Renal cancer cells lacking hypoxia inducible factor (HIF)-1α expression maintain vascular endothelial growth factor expression through HIF-2α

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Recent efforts have been aimed at targeting the hypoxia inducible factor (HIF)-mediated hypoxia-induced gene pathway for renal cell carcinomas (RCC) therapy. Among the various genes induced by HIF, vascular endothelial growth factor (VEGF) is one of the critical mediators in angiogenesis, tumor growth and metastasis. To date, however, limited information is available on the functional differences regarding VEGF transcription between the HIF subunits, namely HIF-1α and HIF-2α. To investigate the HIF-1α and HIF-2α-dependent effect on VEGF gene induction in RCC, a panel of human RCC cell lines was analyzed. We found that a loss of HIF-1α protein expression was a common event in RCC cell lines, which was associated not only with truncated HIF-1α mRNA transcripts but also with transcriptional silencing. Since the CpG rich promoter region of the HIF-1α gene contained a similar frequency of methylated CpG dinucleotides in RCC cell lines, a complex and non-uniform mechanism may be involved in this phenomenon. In these HIF-1α defective cell lines, the knockdown of the HIF-2α gene demonstrated that HIF-2α regulated the VEGF production, irrespective of the VHL gene mutation status. In contrast, HIF-1α played a predominant role in VEGF secretion in the cells expressing both wild-type HIF-1α and HIF-2α proteins. HIF-1α may therefore represent an important target molecule for RCC therapy; however, HIF-2α should be targeted in HIF-1α defective renal cancer cells.

Introduction

Renal cell carcinomas (RCC) is the most common malignancy of the adult kidney, accounting for ~2% of all adult malignancies (1). Approximately 33% of all such patients have metastatic disease at presentation, and 40% of the individuals undergoing a surgical resection eventually develop metastases (2). RCC has been reported to be resistant to radiation or chemotherapy (3) and the prognosis for these patients remains poor. Although the use of cytokines such as interferon-α, interleukin-2 and combinations of these agents represents an encouraging pathway for the treatment of metastatic RCC, patients who do not respond to such cytokine-based therapy also have a very poor prognosis with a 5 year survival of <2% (2). The ideal therapeutic targets for an alternative treatment strategy in advanced RCC is thus hypothesized to be genes or gene products critical to the development or maintenance of the cancer. Histopathological evaluations of RCC have revealed it to be a highly vascularized neoplasm demonstrating clear evidence of abundant angiogenesis and abnormal blood vessel development (4). This notion has thus raised considerable concerns regarding the development of angiostatite therapies for RCC.

Interest in the role of VHL–hypoxia inducible factor (HIF)-mediated hypoxia-induced gene pathway in solid tumors has recently grown exponentially (5). HIF is a heterodimeric transcriptional factor composed of a constitutively expressed β subunit and an oxygen-regulated α subunit. It plays a key role in the regulation of the glucose metabolism, angiogenesis and erythropoiesis under oxygen-limited conditions (6). In normoxia, HIF-α molecules are subjected to a regulatory process involving the enzymatic hydroxylation of conserved prolyl and asparaginyl residues, thus leading to rapid VHL protein-mediated ubiquitination and proteasomal degradation (7). As a result, intratumoral hypoxia, or genetic mutations that disrupt the function of VHL protein (pVHL), induce the activation of this pathway. Increased HIF-α activity leads to the upregulation of genes that are involved in many aspects of cancer progression, including metabolic adaptation, apoptosis resistance and especially angiogenesis. Among the different HIF-α subunits identified to date, HIF-1α and HIF-2α are known to be widely expressed in primary cancer, in contrast to the lack of detectable staining in most normal tissues (8).

