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REVIEW

HIF Signaling Pathway in Pheochromocytoma and Other Neuroendocrine Tumors

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Summary

Hypoxia-inducible factors (HIFs) are transcription factors controlling energy, iron metabolism, erythropoiesis, and development. Dysregulation of these proteins contributes to tumorigenesis and cancer progression. Recent findings revealed the important role of HIFs in the pathogenesis of neuroendocrine tumors, especially pheochromocytoma (PHEO) and paraganglioma (PGL). PHEOs and PGLs are catecholamine-producing tumors arising from sympathetic- or parasympathetic-derived chromaffin tissue. To date, eighteen PHEO/PGL susceptibility genes have been identified. Based on the main signaling pathways, PHEOs/PGLs have been divided into two clusters, pseudohypoxic cluster 1 and cluster 2, rich in kinase receptor signaling and protein translation pathways. Recent data suggest that both clusters are interconnected *via* the HIF signaling and its role in tumorigenesis is supported by newly described somatic and germline mutations in *HIF2A* gene in patients with PHEOs/PGLs associated with polycythemia, and in some of them also with somatostatinoma. Moreover, HIFα signaling has also been shown to be upregulated in neuroendocrine tumors other than PHEO/PGL. Some of these tumors are components of hereditary tumor syndromes which can be associated with PHEO/PGL, but also in ileal carcinoids or melanoma. HIF signaling appears to be one of the crucial players in tumorigenesis, which could suggest new therapeutic approaches for treatment of neuroendocrine tumors.

Key words

Pheochromocytoma • Paraganglioma • Hypoxia-inducible factor • Oxygen sensing • Therapy

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Introduction

PHEOs and PGLs are rare neuroendocrine catecholamine producing tumors. PHEOs arise from chromaffin cells within the adrenal medulla whereas tumors arising from extra-adrenal sympathetic and parasympathetic paraganglia are called PGLs (Pacak *et al.* 2007, Pacak 2011). The majority of PHEOs/PGLs are benign tumors but metastasis can also occur, especially in patients with a specific genetic background (Brouwers *et al.* 2006, Ayala-Ramirez *et al.* 2011, Eisenhofer *et al.* 2012). In the past, it was postulated that 10 % of these tumors were hereditary, but recent studies show 30-40 % PHEOs/PGLs to be genetically inherited (Neumann *et al.* 2002, Karasek *et al.* 2012). Moreover, 10-24 % of apparently sporadic tumors were found to have somatic mutations (Burnichon *et al.* 2011, Welander *et al.* 2012, Crona *et al.* 2014).

In this review, we focus on the role of hypoxia-

inducible factor signaling in neuroendocrine tumor development.

Update on genetics of PHEO/PGL

To date, 18 genes predisposing to PHEO/PGL development have been identified (Table 1). The susceptibility genes include the *VHL* tumor suppressor gene, the *NF1* tumor suppressor gene, the *RET* proto-oncogene, the SDH complex subunits genes (*SDHA, SDHB, SDHC*, and *SDHD*), and the *SDHAF2* gene (Karasek *et al.* 2012, Vicha *et al.* 2013). The new PHEO/PGL susceptibility genes include *TMEM127*, *MAX*, *HIF2A* and, by anecdotal reports, *KIF1Bβ*, *PHD2/EGLN1*, *H-RAS*, *K-RAS*, *IDH*, *FH*, and *BAP1* genes (Hrascan *et al.* 2008, Ladroue *et al.* 2008, Schlisio *et al.* 2008, Gaal *et al.* 2010, Qin *et al.* 2010, Astuti *et al.* 2011, Comino-Mendez *et al.* 2011, 2013, Jiang and Dahia 2011, Wadt *et al.* 2012, Zhuang *et al.* 2012, Crona *et al.* 2013, Castro-Vega *et al.* 2014).

Two main clusters, based on the main signaling pathways, currently represent almost all hereditary PHEOs/PGLs: 1) cluster 1, a pseudohypoxic cluster, which is mainly represented by *VHL, SDHx* and, probably *HIF2A* and *FH* mutations, and 2) rich in kinase receptor signaling and protein translation pathways, cluster 2 is mainly composed of *NF1, RET, KIF1Bβ, TMEM127*, and *MAX* mutations (Dahia *et al.* 2005, Gimenez-Roqueplo *et al.* 2012, Vicha *et al.* 2013, Castro-Vega *et al.* 2014). Although cell signaling in these two clusters seems to be distinct, recent findings uniquely unify important existing connections between signaling pathways in both clusters. Not only that, the HIF signaling pathways appear to be a crucial, perhaps the most important player, in PHEO/PGL tumorigenesis.

