**Hypoxia-inducible Factor 2α: A Key Player in Tumorigenesis and Metastasis of Pheochromocytoma and Paraganglioma?**

**Authors**
Nicole Bechmann¹, ², ³, ⁴, Graeme Eisenhofer¹, ²

**Affiliations**
1 Institute of Clinical Chemistry and Laboratory Medicine, University Hospital Carl Gustav Carus, Medical Faculty Carl Gustav Carus, Technische Universität Dresden, Dresden, Germany
2 Department of Medicine III, University Hospital Carl Gustav Carus, Medical Faculty Carl Gustav Carus, Technische Universität Dresden, Dresden, Germany
3 German Institute of Human Nutrition Potsdam-Rehbruecke, Department of Experimental Diabetology, Nutheital, Germany
4 German Center for Diabetes Research (DZD), München-Neuherberg, Germany

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**Correspondence**
Nicole Bechmann, PhD  
University Hospital Carl Gustav Carus, Medical Faculty Carl Gustav Carus, Technische Universität Dresden, Fetscherstrasse 74  
01307 Dresden  
Germany  
Tel.: +49 351 458–19687  
Nicole.bechmann@uniklinikum-dresden.de

**ABSTRACT**
Germline or somatic driver mutations linked to specific phenotypic features are identified in approximately 70 % of all catecholamine-producing pheochromocytomas and paragangliomas (PPGLs). Mutations leading to stabilization of hypoxia-inducible factor 2α (HIF2α) and downstream pseudohypoxic signaling are associated with a higher risk of metastatic disease. Patients with metastatic PPGLs have a variable prognosis and treatment options are limited. In most patients with PPGLs, germline mutations lead to the stabilization of HIF2α. Mutations in HIF2α itself are associated with adrenal pheochromocytomas and/or extra-adrenal paragangliomas and about 30 % of these patients develop metastatic disease; nevertheless, the frequency of these specific mutations is low (1.6–6.2 %). Generally, mutations that lead to stabilization of HIF2α result in distinct catecholamine phenotype through blockade of glucocorticoid-mediated induction of phenylethanolamine N-methyltransferase, leading to the formation of tumors that lack epinephrine. HIF2α, among other factors, also contributes importantly to the initiation of a motile and invasive phenotype. Specifically, the expression of HIF2α supports a neuroendocrine-to-mesenchymal transition and the associated invasion-metastasis cascade, which includes the formation of pseudopodia to facilitate penetration into adjacent vasculature. The HIF2α-mediated expression of adhesion and extracellular matrix genes also promotes the establishment of PPGL cells in distant tissues. The involvement of HIF2α in tumorigenesis and in multiple steps of invasion-metastasis cascade underscores the therapeutic relevance of targeting HIF2α signaling pathways in PPGLs. However, due to emerging resistance to current HIF2α inhibitors that target HIF2α binding to specific partners, alternative HIF2α signaling pathways and downstream actions should also be considered for therapeutic intervention.
ABBREVIATIONS

ARNT ary hydrocarbon receptor nuclear translocator
ATRX ATRX chromatin remodeler
ccRCC clear cell renal cell carcinoma
dLST dihydrolipoamide S-succinyltransferase
EMT epithelial-mesenchymal transition
EPAS1 endothelial PAS protein 1
FH fumarate hydratase
FGFR1 fibroblast growth factor receptor 1
GOT2 glutamic-oxaloacetic transaminase 2
HIF2α hypoxia-inducible factor 2α
HREs hypoxia-responsive elements
IDH isocitrate dehydrogenases
NF1 neurofibromin 1
MAX MYC associated factor X
MDH2 malate dehydrogenase 2
neuroendoMT neuroendocrine-to-mesenchymal transition
PNMT phenylethanolamine N-methyltransferase
PPGLs pheochromocytomas and paragangliomas
PHDs prolyl hydroxylases
SDH succinate dehydrogenase
RET Ret proto-oncogene
SLC25A11 mitochondrial 2-oxoglutarate/malate carrier
TET ten-eleven translocation
TMEM127 transmembrane protein 127
VHL von Hippel-Lindau