Since the somatic mutation of the VHL gene is the most frequent genetic alteration observed in RCC (9), intense vascular networks of the tumors can thus be attributed to the inappropriate accumulation of HIF-α via the stimulation of angiogenic gene induction. Vascular endothelial growth factor (VEGF) is one of the most potent pro-angiogenic factors, whose expression is transactivated by HIF-α (6). Elevated VEGF levels have been reported in RCC cell lines, tissues (10) and serum in RCC patients (11). An increased expression of VEGF has also been shown to be associated with malignant progression and a poor treatment outcome (12). Furthermore, a recent phase II trial of anti-VEGF antibodies therapy in RCC patients has shown the favorable results with a significant delay in the disease progression (13). These findings suggest that suppressing the HIF-mediated hypoxia-induced VEGF gene pathway may therefore be an important therapeutic strategy for the treatment of RCC. However, until now the relative contribution of HIF-1α versus HIF-2α to VEGF regulation in RCC has not yet been fully understood. Although several immunohistochemical
analyses on RCC specimens have revealed an overexpression of HIF-α proteins to be significantly associated with the upregulation of VEGF (14,15) and microvessel density (16), their target gene specificity have so far hardly been elucidated. Each HIF-α subunit is known to have a potential to transactivate the VEGF gene through binding to hypoxia response elements (17); however, recent target gene analyses have suggested HIF-1α to play a predominant role in non-RCC cancer cell lines (18,19). It is noteworthy that several groups have clearly demonstrated the tumorigenic potential of HIF-2α, but not HIF-1α, in 786-O cell line with VHL-negative RCC cell background (20,21). These observations have raised question about whether VEGF is an actual downstream target of HIF-2α in RCC. The purpose of this study is to gain a further understanding of the HIF-1α- and HIF-2α-dependent effect on VEGF gene induction in RCC by using a broader panel of RCC cell lines.

Materials and methods

Cell culture

Human RCC cell lines (786-O, 769-P, A-498, SW-839, Caki-1, Caki-2 and ACHN) were obtained from American Type Culture Collection. 293T-17 and KU-20-01 cell lines were established in our institute (22,23). All cells were grown in RPMI 1640 containing 10% FBS, 300 μg/ml streptomycin and 100 U/ml penicillin G at 37°C in a humidified, 5% CO2 containing atmosphere. Hypoxic conditions were generated in a humidified sealed chamber gassed with 1% O2, 5% CO2 and balanced N2.

DNA extraction and VHL mutation analysis

The genomic DNA samples were purified by standard procedures using DNA extraction and VHL mutation analysis previously (24).

Promoter methylation analysis

Genomic DNA was treated with sodium bisulfite as described (25). After promoter methylation analysis on RCC specimens have revealed an overexpression of HIF-1α in RCC, but not HIF-1α, in 786-O cell line with VHL-negative RCC cell background (20,21). These observations have raised question about whether VEGF is an actual downstream target of HIF-2α in RCC. The purpose of this study is to gain a further understanding of the HIF-1α- and HIF-2α-dependent effect on VEGF gene induction in RCC by using a broader panel of RCC cell lines. The genomic DNA samples were purified by standard procedures using DNA extraction and VHL mutation analysis previously (24).

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RNA extraction, RT-PCR and northern hybridization

The cells were lysed with an ISOGEN reagent (Nippon Gene, Toyama, Japan) according to the manufacturer’s directions for total RNA extraction. RNA was quantitated by the ratio of absorbance at 260/280 nm. First-strand cDNA was synthesized from 2 μg RNA by ReverTra Ace (Toyobo, Osaka, Japan) with a random hexamer primer. The primers for PCR amplification of the HIF-1α cDNA sequence between exons 1 and 6 were sense, 5′-AAGCTAGAACTTCATCATCTAG-3′, antisense 5′-GGGACAGTCATTAGTGG-3′; and 5′-AAGCTAGAACTTCATCATCTAG-3′, antisense 5′-GGGACAGTCATTAGTGG-3′.

Immunoblotting

Cell lysates were prepared in RIPA buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 1% Na deoxycholate, 0.1% SDS, 50 mM NaF, 1 mM Na3VO4, 10 μM leupeptin and 10 μg/ml aprotinin) followed by centrifugation (12 000 r.p.m., 10 min). Extracts were quantitated using the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA, USA). For immunoblotting, 20 μg proteins were resolved in 7.5% SDS–PAA gels and transferred to nitrocellulose membrane (Bio-Rad). The membranes were blocked with TBS containing 5% non-fat dried milk. Proteins were detected using monoclonal antibodies to HIF-1α (BD Transduction Laboratories, Lexington, KY, USA), HIF-2α (Novus Biologicals, Littleton, CO, USA) at 1:250 and 1:1000, respectively. Immunoreactive bands were visualized by an amplified alkaline phosphatase system according to manufacturer’s instructions (Bio-Rad).

