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VDUP1 mediates nuclear export of HIF1α via CRM1-dependent pathway

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Abstract

Hypoxia-inducible factor 1α (HIF1α) is a critical transcriptional factor for inducing tumor metastasis, and stabilized under hypoxia but degraded by von Hippel–Lindau protein (pVHL) under normoxia. For the maximal degradation of HIF1α, it must be exported to the cytoplasm via an unidentified transporter. Here, we demonstrate that vitamin D3 up-regulated protein 1 (VDUP1) associates with the β-domain of pVHL and enhances the interaction between pVHL and HIF1α to promote the nuclear export and degradation of HIF1α hypoxia-independently. Blocking of VDUP1 translocation either by leptomycin B or by nuclear export signal mutation inhibited the nuclear export of pVHL/HIF1α and relieved the destabilization of HIF1α. VDUP1 suppressed cell invasiveness and tumor metastasis, which were also recovered by blocking of nuclear export. Taken together, these findings indicate that VDUP1 is a novel tumor suppressor which mediates the nuclear export of pVHL/HIF1α complex to destabilize HIF1α.

Keywords: HIF1α; pVHL; VDUP1; CRM1; Transport

1. Introduction

Hypoxia-inducible factor 1 (HIF1) has a central role in regulating oxygen homeostasis, tumor metastasis, and angiogenesis [1,2]. Recent reports have shown that it is also involved in more diverse physiological responses such as inflammation and immune cell regulation [3,4].

HIF1 is a heterodimeric transcription factor that is composed of HIF1α and HIF1β [5,6]. HIF1α is regulated at two independent levels-transcriptional activation and protein stability. HIF1α protein undergoes proteasomal degradation that is dependent on oxygen. In the presence of oxygen, HIF1α is prolyl hydroxylated at specific residues (P402 and P564) in oxygen-dependent domains by prolyl hydoxylases. Once hydroxylated, von Hippel–Lindau protein (pVHL) binds to HIF1α in a complex with multi-component ubiquitin ligase (pVHL-Elongin BC-Cul2-Rbx) to degrade HIF1α. Under hypoxic condition, unhydroxylated HIF1α accumulates and translocates to the nucleus, where it forms active complex with HIF1β to activate the target genes. Upon reoxygenation, it is exported to the cytoplasm [7]. According to a recent model [8], prolyl hydroxylation of HIF1α induces the binding of pVHL and ubiquitination of HIF1α. Then, ubiquitinylated HIF1α is exported into the cytoplasm to be degraded by 26S proteasome complex.

Shuttling of pVHL bound with HIF1α between the nucleus and cytoplasm is prerequisite for the degradation of HIF1α in the cytoplasm [8,9]. However, little is known about molecular mechanism for pVHL/HIF1α translocation between the nucleus and cytoplasm. In this study, we found that Vitamin D3 up-

Abbreviations: CRM1, chromosome region maintenance 1; HDAC, histone deacetylase; HIF1α, hypoxia-inducible factor 1α; NES, nuclear export signal; pVHL, von Hippel–Lindau protein; SAHA, suberoylanilide hydroxamic acid; VDUP1, vitamin D_3 up-regulated protein 1

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regulated protein 1 (VDUP1), a novel tumor and metastasis suppressor [10,11], decreased HIF1α stability by enhancing nuclear export of pVHL/HIF1α complex and this process was mediated by chromosome region maintenance 1 (CRM1) nuclear receptor-dependent pathway.

2. Materials and methods

2.1. Human tumor tissues and patient information

We used human gastric cancer tissue specimen preserved in the Department of Pathology, Seoul National University Hospital. Primary gastric carcinoma in these patients was diagnosed and treated at Seoul National University Hospital between January 1, 1995 and June 30, 1995. The age, sex, tumor location, gross type, tumor size, lymphatic invasion, and pTNM classification were evaluated by reviewing the medical chart and pathologic report.

2.2. Cell lines and hypoxia conditions

293T, HeLa, H-1299, B16-F10 melanoma, and lung fibroblast cells were maintained in DMEM supplemented with 10% fetal bovine serum. Lung fibroblast cells were prepared from VDUP1 knockout mice and used in the stages from passage number 3 to 7. For hypoxic condition, cells were incubated at 1% O_2 balanced with N_2 in hypoxic chamber (Forma) for 4 h in HIF1α-protein analyses and for 16 h in HIF1α-transactivation assay.

2.3. In vitro invasion assay

Cell invasiveness was assayed using BioCoat Matrigel invasion chamber (BD Biosciences) according to the manufacturer's instructions. In brief, HeLa cells were transiently transfected with indicated plasmids using LipofectAMINE Plus reagent (Invitrogen). After 24 h transfection, cells were detached using trypsin-EDTA, resuspended in 750 μl of DMEM without serum, and plated at 3×10^4 number per upper chamber. DMEM containing 5% FBS was added to the lower chamber and the plates were incubated at 37 °C. After 12 h, the plates were moved into hypoxic condition and incubated more for 16 h. At completion, cells were removed from the upper side of the membrane with a cotton swab, the lower cells were fixed and stained with Toluidine Blue (Sigma), and then mounted in mounting medium (DAKO). Stained cells in the lower side were counted and data was expressed as the percent invasion of the mean of cells invading relative to that of empty control cells.