Table 1. PHEO/PGL susceptibility genes and genotype-phenotype correlations.

Gene	Syndrome	Locus	Inheritance	Biochemical phenotype	Common PHEO/PGL sites	Malignant potential
SDHA	-	5p15	AD	Unknown	HNPGL/TAPGL	0-14 %
SDHB	PGL4	1p36.13	AD	MN, NMN, MTY, NS	TAPGL/HNPGL/adrenal	43 %
SDHC	PGL3	1q21	AD	MN, NMN, NS	HNPGL/TAPGL/adrenal	Low
SDHD	PGL1	11q23	AD PI	MN, NMN, MTY, NS	HNPGL/TAPGL/adrenal	5 %
SDHAF2	PGL2	11q13.1	AD PI	Unknown	HNPGL	Unknown
VHL	VHL	3p25-26	AD	NMN	adrenal/TAPGL/HNPGL	Low (<5 %)
NF1	NF1	17q11.2	AD	MN, NMN	Adrenal	11 %
RET	MEN	10q11.2	AD	MN, NMN	Adrenal	Low (<1-5 %)
MAX	-	14q23.3	AD PI	NMN and MN	Adrenal	10-25 %
TMEM127	-	2q11.2	AD	MN and NMN	Adrenal/TAPGL/HNPGL	Rare (4 %)
HIF2A	Pacak-Zhuang	2p21-p16	Somatic Germline*	NMN	TAPGL/adrenal	Unknown
H-RAS	NA	11p15.5	Somatic	Unknown	Adrenal	Unknown
K-RAS	NA	12p12.1	Somatic	Unknown	Adrenal	Unknown
IDH1	NA	2q33.3	Somatic	Unknown	Unknown (HNPGL?)	Unknown
KIF1Bβ	NA	1p36.2	Germline	Unknown	Unknown (Adrenal?)	Unknown
PHD2/EGLN1	NA	1q42.1	Germline	Unknown	Unknown (TAPGL?)	Unknown
FH	NA	1q43	Germline	Unknown	Adrenal/TAPGL	Unknown/ potentially high
BAP1	NA	3p21.1	Germline	Unknown	Unknown (TAPGL?)	Unknown

*Presenting only with PHEO/PGL together with polycythemia; most likely different from the typical somatic type of HIF2A mutation. AD, autosomal dominant; HNPGL, head and neck paraganglioma; MN, metanephrine; MTY, metoxytyramine; NMN, normetanephrine; NS, nonsecreting; PGL, paraganglioma; PHEO, pheochromocytoma; PI, paternal inheritance; TAPGL, thoracic or abdominal paraganglioma.

Hypoxia-inducible factors (HIFs)

HIFs are transcription factors which are activated under hypoxic or pseudohypoxic conditions and mediate adaptive responses of cells to these states, an idea originally pioneered by Semenza (2011) then later further studied and advanced by others (Schofield and Ratcliffe 2004, Kaelin and Ratcliffe 2008). Hypoxia reflects the lack of sufficient oxygen supply (O_2 concentration drops below 21 %); pseudohypoxia is the state where oxygen is present in a sufficient amount in cells but cannot be processed and further utilized due to an alteration in oxygen-sensing pathways. HIFs are composed of stable, constitutively expressed β-subunit, and ubiquitously expressed, oxygen-sensitive α-subunits, consisting of three isoforms; HIF-1α, HIF-2α, and HIF-3α (Schofield and Ratcliffe 2004, Kaelin and Ratcliffe 2008, Keith *et al.* 2012).