Proteasome inhibition assay

To determine whether a loss of HIF-α protein expression was caused by abnormal degradation, we incubated cells with MG132 (carbobenzoxyl-leucinyl-leucinyl-leucinal; Sigma Chemical Co., St Louis, MO, USA), a potent proteasome inhibitor, as described previously (24). The HIF-α protein levels were detected by a subsequent western blot analysis.

Small interference RNA preparation and transfection

Small interference RNA (siRNA) were synthesized at Dharmacon Research (Lynwood, CO, USA) at 1:250 and 1:1000, respectively. Immunoreactive bands were visualized by an amplified alkaline phosphatase system according to manufacturer’s instructions (Bio-Rad).

Purchased primers for VHL were as follows: VHL sense 5′-TGTGGAGATT-ITTTGGTGATGTT-3′ and 5′-CCCAAGCACCAACACACACACAC-3′ for VHL US. To elucidate the methylation status of CpG site within the HIF-1α promoter region, bisulfite sequencing was carried out. The treated DNA was amplified at an annealing temperature of 56°C using bisulfite-specific primers 5′-GGAATTTAAGGAAAGTTTGG-3′ and 5′-GACCCCGCACTCCATC-3′. The PCR products were blunted by T4 DNA polymerase and then cloned with Zero Blunt TOPO PCR Cloning Kit (Invitrogen, Carlsbad, CA, USA). Several clones for each cell line were sequenced with the following primers, 5′-GCCAAGCTATTAGGAGACATTAG-3′, 5′-GTTGATTG-TAAATACGACTCATAAGG-3′ and 5′-AAGATATAATCTCCTCATTCCGCG-3′. The first two and the last primers are corresponding to the vector and internal sequence of the PCR product, respectively.

DNA extraction, RT-PCR and northern hybridization

The cells were lysed with an ISOGEN reagent (Nippon Gene, Toyama, Japan) according to the manufacturer’s directions for total RNA extraction. RNA was quantitated by the ratio of absorbance at 260/280 nm. First-strand cDNA was synthesized from 2 μg RNA by ReverTra Ace (Toyobo, Osaka, Japan) with a random hexamer primer. The primers for PCR amplification of the HIF-1α cDNA sequence between exons 1 and 6 were sense, 5′-AAGCTAGAACTTCATCATCTAG-3′, antisense 5′-GGGACAGTCATTAGTGG-3′; and 5′-AAGCTAGAACTTCATCATCTAG-3′, antisense 5′-GGGACAGTCATTAGTGG-3′. Other primers used in this study are as follows: HIF-1α exons 3 and 10 (sense 5′-AATGTTGAACAAATCATAGGAATTA-3′, antisense 5′-CTCAGTGGATTTGTCTAGT-3′), exons 7 and 12 (sense 5′-TCTAGTCAGCTTTAGTCTAGT-3′, antisense 5′-TCTAGTCAGCTTTAGTCTAGT-3′), and 5′-AAGCTAGAACTTCATCATCTAG-3′, antisense 5′-GGGACAGTCATTAGTGG-3′. HIF-2α exons 1 and 6 (sense 5′-TGCAGTGCAAGGAAAGGAA-3′, antisense 5′-CAGCTCTCTCAGGTTAAG-3′), exons 3 and 8 (sense 5′-AACGCTTTGGGATTTTTTCTT-3′, antisense 5′-TGAGAAGACACGTCATTAC-3′), exons 7 and 9 (sense 5′-AGGGAGCTACCTCTCTCTCTT-3′, antisense 5′-AGGGAGCTACCTCTCTCTCTT-3′), and 5′-AAGCTAGAACTTCATCATCTAG-3′, antisense 5′-GGGACAGTCATTAGTGG-3′. The products of PCR reactions, using a human cDNA library as a template, were cloned and sequenced to confirm the correct amplification. The sequenced fragment was then amplified by PCR again. The resulting PCR fragment was labeled with32P-dCTP using the Random Primed DNA Labeling Kit (Roche Diagnostics, Tokyo, Japan). Hybridization in formamide-containing buffer was carried out overnight at 42°C. After washing, radioactive hybrids were then visualized with a FluorImager FLA3000 (Fuji, Tokyo, Japan).
immunoassay kit (BioSource, Camarillo, CA, USA). VEGF levels were normalized to the final cell numbers that were counted with a hemocytometer.