2.4. In vivo metastasis assay

B16-F10 cells were transfected with empty, VDUP1, or VDUP1-L294A vectors using CytoPure-*cmn* reagent (Qbiogene). After 48 h later, cells (3×10^5 number in 0.1 ml of PBS) were injected i.v. into the lateral tail veins of 6-week-old C57BL6 female mice. Five mice were used for each sample. Animals were killed after 2 weeks injection and their lungs were removed for quantification of metastases.

2.5. Short interference knockdown of VDUP1

SiRNA of VDUP1 was designed and synthesized by Samchully Pharm. (South Korea), with the following sequences. VDUP1 (sense); 5′-CCA UCC AUG CUG ACU UUG ATT-3′, and VDUP1 (antisense); 5′-UCA AAG UCA GCA UGG AUG GTT-3′. For negative control of siRNA, luciferase sequence was used. GL2 (sense); 5′-CGU ACG CGG AAU ACU UCG ATT-3′ and GL2 (antisense); 5′-UCG AAG UAU UCC GCG UAC GTT-3′. 100 nM of annealed duplex was transfected into the cells using LipofectAMINE Plus or 2000 (Invitrogen) according to the manufacturer's protocol.

2.6. Co-immunoprecipitaton assay

In order to evaluate the *in vivo* binding between pVHL and full- or partial-sized VDUP1, 293T cells were transfected with HA-pVHL and GST-VDUP1 or -partial VDUP1 forms, as indicated. After 24 h of transfection, the lysate supernatants were incubated for 1 h at room temperature with Glutathione Sepharose 4B (Amersham Biosciences), washed three times in washing buffer (lysis buffer containing 0.1% Triton X-100), then subjected to SDS-PAGE. In order to assess binding between pVHL and HIF1α, the same cells were transfected with Flag-pVHL, GST-HIF1α, and Flag-VDUP1 as indicated. After 24 h of transfection, the cells were incubated for 4 h with 10 μM MG-132 (Sigma) under hypoxic conditions, and then released into normoxic conditions for 15 min. The cell lysates were immunoprecipitated via the same technique.

2.7. Immunofluorescence staining

HeLa cells were transfected with GFP-HIF1α, HA-VHL, and/or GST-VDUP1 as indicated and were grown on coverslips for 24 h, and further incubated under hypoxia or normoxia for 6 h. In some cases, cells were treated with 1 ng/ml of leptomycin B (A.G. Scientific) for 2 h before fixation. Cells

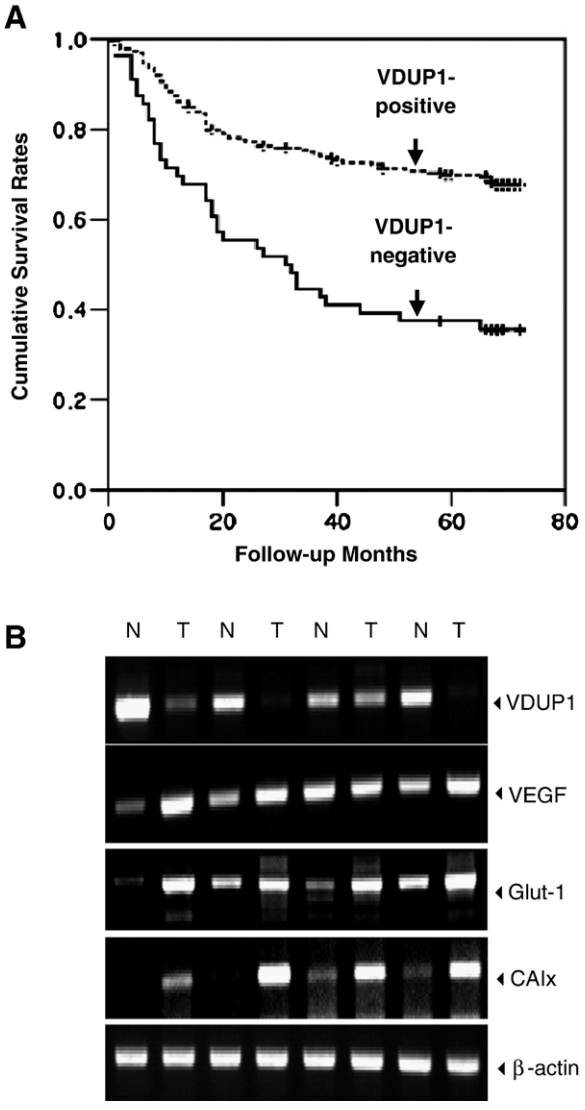

Fig. 1. A. Kaplan–Meier survival curves for patients with gastric cancer. The patients with VDUP1 positive cancers (dotted line) showed better prognosis compared to those with VDUP1 negative cancers (solid line). The differences between the two groups are statistically significant ($p<0.001$). B. Expression of VDUP1 and HIF1α target genes in gastric cancer tissues was analyzed. Gastric cancer tissues were prepared and analyzed by RT-PCR. N; normal tissue, and T; tumor tissue.