Hypoxia Inducible Factor-1 α Inhibition in Von Hippel Lindau-mutant Malignant Pleural Mesothelioma Cells

TAKEHITO SHUKUYA^{1,2,3}, JUN OYANAGI⁴, MASAKUNI SERIZAWA²,
MASARU WATANABE^{2,4}, NOBUYUKI YAMAMOTO^{1,4} and YASUHIRO KOH^{2,4}

¹Division of Thoracic Oncology, Shizuoka Cancer Center, Shizuoka, Japan;

²Drug Discovery and Development Division, Shizuoka Cancer Center Research Institute, Shizuoka, Japan;

³Department of Respiratory Medicine, Juntendo University, Tokyo, Japan;

⁴Internal Medicine III, Wakayama Medical University, Wakayama, Japan

Abstract. *Background/Aim:* Molecular targeted agents have been successfully developed against solid tumors and their use is also being investigated for the treatment of malignant pleural mesothelioma (MPM). We have previously reported von Hippel Lindau (VHL) mutations detected by massive parallel sequencing technology in samples of patients with MPM. Here, we conducted an in vitro study to investigate the therapeutic approaches in VHL-mutant MPM. *Materials and Methods:* Three MPM cell lines with or without a VHL mutation were used and the effects of molecular-targeted agents on growth inhibition were evaluated. Based on the characteristics of the molecular targeted agents that exhibited growth inhibitory effect, the effects of knockdown by siRNA were also evaluated. *Results:* NCI-H28 MPM cells harboring the VHL L89H mutation were sensitive to YC-1, known as an inhibitor of hypoxia inducible factor (HIF)-1 α , and YC-1 treatment induced massive apoptosis in a dose-and-time-dependent manner. Knockdown of HIF-1 α by siRNA partially inhibited the growth of NCI-H28 cells, suggesting that an additional blockade may be required to completely inhibit growth signaling. *Conclusion:* The VHL mutation may predict tumor responses to YC-1, a HIF-1 α inhibitor.

Malignant pleural mesothelioma (MPM) is an aggressive type of neoplasms arising from the pleural cavity (1, 2). It has been reported that about 80% of MPM cases are related to asbestos exposure (3). Genetic predisposition, simian Virus 40 infection

(SV40), radiotherapy, and exposure to fibers with physical properties similar to asbestos, may trigger to MPM development (4-7). As MPM patients are typically diagnosed at advanced stages of cancer, which are mostly unresponsive to conventional chemotherapy, the prognosis remains poor. The median survival of patients with MPM ranges from 9 to 12 months, regardless of treatment with chemotherapy combining cisplatin and pemetrexed or raltitrexed (8, 9).

Recently, molecular targeted therapy has gained much attention. In cancers such as non-small-cell lung cancer and breast cancer, somatic mutations and gene amplifications are thought to be oncogenic, and treatment with inhibitors specific to tumors with such drivers are beneficial for survival (10) and efforts are being undertaken to assess the genetic abnormalities in solid tumors (11-13).

Recently, immune checkpoint inhibitors have shown immense efficacy in various malignancies. Nivolumab elicits significant effects in patients with MPM and has been approved for the treatment of MPM in Japan (14, 15). Despite these advances, improvements in chemotherapy, especially with respect to molecular targeted agents for MPM treatment, are quite slow, partly because the number of patients is small. However, thus far, several studies on the genetic alterations in MPM have been published. A homozygous deletion in *PTEN* was detected in two of 21 MPM cell lines and this mutation was related to Akt activation (16, 17). Thomas *et al.* have reported *NRAS* mutations in three of 37 patients with mesothelioma (18). Approximately 40% of MPM cases harbor inactivating mutations in neurofibromatosis 2 (*NF2*) gene, and *BAP1* loss and/or mutation have been detected in 42% of MPM patients (19). We have previously reported genetic alterations detected by massive parallel sequencing technology, and 16 genetic mutations including two *VHL* mutations were detected in 21 specimens from patients with MPM (20). More comprehensive genome-based mutational analysis was also conducted, and loss of *CDKN2A/B* was reported in MPM and malignant peritoneal mesothelioma

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Correspondence to: Yasuhiro Koh, MD, Ph.D., Internal Medicine III, 811-1 Kimiidera, Wakayama 641-8509, Japan. Tel: +81 734410619, Fax: +81 734462877, e-mail: ykoh@wakayama-med.ac.jp

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(21). Inflammation-related biomarkers on survival of MPM patients were also reported (22).

