Bedford, MA). After blocking the membrane with 5% albumin, Western blotting was done using an antibody of interest and detected with enhanced chemiluminescence detection system (Amersham International, Buckinghamshire, United Kingdom).

**RNA and reverse transcription-PCR.** A standard TRizol preparation protocol (Life Technologies, Inc., Rockville, MD) and reagents were used for total RNA isolation from cells. Reverse transcription was done using the Reverse Transcription System (Promega, Madison, WI) according to the manufacturer's instruction. The following primers were used: HGF, 5'-GGAGGGCACCTGTGCTACCT-3' (forward primer) and 5'-CCGCGATGATTGATC-TGCC-3' (reverse primer) and β-actin, 5'-ACCTGGCAGGCGGGT-3' (forward primer) and 5'-CCACGTCAACTT-CATGATG-3' (reverse primer). PCR amplification was done with the GeneAmp Gold PCR Reagent kit according to the manufacturer's instruction using the PTC-200 DNA Engine Thermal Cyclers (MJ Research, Waltham, MA). The PCR started with denaturation at 95°C for 10 minutes followed by 30 cycles at 95°C for 30 seconds, 60°C for 30 seconds, and at 72°C for 30 seconds.

**Reagents.** Anti-human MET antibodies (c-28 for immunoprecipitation and c-12 for Western blotting), anti-β-catenin antibodies, anti-N-cadherin antibodies, control mouse IgG, and control rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphotyrosine antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-phospho–mitogen-activated protein kinase (MAPK) antibodies and anti-MAPK antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-VHL protein antibodies were purchased from PharMingen (San Jose, CA). K252a was purchased from Alomone Labs (Jerusalem, Israel). HGF was purchased from Calbiochem (La Jolla, CA). Cultured cells were stimulated with 10 ng/mL HGF for 10 minutes.

**Cell growth analysis.** Cells were seeded onto 60-mm dishes at a density of 5 × 10^4 per dish. Cells were cultured in appropriate medium with 10% FCS and then treated with K252a dissolved in DMSO at 25 or 50 mmol/L concentration daily. Simultaneously, the cells were cultured in appropriate medium with 10% FCS and then treated daily with DMSO. Number of cultured cells was counted manually daily.

**Figure 1.** Inactivation of VHL gene induces constitutive phosphorylation of MET protein in CCRC cell lines at high cell density. A. Cell lysate (400 µg) from each cell line after 24 hours in serum-free culture under subconfluence condition was immunoprecipitated with anti-MET antibody, resolved by 8% SDS-PAGE, and analyzed by Western blotting using anti-phosphotyrosine antibodies (top). The filter was stripped and reprobed with anti-MET antibody (bottom). B. Total RNA from CCRC cell lines was extracted and reverse transcription-PCR of HGF mRNA or β-actin mRNA was done. Control: MRC-5, parental cells without transfection; lanes 4, 8, and 12, cells transfected with empty vector. The cells were harvested under confluence (lanes 1–4), subconfluence (lanes 5–8), and sparse condition (lanes 9–12). Cell lysate (400 µg) from each cell line was immunoprecipitated with anti-MET antibody, resolved by 8% SDS-PAGE, and analyzed by Western blotting using anti-phosphotyrosine antibodies (top) and reprobed with anti-MET antibody after stripping (top middle). Cell lysate (30 µg) was blotted by anti-pVHL antibody (bottom middle) or anti-β-actin (bottom).
Results

At first, we monitored expression levels and phosphorylation levels of MET protein in established CCRC cell lines and in RPTEC, which is thought to be the normal origin for CCRC. The cells were cultured at subconfluence in serum-free medium for 24 hours to eliminate the effects of HGF/SF in serum. The 170-kDa product detected corresponds to the intracellular single-chain MET precursor, whereas the 140-kDa product corresponds to the β-chain of the mature, cell surface–associated 190-kDa disulfide-linked MET heterodimer (26, 39, 40). MET protein was overexpressed in CCRC cell lines compared with RPTEC (Fig. 1A, bottom). Although MET protein in RPTEC was not phosphorylated without HGF/SF, MET protein in CCRC cells was constitutively (Fig. 1A, top). The expression of HGF/SF was not detected in cancer cells and RPTEC (Fig. 1B).

It is well known that inactivation of VHL gene predisposes CCRC, and there were VHL gene mutations in three cell lines, UMRC-3, 786-O, and UMRC-6, except Caki-1. To evaluate the effect of the VHL gene on MET phosphorylation, exogenous wild-type VHL genes or the naturally hotspot mutant VHL R167W genes were infected to 786-O cells transiently by adenoviral vectors. We compared the phosphorylation level of MET protein in these cells under confluent, subconfluent, and sparse conditions, because we reported previously that the VHL protein content in RPTEC was increased dramatically in dense culture and that the VHL protein modified the expression of some genes and suppressed growth suppressor at high cell density (16, 41). The constitutive phosphorylation of MET detected in 786-O was depressed by exogenous wild-type VHL genes only under confluent conditions without a decrease of MET protein expression levels (Fig. 1C, top, top middle, lane 2). This depression was not detected under subconfluent or sparse conditions. Exogenous mutant VHL R167W gene did not depress the phosphorylation of MET protein.