Under normoxic conditions, HIF-1α and HIF-2α are degraded *via* the ubiquitin-proteasome pathway, which is controlled by several key enzymatic reactions, the first including prolyl hydroxylation by PHDs (also called as the main O_2 sensors) and the second represented by subsequent bindings to the VHL protein (for a review, see Kaelin and Ratcliffe 2008, Koh and Powis 2012). When these enzymatic reactions occur, HIFs do not stabilize well and they are degraded quickly by the proteasome. Additional and less important negative regulation of HIF is mediated *via* the HIF-3α2 splice variant (IPAS), which forms inactive complexes with HIF-1α, and also *via* the FIH1 that hydroxylases HIF on the asparaginyl residue of the C-terminal domain. FIH1 blocks HIF-1α interaction using coactivators with proteasome ultimately breaking down the complex (Makino *et al.* 2001, Lando *et al.* 2002, Jang *et al.* 2005, Heikkila *et al.* 2011).

Hypoxia or pseudohypoxia lead to HIFα stabilization and its heterodimerization with HIF-β, binding of coactivators, and the transactivation of HIF target genes, including those associated with angiogenesis, hematopoiesis, glycolysis, cell growth, and cell migration (Maxwell *et al.* 2001, Lau *et al.* 2007, Kaelin and Ratcliffe 2008).

HIF-1α and HIF-2α are the best studied isoforms and were found to have mostly complementary, but also opposite functions (Hu *et al.* 2003, Carroll and Ashcroft 2006, Rankin *et al.* 2007). HIF-1α is activated during short periods of severe hypoxia, while HIF-2α is active under mild hypoxia for more prolonged periods of time (Holmquist-Mengelbier *et al.* 2006). Term mild hypoxia is used for oxygen concentrations in tissues of 1-5 % and severe hypoxia is defined as below 1 % (Koh and Powis 2012). The distinct effect of different oxygen concentrations on HIFα activation is mediated by HAF, which marks HIF-1α for degradation but transactivates HIF-2α by binding to a different protein site than in HIF-1α (Koh *et al.* 2008, 2011). Moreover, both HIF-1α and HIF-2α functionally interact with many other signal transduction and transcriptional systems, including NOTCH, WNT, and MYC pathway; thus, HIFs can also regulate gene expression *via* a hypoxia-independent mechanism (Kaelin and Ratcliffe 2008).

Distinct regulation of HIFα isoforms leads to distinct expression patterns of these transcription factors. HIF-1α is expressed in all cells, whereas HIF-2α is preferentially expressed in the endothelium, kidney, heart, lung, gastrointestinal epithelium, and neural crest cell derivatives, including chromaffin cells (Wiesener *et al.* 2003, Keith *et al.* 2012). HIF-2α was shown to play an important role in the regulation of developmental processes of sympathoadrenal lineage; it also regulates catecholamine synthesis and secretion reflected by a typical noradrenergic phenotype (for a review, see Richter *et al.* 2013).

HIF signaling in tumorigenesis

HIF interaction with many signaling and transduction pathways and the presence of (pseudo)hypoxia in cancer lead to the presumption that HIF is one of the most important players in tumorigenesis. It was found that both HIF-1α and HIF-2α are overexpressed in most human cancers (Zhong *et al.* 1999, Talks *et al.* 2000, Hockel and Vaupel 2001). HIF-1α and HIF-2α expression in tumors vary among cell types and these differences most probably reflect the consequences of preferential HIF-1α or HIF-2α function in different cancer cell subtypes, varying stages of tumor progression (including a degree of hypoxia), and within microenvironmental changes of the tumor (Blouw *et al.* 2003, Raval *et al.* 2005, Semenza 2010, Keith *et al.* 2012). Although both HIFα subunits are often overexpressed in many tumors, HIF-2α is preferentially linked to more aggressive lesions with a higher metastatic potential or already present metastases (Warnecke *et al.* 2004, Koh and Powis 2012).

Thus, HIF-2α overexpression was found to be associated with metastatic progression and poor

prognosis in specific cancer types, especially astrocytoma, glioma, neuroblastoma, head and neck cancers, and melanoma (Keith *et al.* 2012). In renal cell carcinoma, in which VHL mutations lead to decreased HIFα degradation by the defective bindings of hydroxylated HIFs to pVHL, HIF-2α stabilization leads to the tumor formation in mice (Kondo *et al.* 2003) and protumorigenic genes were shown to be more HIF-2α dependent (Raval *et al.* 2005). However, it was also shown that both HIF-2α upregulation and downregulation can promote tumor development and progression (Kim *et al.* 2009, Mazumdar *et al.* 2010) therefore, a certain balance between HIF-1α and HIF-2α is needed for optimal function, specifically in tumors. Moreover, HIF-2α has been shown to regulate HIF-1α target genes expression in the absence of HIF-1α and vice versa (Keith *et al.* 2012, Koh and Powis 2012). Thus, it is not surprising that renal cell carcinomas may be divided into two major subgroups; those preferentially expressing HIF-2α and those with combined expression of HIF-1α and HIF-2α.