VHL, known as a tumor suppressor gene, is located on chromosome 3p25 and the protein product of *VHL*, *i.e.*, pVHL, has multiple functions. The most well-studied function of *VHL* is its ability to target HIFs for polyubiquitination and proteasomal degradation via substrate recognition as part of the ubiquitin ligase complex (23). Sekido *et al.* have reported a *VHL* mutation in NCI-H28, a mesothelioma cell line (24). Based on these findings, we investigated *VHL* mutations as potential targets for HIF-1 α inhibition in MPM treatment.

Materials and Methods

Cell culture and reagents. Human malignant mesothelioma cell lines MSTO-211H, NCI-H2052, and NCI-H28 were purchased from ATCC (Manassas, VA, USA). These cell lines were maintained in RPMI1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen) in humidified air containing 5% CO₂ at 37°C. The cells were incubated under anaerobic conditions with Anaeropack Kenki 5% (Mitsubishi Gas Chemical Co., Tokyo, Japan), providing a hypoxic condition with approximately 5% CO₂ and less than 1% O₂. Cobaltous chloride (CoCl₂) was purchased from Wako Pure Chemical Industries (Osaka, Japan). The following anti-cancer drugs were purchased from the indicated companies: gefitinib (Tocris Bioscience, Ellisville, MO, USA); erlotinib, lapatinib, crizotinib, vandetanib selumetinib, and trametinib (LC Laboratories, Woburn, MA, USA); afatinib, dabrafenib, and MK-2206 (Selleck Chemicals, Houston, TX, USA); and YC-1 (Calbiochem, La Jolla, CA, USA).

DNA and RNA extraction. Genomic DNA was extracted from the cell lines using the QIAamp DNA FFPE kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The DNA concentration was quantified using the Quant-iT PicoGreen dsDNA assay kit (Invitrogen).

Amplicon-based next-generation sequencing and variant call. A multiplexed amplicon-based next generation sequencing was performed using the TruSeq amplicon-cancer panel (TSACP; Illumina, San Diego, CA, USA), designed to identify somatic mutations in 48 cancer-related genes according to the manufacturer's instructions. Paired-end sequencing was carried out on an Illumina MiSeq platform with the MiSeq reagent kit v1 or v2 (300–cycles) (Illumina) with a 1,900 $\times$ average coverage.

Data processing was conducted using CLC Genomics Workbench version 6.0.3 (CLC bio, Aarhus, Denmark). Quality-trimmed sequence reads were mapped to the human genome (hg19). Non-synonymous somatic mutations were extracted using COSMIC and hg19 CDS file followed by the elimination of germline mutations using HapMap, 1000 genomes, and dbSNP Final variant calls were made based on the following criteria: frequency $\geq 5\%$ or over 40 counts of a mutated allele with coverage 500 or more.

In vitro growth-inhibition assay. The growth-inhibitory effect of each inhibitor was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay as described previously (25). Briefly, an exponentially growing cell suspension was seeded into each well of

a 96-well plate at the density of 2,000–3,000 cells per well. After overnight culture, the cells were treated with each inhibitor at various concentrations and cultured at 37°C in a humidified atmosphere for 72 h. Then, MTT solution was added and the plates were further incubated for 4 h at 37°C. After centrifuging the plates, cells were dissolved in dimethyl sulfoxide. Optical density was measured at 562 and 630 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