**Results**

**Mutations of VHL gene in RCC cell lines**

We analyzed nine RCC cell lines for any mutation of the VHL tumor suppressor gene (Figure 1A). The sequence analysis revealed 4 of 9 cell lines to have a mutation including frame shift and missense. A methylation analysis demonstrated that 769-P cells had hypermethylated of promoter region CpG island of this gene (Figure 1B).

**Qualitative analysis of VHL and HIF-1α/-2α mRNA expression in RCC lines**

We next investigated the expression of VHL mRNA by RT–PCR. No VHL mRNA could be detected in the 769-P cells (Figure 2). Since treatment with the demethylating agent 5′-aza-2′-deoxycytidine (5-aza-dC) restored the expression of VHL mRNA in 769-P cells (data not shown), aberrant CpG island promoter methylation was thus suggested to be associated with the inactivation of VHL gene in 769-P.

Some renal carcinoma cells (786-0, A-498) are known to express HIF-2α protein, but no HIF-1α protein (28). One recent RNase protection assay analysis showed the expression of a HIF-1α-related mRNA in 786-O cells (19), while other studies did not (29,30). These previous findings suggest that HIF-1α mRNA may be truncated in 786-O cells. To identify the truncated forms of HIF-1α mRNA by RT–PCR, four pairs of PCR primers were designed. Complete HIF-1α mRNA was present in A-498, SW-839, Caki-1, Cali-2, ACHN and KU-19-20, whereas 786-O expressed a truncated HIF-1α mRNA. No HIF-1α-related mRNA could be detected in 769-P and KU-20-01. In contrast to HIF-1α mRNA, a full-length HIF-2α mRNA expression was observed in every cell line investigated.

**Bisulfite sequencing of the 5′-CpG-rich promoter region of the HIF-1α gene in RCC lines**

Examining the HIF-1α gene sequence (Accession No. AH_006957), we found that the promoter and 5′-first exonic region met the criteria for a CpG island (31) (Figure 3A). To examine the possibility that methylation change in the CpG island of HIF-1α gene may account for the loss of expression of this mRNA, bisulfite sequencing was carried out. Focusing on four Sp1 sites in the HIF-1α promoter region, we found the 3rd and the 4th Sp1 sites (the 49th and the 50th CG, respectively) to be completely free from methylation in all cell lines (Figure 3B). The 2nd Sp1 site (the 42th CG) and their boundaries were frequently methylated; however, this did not seem to affect the gene expression. Notably, the methylation of the 1st Sp1 site (the 5th CG) and its adjacent region were only observed in 769-P cells, which lack HIF-1α mRNA. We had speculated that the methylation of this region might result in gene silencing, but it could not confirm the restoration of the HIF-1α mRNA expression by treatment with 5-aza-dC (data not shown). In general, this sequence analysis revealed that the promoter contained a similar frequency of the methylated CpG dinucleotides in all renal cancer cell lines investigated.
The expression of HIF-1α and HIF-2α protein under normoxic/hypoxic conditions in RCC cell lines

To examine the HIF-1α and HIF-2α protein regulation, we conducted a western blot analysis. Whole cell extracts were prepared following normoxic or hypoxic incubation for 6 h. In the cell lines with wild-type VHL, a strong upregulation of HIF-1α protein was observed under hypoxic stimulation, whereas the VHL-mutated cell lines showed a similar level of HIF-1α protein under both conditions (Figure 4). No HIF-1α protein was detected in the cell lines with HIF-1α mRNA truncated or deficient. However, even with the complete expression of mRNA, A-498 and SW-839 did not express any detectable HIF-1α protein. Neither isoform of HIF-2α protein was observed in 769-P cells.