At the same time, we compared the phosphorylation of MET protein in 786-O cells and their stable transformants expressing exogenous wild-type VHL gene. The constitutive phosphorylation of MET detected in 786-O cells was also depressed under confluent conditions without a decrease in MET expression level of 786-O stable transformants expressing wild-type VHL gene (Fig. 2A, lanes 2 and 5). This depression of MET phosphorylation induced by VHL gene was detected in experiments using UMRC-6 (Fig. 2B, lanes 2 and 5). Wild-type VHL gene did not depress MET phosphorylation at low cell density (Fig. 2A and B, lanes 1 and 4). The sensitivities of MET protein to HGF/SF were not inhibited by VHL gene expression (Fig. 2A and B, lanes 3 and 6).

To determine whether inactivation of VHL gene induces phosphorylation of intracellular protein nonspecifically, we immunoblotted total cell lysate by anti-phosphotyrosine antibodies. Most bands of proteins containing phosphotyrosine in CCRC cells...
were not affected by expression of VHL genes, but the bands located at 140 kDa were depressed by expression of VHL genes (Fig. 3A, lanes 2 and 5). We confirmed that these depressed bands represented MET protein, because these bands were recovered after addition of HGF/SF (Fig. 3A, lanes 3 and 6) and were consistent with the bands reblotted by anti-MET antibodies (Fig. 3B).

To investigate the role of activated MET protein in CCRC oncogenesis, the activation of MET protein was inhibited by K252a, a specific inhibitor of MET protein (42). K252a inhibited the activation of MET protein and also activation of MAPK dose-dependently in UMRC-3 cells, one of established CCRC cell lines lacking wild-type VHL genes (Fig. 4A). Most protein containing phosphotyrosine were not affected (Fig. 4B). The growth of UMRC-3 cells in vitro was inhibited by K252a at 25 nmol/L (Fig. 4C). Additionally, the growth of other CCRC cells, 786-O and UMRC-6, was inhibited by K252a at 25 nmol/L (data not shown). The growth of RPTEC was not inhibited when the concentration of K252a reached 50 nmol/L (data not shown).

To determine whether the in vitro antiproliferative activity of K252a could be translated into antitumor activity in vivo, K252a was given to athymic nude mice with tumor xenografts of UMRC-3 cells. When the tumor volume reached 200 mm³, the animals were given K252a dissolved in physiologic saline i.p. at 500 μg/kg every 3 days. The control group received the same volume physiologic saline. At 4 weeks, control animals developed large tumors of 5.7 ± 1.6 relative volume compared with those at 0 week. Mice treated with K252a showed inhibition of relative tumor volume at 4 weeks by 3.6 ± 0.7 with partial depression of MET phosphorylation (Fig. 4D). There were statistically significant differences in relative tumor volume between control and K252a-treated mice (P < 0.01). The pathologic examination did not detect specific findings like necrosis or apoptosis in tumors in mice treated with K252a. The body weight of nude mice treated with K252a at 4 weeks was 94.1 ± 2.9% of that of control mice (P < 0.01).

We stimulated the CCRC transformants expressing wild-type VHL genes with the medium culturing the CCRC cells lacking VHL.
genes to evaluate the possibility that the autocrine loop of some growth factors or cytokine produced by inactivation of VHL gene regulated the phosphorylation of MET protein. MET protein in CCRC transformants was not phosphorylated by the medium culturing parental cells lacking wild-type VHL genes (Fig. 5).

We focused on the effect of VHL gene on β-catenin and N-cadherin, which were associated with cell-cell adhesion. N-cadherin, which expressed in both CRCC and RPTEC, was suggested to be associated with oncogenesis of CCRC (33–36). β-Catenin was known to have a potency to bind cadherin and MET protein (43, 44).

When the lysate of 786-O cells was immunoprecipitated, N-cadherin and β-catenin were coprecipitated (Fig. 6B and C, lane 6). Exogenous wild-type VHL gene inhibited this coprecipitation (Fig. 6B and C, lane 5).

To determine whether the modifications of binding by VHL gene were dependent on the phosphorylation of MET protein, we inhibited the phosphorylation of MET protein in cancer cells by K252a (Fig. 6A, top, lane 1). The binding of N-cadherin and β-catenin was dependent on MET phosphorylation (Fig. 6B and C, lane 4). The expression levels of MET protein, N-cadherin, and β-catenin were not affected by VHL gene or K252a (Fig. 6D).

The result that VHL gene inhibited the phosphorylation of MET protein only at dense culture suggested that intercellular adhesion was necessary for the dephosphorylation of MET protein by VHL gene. To confirm this hypothesis, the CCRC transformants expressing wild-type VHL genes were cultured at high density in a calcium-free medium, because intercellular adhesion is dependent on the calcium ion. The phosphorylation of MET protein in CCRC cells inhibited by expression of wild-type VHL genes was recovered immediately when cell-cell adhesion was blocked (Fig. 6E, top), and MAPK was activated following MET phosphorylation (Fig. 6E, bottom middle).