Immunoblot analysis. Cells were washed with iced-cold PBS and lysed with M-PER lysis buffer supplemented with Halt protease inhibitor cocktail and Halt phosphatase inhibitor cocktail (Pierce Chemical Co., Rockford, IL, USA). The protein concentration of the lysate was measured using the BCA protein assay reagent (Pierce). Equal amounts of protein (20 μ g) were separated by SDS-PAGE and then transferred onto nitrocellulose membranes (Bio-Rad) and detected as previously described (20). Antibodies against phospho-EGFR, Akt, phospho-Akt, ERK1/2, phospho-ERK1/2, S6 ribosomal protein, phospho-S6 ribosomal protein, PARP, cleaved-PARP, caspase-3, cleaved-caspase-3, and phospho-MET were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies specific for GLUT1 and ENO, HIF-1 α , and MET were purchased from Abcam (Cambridge, UK), BD Biosciences (Franklin Lakes, NJ, USA), and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively.

APO-BRDU assay. Apoptosis was measured by the TUNEL assay using the APO-BRDU assay kit (Phoenix Flow Systems, San Diego, CA, USA). The cells were treated with or without YC-1 for 24 or 48 h. After the treatment, the cells were harvested, fixed, and stained according to the manufacturer's instructions. Data were acquired using an AccuriC6 flow-cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Small interfering RNA (siRNA) transfection. siRNA targeting HIF-1 α and siRNA Negative Control Med GC Duplex #2 were purchased from Invitrogen. The target sequence of HIF-1 α was 5'-CCAGCCGCTGGAGACACAA TCATAT-3'. These siRNAs were diluted to 0.4 μ M with 250- μ l Opti-MEM medium (Invitrogen). Five microliters of Lipofectamine 2000 (Invitrogen) was diluted with 250- μ l Opti-MEM medium (Invitrogen). Diluted siRNA molecules were gently mixed with diluted Lipofectamine 2000 (Invitrogen) and incubated for 20 min at 25°C. Subsequently, 1.25 $\times 10^5$ cells in 500 μ l of 10% FBS medium were gently mixed with 500 μ l of siRNA-Lipofectamine 2000 complexes and incubated for 10 min at 25°C. The resulting mixtures were seeded into 6-well plates. After incubation for 24 h, the medium was replaced with 10% FBS medium. The growth-inhibitory effect of HIF-1 α knockdown was assessed with cell counting. The cells were trypsinized and the cell number was counted using the Scepter 2.0 handheld automated cell counter (Millipore, Bedford, MA, USA).

Statistical analysis. *p*-values < 0.05 were set to indicate statistically significant differences. All statistical analyses were conducted using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA, USA).

Results

Confirmation of VHL mutation in MPM cell lines. Previously, we have found two *VHL* mutations in 21 samples from patients with MPM (16). To investigate whether the prevalence of *VHL* mutations is a potential target for MPM

Figure 1. A VHL mutation was found in the NCI-H28 cell line. (A) Detection of the VHL L89H mutation in the NCI-H28 cell line by amplicon-based massive parallel sequencing. (B) VHL gene and protein structure. The missense mutation found in the NCI-H28 cell line is shown in red.

treatment, we first investigated actionable mutations in 48 cancer-related genes by massive parallel sequencing. As shown in Figure 1A, the missense mutation changing the leucine-89 residue to histidine (p.Leu89His) was confirmed in NCI-H28 cells. The mutation was located in the middle of

Figure 2. NCI-H28 cells overexpressing HIF-1 α protein under normoxic conditions were sensitive to YC-1, a HIF-1 α inhibitor. (A) Expression levels of HIF-1 α in MPM cell lines. Overexpression of HIF-1 α was inducible under hypoxic conditions and CoCl₂-induced hypoxia-mimicking conditions in NCI-H28 cells with the VHL mutation and in MSTO-211H and NCI-H2052 cells without the VHL mutation. Cells were incubated under hypoxic conditions for 2 h or treated with CoCl₂ for 18 h. (B) Molecular-targeted agents including YC-1, a HIF-1 α inhibitor, were tested in the NCI-H28 MPM cell line and the IC₅₀ values of 11 inhibitors are shown. (C) The growth-inhibitory effect of YC-1 in three MPM cell lines. Significant growth inhibitory effect of YC-1 was observed only in NCI-H28 cells and not in MSTO-211H and NCI-H2052 cells.