Association between HIF-α protein and mRNA level

To explain the discrepancy between the lack of a HIF-α protein expression but a positive mRNA expression, a northern hybridization analysis was performed. As shown in Figure 5A, the levels of mRNA were quite weak or absent in the cell lines lacking measurable protein expression (Figure 5B). For example, A-498 and SW-839 cells, which had been shown by RT–PCR to have a complete form of HIF-1α mRNA, did not express any detectable mRNA based on this analysis. Likewise, the HIF-2α mRNA level of 769-P cells was very low. To rule out the possibility that the HIF-α protein is rapidly degraded by the proteasome, these three cell lines were cultured in the presence of the proteasome inhibitor MG132. This treatment did not cause any change in the detection of HIF-α protein based on a western blot analysis (data not shown), thus arguing against an aberrant degradation of the HIF-α in these cells. Since the densitometric graphs demonstrate a correlation between HIF-α mRNA and protein levels (Figure 5C), the differences in the HIF-α protein levels among RCC cell lines is thought to mainly depend on the different levels of mRNA expression.
Stimulation of VEGF production under hypoxia in RCC cell lines

To evaluate the regulation of VEGF by HIF-1α, secreted VEGF was measured by ELISA in normoxic or hypoxic incubation for 24 h. In all wild-type VHL cell lines, the VEGF production level was relatively weak, under normoxia, but it was significantly stimulated under hypoxia (Figure 6). This VEGF upregulation was similar in HIF-1α deficient and wild-type cell lines. In all VHL-mutated cell lines examined, VEGF production was not stimulated by hypoxia. Compared with HIF-2α-negative 769-P cells, the other three HIF-2α positive cell lines showed significantly higher levels of VEGF production. These findings suggest that in situations where HIF-1α protein is deficient, HIF-2α protein can regulate the VEGF production in both VHL-mutant and wild-type cell lines.

siRNA knockdown model

To elucidate the molecular basis of the HIF-1α- or HIF-2α-specific effect on VEGF regulation, we analyzed the expression of VEGF after treatment with siRNA complementary to each HIF-α. HIF-1α targeted siRNA inhibited the expression of HIF-1α protein with minimal effect on HIF-2α and beta-actin expression; HIF-2α siRNA decreased HIF-2α expression but it had no effect on the levels of HIF-1α. This treatment did not significantly influence cancer cell viabilities (data not shown.) In KU-20-01, 786-O and A-498 cells, which expressed HIF-2α protein, but lacked functional HIF-1α, silencing the HIF-2α protein significantly reduced VEGF expressions (Figure 7). In contrast, siRNA directed against HIF-1α, but not against HIF-2α, inhibited VEGF production in Caki-2 and KU-19-20 cells that expressed both HIF-α proteins (Figure 8). These results indicate that the expression status of HIF-α subunits may lead to a loss of target gene specificity, and VEGF transcription is therefore possibly regulated by each HIF-α isoform in RCC.

Discussion

In this study, we demonstrated the functional redundancy of the two HIF-α subunits HIF-1α and HIF-2α in VEGF expression and secretion.
transcription in human RCC cell lines. The upregulation of HIF-2α was associated with VEGF production in the cell lines lacking HIF-1α, yet a functional dominance of HIF-1α was observed in the cells expressing both HIF-α subunits. These results suggest the potential significance of each HIF-α subunit in tumor angiogenesis and provide insights into developing treatment strategies targeting the VHL/HIF pathway in RCCs. We propose that HIF-1α represent an important target molecule for RCC therapy; however, HIF-2α should be targeted in HIF-1α defective renal cancer cells.