HIF activation in cancer is mediated not only *via* the (pseudo)hypoxia signaling pathway but also *via* multiple oxygen-independent oncogenic pathways, including growth factor signaling pathways and a loss of tumor suppressor genes (for a review, see Jochmanova *et al.* 2013, Richter *et al.* 2013).

HIF in PHEO/PGL development

The role of HIF in the PHEO/PGL pathogenesis has been anticipated for a long time since HIF pathway was previously found to be dysfunctional in these tumors (Maxwell *et al.* 2001, Dahia *et al.* 2005, Favier and Gimenez-Roqueplo 2010). Interestingly, most of hereditary PHEOs/PGLs are indeed related to the hypoxia signaling pathway, mainly through the mutations within the *VHL, PHD2, FH, SDHx*, and *SDHAF2* genes (Jafri and Maher 2012, Toledo *et al.* 2013, Castro-Vega *et al.* 2014), which lead to increased stability of HIF (Gimenez-Roqueplo *et al.* 2001). In the *RET*- and *NF1*-associated PHEOs/PGLs, the activation of the RAS-MAPK-mTORC pathway is present, which leads to HIF-1α activation (Eisenhofer *et al.* 2004a, Dahia *et al.* 2005, Johannessen *et al.* 2005, Foster and Fingar 2010, Lopez-Jimenez *et al.* 2010). In tumors associated with *TMEM127* and *MAX* mutations, HIFα levels seem to be increased also due to mTORC activation (Brugarolas *et al.* 2004, Land and Tee 2007; for a review, see Jochmanova *et al.* 2013). *K-RAS* and recently described *H-RAS* mutations in PHEOs/PGLs lead to the activation of the RAS/RAF/ERK pathway, a part of the RAS/MAPK pathway (Hrascan *et al.* 2008, Crona *et al.* 2013), and to the increased HIFα signaling. *FH* mutations result in a loss of fumarate hydratase activity, an enzyme important in the Krebs cycle. *FH*-related PHEOs/PGLs had widespread alterations in protein succination and displayed the same epigenetic changes as SDHB-related tumors (Castro-Vega *et al.* 2014).

One of the most recently described genes associated with PHEO/PGL development is *HIF2A*. Initially, somatic gain-of-function *HIF2A* mutations were found in tumors from two female patients with multiple PGLs and polycythemia. One of these patients also presented with somatostatinoma (Zhuang *et al.* 2012). Subsequently, two other patients with multiple PGLs and somatostatinomas associated with polycythemia were also found to have somatic *HIF2A* mutations, suggesting the existence of a new syndrome described by Pacak and Zhuang (Pacak *et al.* 2013). *HIF2A* mutations result in HIF-2α protein stabilization by affecting its hydroxylation, which prevents recognition of HIF-2α by VHL and results in increased HIF-2α protein half-life followed by changes in transcriptional activity of many hypoxia-related genes (Zhuang *et al.* 2012, Yang *et al.* 2013). Further investigations revealed other patients with PHEO and this syndrome, extending the clinical characteristics of the Pacak-Zhuang syndrome to multiple PHEOs (Taieb *et al.* 2013). Subsequently, Lorenzo *et al.* (2013) found a male patient presenting with PHEO and PGLs associated with polycythemia due to a germline *HIF2A* mutation and Favier *et al*. (2012) identified a *HIF2A* mutation in PHEO resected from a 24-year-old female without polycythemia. However, none of these presented with somatostatinoma. Comino-Méndez *et al.* (2013) described three additional female patients with multiple PHEOs/PGLs and polycythemia and three patients with apparently sporadic disease. The other patient with somatic *HIF2A* mutation – female presenting with polycythemia, PHEO, PGLs and somatostatinoma – has been described recently by Buffet *et al*. (2014). They also found a *HIF2A* heterozygous mutation in a male patient with polycythemia and PGL; the mutation was present in the PGL tissue but also as a mosaic in leukocyte DNA and DNA extracted from buccal cells. HIF-2α stabilization and its involvement in the pathogenesis of PHEOs/PGLs is also supported by the presence of only the noradrenergic biochemical