the β -domain, which is related to the tumor suppressive function of pVHL (Figure 1B). No actionable mutations were detected in the other 47 cancer-related genes (data not shown). Furthermore, we confirmed that the other two mesothelioma cell lines, MSTO-211H and NCI-H2052, did

Figure 3. YC-1 induced apoptosis in NCI-H28 cells. (A), (B) The effects of YC-1 were evaluated in three mesothelioma cell lines by western blot analysis. Exposure to YC-1 induced cleavage of both caspase 3 and PARP only in NCI-H28 cells. (C), (D) Induction of apoptosis by YC-1 treatment was measured using the Apo-brdU assay. Cells were treated with YC-1 (0.2, 1, and 5 μmol/l) for 24 or 48 h and their number was quantitated using a flow cytometer. The percentage of apoptotic cells (Brd-UTP positive cells) is indicated in the gated area in each panel. The relative numbers of Brd-UTP positive cells are plotted. (E) The effect of YC-1 on molecules downstream of HIF-1α by western blot analysis NCI-H28 cell extracts.

not exhibit actionable mutations, including the *VHL* mutation (data not shown). Thus, the NCI-H28 cells were suitable for investigating whether cancer cells with the *VHL* mutation are dependent on the *VHL*-related signaling pathway.

HIF-1α expression and in vitro growth inhibition via the HIF-1α inhibitor. The *VHL* tumor suppressor regulates the degradation of HIF-1α by the pVHL/E3 ubiquitin ligase complex, which is crucial for the inhibition of the malignant transformation of cells (23). Thus, if there is loss of functional pVHL due to a genetic disorder, HIF1-α is not targeted for degradation and is consequently up-regulated constitutively (23). Indeed, overexpression of HIF1-α even under normoxic conditions was observed in *VHL*-mutated NCI-H28 cells (Figure 2A). Meanwhile, HIF-1α overexpression was also

induced under hypoxic conditions and CoCl₂-induced hypoxia-mimicking conditions in the other two *VHL* wild-type MPM cell lines (Figure 2A).

The HIF-1α inhibitor YC-1 [3-(5'-hydroxymethyl-2'-furyl)-1-benzyl indazole] accelerates HIF-1α degradation and inhibits the *de novo* synthesis of HIF-1α by inactivating the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin (mTOR) pathway (26). We examined whether HIF-1-overexpressing NCI-H28 cells are sensitive to the HIF-1α inhibitor YC-1. The half-maximal inhibitory concentration (IC₅₀) values of 11 molecular targeting agents including YC-1 are shown in Figure 2B. The growth of NCI-H28 cells was inhibited at the IC₅₀ value of 337 nM, which was more than two-fold lower than the concentration of other agents, including tyrosine and serine/threonine

Figure 4. Effect of HIF-1 α silencing using HIF-1 α siRNA in NCI-H28 cells. (A) Expression levels of HIF-1 α in non-treated, control siRNA transfected, and siRNA against HIF-1 α -transfected NCI-H28 cells. HIF-1 α expression was successfully abolished by the siRNA targeting HIF-1 α . HIF-1 α silencing induced cleaved PARP in NCI-H28 cells. (B) The effect of HIF-1 α silencing on the growth of NCI-H28 cells. Knockdown of HIF-1 α by siRNA showed a modest growth inhibitory effect. *Student's two-sided t-test.

kinase inhibitors (Figure 2B). This significant growth inhibitory effect of YC-1 was observed only in NCI-H28 cells and not in VHL wild-type MSTO-211H and NCI-H2052 cells (Figure 2C).

YC-1 induce apoptosis of the VHL-mutated MPM cell line. Exposure to YC-1 for 24 h induced the cleavage of caspase 3 and PARP protein in NCI-H28 cells in a dose-dependent manner (Figure 3A), indicating the induction of apoptosis. This induction of apoptosis was not observed in the other two VHL wild-type cell lines (Figure 3A). Treatment of YC-1 did not affect the phosphorylation of EGFR, ERK1/2, Akt, and S6 kinase in MSTO-211H and NCI-H2052 cells (Figure 3B). In contrast, YC-1 induced the expression of phospho-ERK1/2, -Akt, -S6 but did not affect phospho-EGFR expression in NCI-H28 cells (Figure 3B).