Our results indicate that a loss of HIF-1α protein expression is a common event in human RCC cell lines. Although post-translational modifications of HIF-1α protein have been extensively studied, no definitive mechanism has yet been elucidated to account for the loss of this gene expression. From this study, two distinctive patterns of HIF-1α mRNA expression were seen in HIF-1α-negative cells, namely, either undetectable or extremely low mRNA expression levels, and a truncated mRNA expression. Because transcriptional silencing is often associated in cancers with an aberrant methylation of gene promoter region, we hypothesized that the mechanism of undetectable or low mRNA expression levels involves the hypermethylation of the HIF-1α gene in some renal cancer cells. To our knowledge, this is the first study that has examined the CpG island methylation status of the HIF-1α gene. A previous study (32) determined Sp1 (5'-CGGCCC-3') and E-box (5'-CANNTG-3') transcription factor-binding sites in the promoter region. Methylation occurring at the CG of the Sp1 binding element has also been shown to sterically interfere with binding of transcription factors to DNA (33). However, the methylation status was not significantly altered between the renal cancer cell lines; therefore, abnormalities of trans regulatory factors might contribute to such gene silencing. Next, truncated HIF-1α mRNA was observed in 786-O,
which has been well known to express HIF-2α but not HIF-1α (34). Alternative splicing, estimated to occur in the pre-mRNAs of 34% of human genes (35), is one possible mechanism to explain this selective exclusion of mRNA sequence. In human cells, three splicing events of HIF-1α mRNA have been previously described with alternative splicing between exons 1 and 2, between exons 13 and 14 (36), and also between exons 11 and 12 (37). In our study of RCC cells, the 3’-terminal exons were constitutively excluded in 786-O cells. Many studies have reported cancer-specific aberrant splicing in the presence or absence of genomic mutations (38); however, the molecular mechanism that regulates such aberrant splicing of HIF-1α mRNA remains unclear.

Tumor growth and the development of metastases are partly dependent upon angiogenesis. The role of HIF in the regulation of VEGF therefore provides a potential link between HIF activation and the promotion of tumor growth. However, the relative contribution of HIF-1α versus HIF-2α in RCC is still not fully understood. Previous studies on VHL-defective 786-O cells have shown HIF-2α to regulate VEGF expression (18,19) while it is also a key player in renal tumorigenesis in vivo (20,21). Surprisingly, a larger panel of VHL-defective RCC cell lines in our study were similarly lacking the functional HIF-1α. HIF-1α has been shown to induce cell death by stabilizing p53, through a Bnip3/Nix-dependent mechanism, by altering the expression of Bel-2 family members or by enhancing caspase 8 activity (33). We speculate that this pro-apoptotic effect of HIF-1α may therefore negatively influence the VHL-mutant clones’ survival during primary tumor culture, and it may thus be difficult to constitutively establish HIF-1α-activated cell lines. Despite the deficiency of functional HIF-1α protein, the VHL-defective cells showed a relatively high level of VEGF secretion when the cells had HIF-2α expression. Combining the results from siRNA knockdown experiments, HIF-2α is thus suggested to play a crucial role in VEGF transcriptional regulation in these cells.

Several studies in RCC tissue samples reveal that, when VHL mutations are not found in the tumor, the frequency of HIF-1α protein expression varies from 14 to 75% (14–16). Although the HIF-1α expression is suggested to correlate with higher levels of VEGF production in the renal tumor, the possible contribution of HIF-2α has not yet been determined from these studies. By using siRNA specifically directing against HIF-1α or HIF-2α, the present study demonstrated, in a subset of VHL-positive RCC cell lines, that hypoxia-induced upregulation of VEGF mainly depended on HIF-1α in cells expressing both HIF-α subunits. During the course of this study, Raval and coworkers (39) published data demonstrating that VEGF production was downregulated by HIF-1α-directed siRNA in Caki-1 cells, which thus correlated with our observations in Caki-2 and KU-19-20 cells. We had performed selective knockdown experiments in all four of the cell lines with wild-type VHL and functional HIF-1α mRNA, but could not achieve a sufficient inhibition of HIF-α protein expression in Caki-1 and ACHN cells. In addition, however, we further showed that HIF-2α played a pivotal role during hypoxic VEGF induction in HIF-1α defective cells (KU-20-01). Numerous studies have revealed that not only HIF-1α but also HIF-2α can transactivate hypoxia response element (HRE)-containing promoter of VEGF gene (17,40,41). The loss of target gene specificity by the transient overexpression of either HIF-α is also suggested (19). Despite this potentially redundant function, two recent reports using a similar siRNA knockdown methods describe the predominance of HIF-1α in this role in human cancer cell lines of hepatoma, neuroblastoma and breast cancer; expressing both HIF-α isoforms and probably functional pVHL (18,19). Our results also support the speculation that VEGF may actually be a HIF-1α target gene, regardless of the cell type, even in RCC. However, the molecular mechanism that explains the predominant transcriptional function of HIF-1α in VEGF regulation should be investigated in future studies.

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Conflict of Interest Statement: None declared.
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