Introduction

Pheochromocytomas and paragangliomas (PPGLs) are rare neural crest-derived tumors (2–8 cases per million per year) that respectively originate from chromaffin cells of the adrenal medulla or the extra-adrenal sympathetic paraganglia [1]. Paragangliomas of the head and neck on the other hand are derived mainly from non-chromaffin cells associated with parasympathetic ganglia. Most PPGLs do not metastasize, but up to 20% of patients with PPGLs present with distant metastases at sites where chromaffin cells are normally absent, such as lymph nodes, lung, liver, and bones [2–4]. Metastatic disease may be identified at either initial diagnosis (synchronous disease) or only become apparent at follow-up (metachronous disease). Whether the metastatic disease is synchronous or metachronous, the prognosis is poor, though highly variable. An early diagnosis or even prediction of metastatic disease in metachronous cases or patients screened due to hereditary risk may nevertheless be useful for earlier intervention and an improved outcome for any affected patient; to this end, several risk factors have been well established to be associated with metastatic disease. These include germline mutations in succinate dehydrogenase (SDH) subunit B (SDHB), large tumor size, extra-adrenal location, and elevated plasma methoxytyramine concentrations [2, 5, 6]. Telomerase activation and ATRX chromatin remodeler (ATRX) mutations are also described as independent risk factors for metastatic PPGLs [7]. Our recent findings further demonstrate that patients with PPGLs characterized by activation of pseudohypoxic pathways due to an increased expression and stabilization of HIF2α are also at high risk of metastatic disease [8]. This association also appears to relate to some other established risk factors.

Hypoxia signaling has a far-reaching impact on cellular differentiation and tumorigenesis mainly driven by the two main HIFα isoforms: HIF1α and HIF2α, which are structurally comparable and mostly have complementary functions [9, 10]. In presence of oxygen, proline residues within HIFα subunits are hydroxylated by α-ketoglutarate- and oxygen-dependent prolyl hydroxylases (PHDs); this then allows for von Hippel-Lindau (VHL) tumor suppressor-mediated proteasomal degradation of the HIF proteins (> Fig. 1a). In absence of oxygen (hypoxia), HIFα subunits form transcriptionally active complexes with aryl hydrocarbon receptor nuclear translocator (ARNT, also known as HIFB) and co-factors (e.g., CREB-binding protein and p300) followed by C-terminal transactivation of genes possessing hypoxia-responsive elements (HREs) (> Fig. 1b). In addition to HRE-mediated mechanisms, both HIFα subunits show interactions with NOTCH, WNT, and MYC pathways, thereby regulating gene expression through these additional pathways [11]. During cellular adaptation to hypoxia, the activity of HIFα differs temporally; while HIF1α primarily mediates the acute response to severe hypoxia, HIF2α mediates the response to chronic, even mild hypoxia [12]. This differential regulation is mediated by different hypoxia-associated factors and involves distinct cellular functions [13]. Moreover, several studies have indicated an oncogenic activity of HIF2α, and tumor suppressor role of HIF1α [14, 15]. HIF1α is ubiquitously expressed in many cell types, while HIF2α expression is restricted to specific cellular populations, including endothelial cells, neural crest cells, and glial cells [16].