Apoptosis induction by YC-1 treatment was confirmed using the APO-BrdU TUNEL assay. Incorporation of BrdUTP was observed in the NCI-H28 cell line in a dose-and-time-dependent manner, but not in VHL wild-type MSTO-211H and NCI-H2052 cells (Figure 3C and D).

We next confirmed the effect of YC-1 treatment on HIF-1 α expression and molecules downstream of HIF-1 α in NCI-H28 cells. Western blot analysis showed the inhibition of HIF-1 α accumulation by treatment with 5 μ M YC-1 (Figure 3E). With respect to molecules downstream of HIF-1 α , YC-1 treatment abolished the expression of MET protein as well as the phosphorylation of MET (Figure 3E). By contrast, YC-1 had little effect on other downstream molecules such as GLUT1 and ENO1 (Figure 3E).

Silencing HIF-1 α with siRNA. To investigate whether the effects of YC-1 were due to the inhibition of HIF-1 α , siRNA targeting HIF-1 α was transfected into NCI-H28 cells, and its effects on cell growth and apoptosis induction were evaluated. Figure 4A shows the successful knockdown of HIF-1 α by the siRNA targeting HIF-1 α . The knockdown of HIF-1 α by siRNA induced the cleavage of PARP, indicating the induction of apoptosis (Figure 4A) and significant growth inhibitory effect in NCI-H28 cells, although the effect was not as potent as that of YC-1 (Figure 4B).

Discussion

Molecular targeted agents have been successfully developed against solid tumors and their use as a treatment for MPM is under investigation. To date, despite preclinical data demonstrating the overexpression of epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR) in MPM tumor cells, clinical trials have shown no significant benefit from the use of single-agent inhibitors of EGFR, PDGFR, or VEGF. In a phase-I trial examining the efficacy of the histone deacetylase (HDAC) inhibitor, vorinostat, four (30%) and two (15%) of the 13 patients with MPM demonstrated stable disease and partial response, respectively. However, in the following phase-III trial that compared vorinostat monotherapy and placebo as second-line chemotherapy for MPM, vorinostat did not improve survival (27). Nevertheless, in these studies, some patients responded well to molecular targeted agents. Based on these findings, molecular targeted agents may be effective when administered

after optimal patient selection, such as selection based on the type of genetic alteration. This study showed that YC-1 might be a potential therapeutic agent for patients with MPM who have the p.Leu89His *VHL* mutation.

Disruption of *VHL* by a somatic mutation, hypermethylation of its promoter, or chromosomal loss has been observed in up to 90% of sporadic clear-cell renal-cell carcinoma cases and pVHL-defective ccRCCs that overexpress mRNAs that are under the transcriptional control of HIF (23). Moreover, overexpression of HIF-1 α in human tumors is associated with poor prognosis and poor therapeutic outcomes (28).

In this study, constitutive overexpression of HIF-1 α was observed in the absence of hypoxia in NCI-H28 cells, which is a mesothelioma cell line carrying the p.Leu89His mutation in *VHL*. Inhibition of HIF-1 α using YC-1 showed a significant growth inhibitory effect and induction of massive apoptosis in NCI-H28 cells but not in other wild-type *VHL* MPM cell lines. Whether the effect of YC-1 on growth inhibition in *VHL*-mutated MPM cells is mediated through the inhibition of HIF-1 α necessitates further study. In the present study, knockdown of HIF-1 α by siRNA resulted in induction of apoptosis and inhibition of growth of NCI-H28 cells; however, the effect was not as potent as that seen after YC-1 treatment. Based on these results, we hypothesize that HIF-1 α blockade alone may not be sufficient to produce a substantial growth-inhibitory effect in *VHL* mutation-induced HIF-1 α -overexpressing cells. YC-1 is not a selective HIF-1 α inhibitor, and it has been reported that YC-1 can potentially modulate other molecules (29). Although the *VHL* mutation appears to be a potential therapeutic target of YC-1 treatment in MPM, based on the findings in this study, further investigation is warranted to identify additional molecules that are inhibited by YC-1 and that may serve as more selective therapeutic agents.