phenotype, which reflects the involvement of HIF-2α in preferential norepinephrine synthesis as described previously (Tian *et al.* 1998, Eisenhofer *et al.* 2004b, Nilsson *et al.* 2005). In summary, the presence of multiple PHEOs/PGLs and duodenal somatostatinomas associated with polycythemia currently seem to only be present in female patients.

Previously, dominantly inherited gain-of-function *HIF2A* mutations were found to be associated with congenital polycythemia (Percy *et al.* 2008, Prchal and Gordeuk 2008), which stresses the role of HIF-2α in the regulation of erythropoiesis. HIF-2α stabilization and PGL-associated erythropoietin production have been also found in patients with *PHD2* and *VHL* mutations (Ladroue *et al.* 2008, Capodimonti *et al.* 2012).

The role of HIF in PHEO/PGL pathogenesis is further supported by the gene expression profiling study, which revealed distinct differences in gene expression between both hereditary and sporadic norepinephrine- and epinephrine-producing PHEOs/PGLs including detection of genes involved in hypoxia-angiogenic pathways, especially *HIF2A* (Eisenhofer *et al.* 2004b). Later on, in the study of Dahia *et al*. (2005), cluster 1 tumors were found to display a gene signature of the activated hypoxia pathway, associated with enhanced angiogenesis and extracellular matrix processes, which are known to be HIF-1α signaling dependent. Subsequent other gene expression profiling studies confirmed strong *HIF2A* expression in VHL and SDHx tumors (Lopez-Jimenez *et al.* 2010, Burnichon *et al.* 2011). Moreover, Lee *et al*. (2005) proposed that the PHEO/PGL mutations of *NF1, RET, VHL* and *SDHx* all act on the same signaling network, resulting in decreased apoptosis (also dependent on the HIF signaling pathway) during chromaffin cell development and tumor formation; additionally, cluster 2 gene mutations seem to enhance HIF-2α signaling in cells (for a review, see Jochmanova *et al.* 2013, Richter *et al.* 2013).

HIF and other neuroendocrine tumors

The HIFα signaling has also been shown to be upregulated in neuroendocrine tumors other than PHEO/PGL. Some of these tumors are components of hereditary tumor syndromes which can be associated with PHEO/PGL, e.g. VHL disease caused by the mutation of *VHL* or MEN due to the mutations in the *RET* proto-oncogene. In MEN type 1, pituitary, parathyroid and pancreas tumors are present, while in MEN type 2, there are medullary thyroid carcinomas, PHEO, and parathyroid tumors or, simply with mucosal neuromas (Gaztambide *et al.* 2013, Lodish 2013). Medullary thyroid cancer is rare and typically occurs as a part of MEN2. A recent study by Takacova *et al.* (2014) brought the experimental evidence for the crosstalk between RET and HIF-1.

Speisky *et al.* (2012) studied the expression of 52 genes in patients with VHL and sporadic pancreatic neuroendocrine tumors. They found upregulation of genes directly related to HIF signaling (*CA9*, *HIF2A*, *GLUT1*), angiogenesis, epithelial to mesenchymal transition, and metastasis in VHL pancreatic neuroendocrine tumors.

Tumor hypoxia and overexpression of HIF-1α and HIF-2α were also found in ileal carcinoids. HIF-2α expression was significantly higher in distant metastasis compared to a primary tumor in the same patient. Immunohistochemical analysis demonstrated expression of VEGF and CA9. Expression profiling of hypoxic carcinoid cells revealed significant regulation of a large number of genes, including the chemokine receptor CXCR4 (Arvidsson *et al.* 2010).

It has also been demonstrated that hypoxia can induce NOTCH downstream signaling in several tumor cell lines. Accumulation of HIF-2α promotes an aggressive phenotype with dedifferentiation and activation of NOTCH signaling in neuroblastoma cells (Jogi *et al.* 2004, Holmquist-Mengelbier *et al.* 2006, Sahlgren *et al.* 2008).