**Discussion**

It was reported that MET protein was overexpressed in CCRC (23, 27, 28), but it is still unknown if the overexpressed MET protein in CCRC is activated without the stimulations of HGF/SF. In this report, we showed that MET protein in CCRC cells was activated constitutively without HGF/SF stimulations. This finding further added to the speculation that MET protein exerts important roles in tumorigenesis of CCRC. We found that the constitutive activation of MET protein in CCRC cells was dependent on the inactivation of VHL gene. This finding suggested that MET protein was activated and acted on the early step of oncogenicity of CCRC, because inactivation of VHL gene was the first event to induce CCRC (5–7). Practically, we found that MET protein in most of early-stage CCRC tumor tissues gained from surgeries showed hyperphosphorylation compared with that in normal tissues. However, Caki-1 cells that express wild-type VHL showed constitutive MET phosphorylation (Fig. 1A). CCRC may harbor another mechanism to induce MET activation (e.g., a mutation in another gene that leads to MET activation).

Our results were contradictory to a previous report by Koochekpour et al. that MET protein in 786-O cells was not phosphorylated without HGF/SF stimulation (45). They showed that VHL genes suppressed the morphologic change and cell motility of CCRC cells induced by HGF/SF stimulation. Our result that constitutive phosphorylation of MET protein in 786-O was inhibited by wild-type VHL gene corresponded to a previous report that branching and invasion of 786-O cells in the absence of exogenous HGF/SF were suppressed by VHL gene (36). As shown in Fig. 2A (lane 3), the phosphorylation level of MET protein in 786-O cells was enhanced by the stimulation of HGF/SF. We considered that MET protein in 786-O cells was phosphorylated partially without HGF/SF and hypersensitive to the stimulation of HGF/SF.

To clarify the role of activated MET protein in CCRC oncogenesis, we inhibited the activation of MET protein by K252a, which was reported to inhibit MET autophosphorylation and MET-mediated biological reactions, including scattering, proliferation, and transformation at nanomolar concentration (42). K252a inhibited not only the autophosphorylation of MET protein but also the phosphorylation of MAPK playing a critical role in the cell growth and differentiation (46–48). It was speculated that the constitutive activation of MET protein was necessary for the growth and oncogenesis of CCRC cells. The finding that K252a did not suppress the growth of normal RPTEC at the same concentration as that suppressing the growth of CCRC cells supported the hypothesis that CCRC tumorigenesis is dependent on activation of MET protein. Finally, we showed that tumorigenesis induced by CCRC cells in nude mice was inhibited by i.p. injection of K252a. These important results mean not only that activation of MET is necessary for CCRC tumorigenesis but also that MET protein has the potential as a target molecule for CCRC therapies. The development of novel MET inhibitors, which are more specific and more effective in vivo than K252a, is desired.

Additionally, we found that MET protein activated by the inactivation of VHL gene modified cell adherence, including N-cadherin and β-catenin. Cadherins are transmembrane molecules mediating calcium-dependent cell-cell adhesion and bound to β-catenin. β-Catenin in turn interacts with α-catenin, which bridges the complex to the actin-based cytoskeleton (30). Cadherins are a multigene family and many investigators reported that N-cadherin was expressed in RPTEC and CCRC (33–36). It was recently reported that β-catenin had potency to bind to MET protein (43, 44). We reported previously that the inactivation of VHL gene induced the growth of CCRC cells at high cell density (16, 41). The presented results suggested the possibility that the

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3 Manuscript in preparation.
Constitutive phosphorylation of MET protein induced by inactivation of VHL gene restructured the molecule complex associated with intercellular adhesion. 786-O (VHL−; lane 3), its stable transfectant expressing wild-type pVHL (VHL+; lane 2) were cultured in serum-free medium for 24 hours under confluence conditions and harvested. 786-O treated with K252a (VHL−; K252a−; lane 1) was cultured under the same condition and harvested 30 minutes after addition of 100 nmol/L K252a. Cell lysate (400 µg) from each cells was immunoprecipitated with anti-MET antibodies (A), anti-β-catenin antibody or control mouse IgG (B), and anti-N-cadherin antibodies or control rabbit IgG (C) and analyzed by Western blotting using anti-phosphotyrosine antibodies, anti-MET antibodies, anti-N-cadherin antibodies, or anti-β-catenin antibodies. Control: total cell lysate (30 µg) of 786-O (A-C). D. total cell lysate (30 µg) from each sample was analyzed by Western blotting using anti-MET antibodies, anti-N-cadherin antibodies, or anti-β-catenin antibodies. E. 786-O stable transfectant expressing wild-type pVHL (786-D+ VHL) cultured previously in serum-free medium for 24 hours under confluence condition was cultured in calcium-free medium and harvested at each time. 0m, cells before medium change. Cell lysate (400 µg) was immunoprecipitated with anti-MET antibody and analyzed by Western blotting using anti-phosphotyrosine antibodies (top). The filter was stripped and reprobed with anti-MET antibody (top middle). Total cell lysate (30 µg) was analyzed by Western blotting using anti-phosphorylated MAPK antibodies (bottom middle) or anti-MAPK antibodies (bottom).

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