Kaira *et al.* have reported the HIF-1 α expression levels in MPM tissues by immunohistochemistry (30). Positive expression of HIF-1 α was observed in 19 of 21 samples from patients with MPM. Meanwhile, only NCI-H28 cells were sensitive to YC-1 among the three MPM cell lines. Based on these findings, it is suggested that *VHL*-mutation-induced HIF-1 α overexpression might be the therapeutic target of YC-1, but HIF-1 α overexpression alone may not be a therapeutic target. MPM cases with *VHL*-mutation-induced HIF-1 α overexpression may behave differently than cases with HIF-1 α overexpression without the *VHL* mutation. Further studies are necessary to clarify this hypothesis and the effects of YC-1 on other molecules relevant to the VHL-HIF pathway.

Several clinical trials of HIF inhibitors for cancer treatment have been conducted (31). PX-478, a novel HIF-1 α inhibitor, was tested against advanced solid tumors and lymphomas. In the phase-I trial, 41 patients with advanced cancer were enrolled and 13 of the 37 evaluated patients (35%) had a stable disease (32). The phase-I trial of the novel HIF-1 α RNA antagonist, EZN-2968, has been conducted and interim results have been reported

(33). Prolonged stable disease with clear evidence of tumor shrinkage was observed in one patient with renal cell carcinoma (RCC) and in one patient with hepatocellular carcinoma (34). In the phase-I/IIa trial, CRLX101, a nanoparticle-drug conjugate containing camptothecin and polyethylene glycol that down-regulated HIF-1 α expression, was tested against metastatic RCC in combination with bevacizumab; five out of 22 patients (23%) achieved a partial response (35). The results of a phase-II trial of CRLX101 against recurrent ovarian, fallopian tube, or primary peritoneal cancer have also been reported (36). Digoxin, a cardiac glycoside that inhibits HIF-1 α synthesis, is undergoing a phase-II trial against Kaposi's sarcoma (NCT02212639). Although HIF-1 α inhibitors have been intensely investigated as treatment options for the abovementioned malignancies, thus far, the effect of HIF-1 α inhibitors in patients with MPM has not been reported.

In conclusion, *VHL*-mutant MPM could be targeted by HIF-1 α inhibitors and the *VHL* mutation might be a promising biomarker for YC-1 treatment. Further investigation is necessary to detect these genetic alterations in a larger cohort and to investigate the effects of YC-1 on other molecules relevant to the VHL-HIF pathway.

Conflicts of Interest

TS has received grants and personal fees from MSD, personal fees from Ono pharmaceutical company, Chugai pharmaceutical company, AstraZeneca, and Eli Lilly, outside the submitted work.

Authors' Contributions

Conceptualization: Takehito Shukuya, Yasuhiro Koh; Data curation: Takehito Shukuya, Yasuhiro Koh; Formal analysis: Takehito Shukuya, Masakuni Serizawa, Masaru Watanabe; Funding acquisition: Nobuyuki Yamamoto, Yasuhiro Koh; Investigation: Takehito Shukuya, Masakuni Serizawa, Masaru Watanabe, Yasuhiro Koh; Methodology: Takehito Shukuya, Masakuni Serizawa, Masaru Watanabe, Yasuhiro Koh; Project administration: Yasuhiro Koh; Resources: Yasuhiro Koh; Supervision: Takehito Shukuya, Nobuyuki Yamamoto, Yasuhiro Koh; Visualization: Takehito Shukuya, Yasuhiro Koh; Writing – original draft: Takehito Shukuya, Jun Oyanagi, Yasuhiro Koh; Writing – review & editing: Takehito Shukuya, Jun Oyanagi, Masakuni Serizawa, Nobuyuki Yamamoto, Yasuhiro Koh.

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