High expression levels of HIF-1α, HIF-2α and their target genes have been detected in melanoma cells and they were found to be related to a poor prognosis of patients (Zhong *et al.* 1999, Giatromanolaki *et al.* 2003, Valencak *et al.* 2009). Enhanced expression of HIFs, transcription of their target genes, and altered metabolism in melanoma cells during disease progression may promote malignant reprogramming and acquisition of stem cell-like properties and more aggressive and metastatic phenotypes, reviewed by Mimeault and Batra (2013).

Conclusions and future treatment options

HIFα signaling appears to be a central signaling pathway involved in the pathogenesis of hereditary PHEOs/PGLs, sporadic PHEOs/PGLs, and other neuroendocrine tumors. This designates HIFα as a very promising therapeutic target, especially for more

aggressive and metastatic tumors where the introduction of HIF-2α is of the greatest interest at present. Nowadays, several agents affecting HIF-1α signaling have been introduced, with various results depending on a cancer HIFα phenotype (Liu *et al.* 2014, for a review, see Melillo 2007a,b). Nevertheless, drugs selectively targeting the HIF-2α signaling pathways have not yet been described but are currently under development (Rogers *et al.* 2013, Scheuermann *et al.* 2013). Moreover, it has been shown that HIF-1α and HIF-2α can activate different genes (Keith *et al.* 2012, for a review, see Koh and Powi 2012); therefore, the development of drugs targeting both HIF-1α and HIF-2α is of great interest. It was shown that HAF switches cells from HIF-1α to HIF-2α signaling; in light of that, one therapeutic approach could be to change the balance of HIFα isoforms by modulating HAF signaling in tumors (Koh *et al.* 2011). Further investigations are needed to elucidate the other signaling pathways involved in PHEO/PGL development and crosstalk within these pathways and HIF signaling pathway. The combination of two or three drugs, one of them targeting the HIFα signaling pathway and the other two regulating signaling pathways independently of HIFs, could potentially serve as a promising therapeutic strategy in combatting metastatic neuroendocrine tumors, especially PHEO/PGL.

Finding of novel diagnostic tests based on biomarkers (detected either biochemically or by molecular imaging) associated with hypoxia and altered metabolic pathways (by measurement of various metabolites, specifically within the Krebs cycle) should also help to select patients who are likely to respond to that specific type of therapy (e.g. to the HIF signaling inhibitors) and to personalize anti-cancer treatment. We predict that the introduction of novel HIF-related therapies will soon be integrated into medical practices that are specialized in the treatment of neuroendocrine tumors.

Conflict of Interest

There is no conflict of interest.

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Abbreviations

BAP1, BRCA1-associated protein-1; CA9, carbonic anhydrase-IX; CXCR4, C-X-C chemokine receptor type 4; ERK, extracellular signal-regulated kinase; FH, fumarate hydratase; FIH1, factor inhibiting HIF; GLUT1, glucose transporter 1; HAF, hypoxia-associated factor; HIF, hypoxia-inducible factor; *HIF2A*, hypoxia-inducible factor 2-alpha gene; *H-RAS*, Harvey rat sarcoma viral oncogene; IPAS, inhibitory PAS domain protein; IDH, isocitrate dehydrogenase; KIF1Bβ, kinesin family member 1B, transcript variant β; *K-RAS*, Kirsten rat sarcoma viral oncogene; MAPK, mitogen activated protein kinases; MAX, MYC-associated factor X; MEN, multiple endocrine neoplasia; mTORC, mammalian target of rapamycin complex; MYC, v-myc avian myelocytomatosis viral oncogene homolog; NF1, neurofibromatosis type 1; PGL, paraganglioma; PHD, prolyl hydroxylase; PHD2/EGLN1, prolyl hydroxylase 2; PHEO, pheochromocytoma; pVHL, von Hippel-Lindau protein; RAF, proto-oncogene serine/threonine-protein kinase; RET, rearranged during transfection; SDH, succinate dehydrogenase; SDHAF2, SDH complex assembly factor 2; TMEM127, transmembrane protein 127; VEGF, vascular endothelial growth factor; VHL, von Hippel-Lindau.

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