HIF2α-dependent genotype-phenotype relationships in PPGLs

Currently, germline or somatic driver mutations can be identified in approximately 70% of catecholamine-producing PPGLs [17, 18]. Close links between genotype and phenotype including the risk of metastatic disease, age of onset, syndromic presentation, and a predominant anatomic site have been recognized [19, 20]. PPGLs are most broadly classified into two main clusters in accordance with their transcriptional profiles [21, 22]. Cluster 1 PPGLs comprise those with mutations encoding two groups of genes; these are either directly involved in the stabilization of HIFs or encode components of the tricarboxylic acid (TCA) cycle. The former include VHL, EGLN1/2 (encoding PHD1/2); and HIF2α, whereas the latter include SDH subunits (SDHA, SDHB, SDHC, SDHD), fumarate hydratase (FH), malate dehydrogenase 2 (MDH2), mitochondrial 2-oxoglutarate/malate carrier (SLC25A11), isocitrate dehydrogenases...
All cluster 1 mutations result in the stabilization of HIFα and lead to activation of hypoxia pathways even in the presence of oxygen (pseudohypoxia) [23]. Cluster 1 PPGLs are more prone to metastasize (18.6% excluding patients with SDHB mutation) compared to cluster 2 PPGLs (4.3%) caused by mutations of genes including (Ret proto-oncogene (RET), neurofibromin 1 (NF1), transmembrane protein 127 (TMEM127), H-Ras (HRAS), fibroblast growth factor receptor 1 (FGFR1), MYC associated factor X (MAX) that lead to activation of kinase signaling pathways [8]. However, due to mutations in SDHB, cluster 1 PPGLs are particularly prone to metastasize (75.6% in [8]) [24]. Both higher expression and stabilization of HIF2α are characteristics of cluster 1 compared to cluster 2 PPGLs [21, 25, 26] and this seems to contribute to the pro-metastatic behavior of cluster 1 tumors [8, 27].

HIF2α blocks glucocorticoid-mediated induction of phenylethanolamine N-methyltransferase (PNMT), the enzyme that converts
norepinephrine to epinephrine, providing a direct link between genotype and biochemical phenotype [26]. This, therefore, explains the expression of PNMT and epinephrine production by cluster 2 PPGLs, but not by cluster 1 PPGLs. HIF2α also influences phosphorylation of tyrosine hydroxylase (TH), the rate-limiting step in catecholamine synthesis, thereby affecting cellular dopamine and norepinephrine production [28]. Cluster 1 PPGLs that produce predominantly norepinephrine with varying amounts of dopamine, have lower total tissue catecholamine content than cluster 2 PPGLs; nevertheless, rates of catecholamine secretion and urinary excretion are higher in cluster 1 than cluster 2 PPGLs [29]. This may reflect a more completely developed secretory system in cluster 2 than in cluster 1 PPGLs that acts to restrain otherwise continuous or constitutive secretion [30]. Thus, although cluster 1 tumors tend to secrete catecholamines more actively than cluster 2 tumors, they have lower catecholamine contents per unit of tissue and produce lower amounts of O-methylated metabolites than cluster 2 tumors. The role of HIF2α in these differences remains unclear.

The predisposing role of HIF2α expression may also explain the reason for tumorogenesis of certain neural crest derivatives according to mutations that involve stabilization of HIF2α protein. As mentioned earlier, while HIF1α is ubiquitously expressed, HIF2α exhibits a much more restricted expression pattern [16]. In particular, HIF2α is expressed transiently during the migration of trunk neural crest cells to sympathetic paraganglia and the adrenal medulla, and before further differentiation [31]. This expression impacts migration and proliferation and has also been associated with the development of neural-crest derived sympathoblast precursors to neuroblastoma subtypes with more aggressive and less differentiated features than other subtypes [32]. Furthermore, using single-cell transcriptomics, human embryos were shown to have different neural crest cell derivatives that populate both the sympathetic paraganglia and adrenal medulla and may be responsible for the heterogeneity of associated tumors [33]. It, therefore, seems possible that cluster 1 tumors may be derived from more primitive neural crest derivatives compared to cluster 2 tumors and that this is associated with expression of HIF2α and different phenotypic features including the propensity for metastasis. Similarly, the younger age of patients with cluster 1 noradrenergic than cluster 2 adrenergic tumors, along with findings of higher proportions of multifocal, extra-adrenal, and metastatic tumors in younger than older patients with PPGLs, has also been proposed to reflect the development of cluster 1 tumors from different populations of chromaffin cell precursors [34, 35].

In line with the above considerations and as outlined by Friedner et al. [19], the cellular origins and the location of tumors can also influence tumor behavior in terms of catecholamine phenotype and metastatic behavior. The majority of chromaffin cell tumors arise from the adrenal medulla [35, 36]. About half of these produce norepinephrine exclusively and reflect cluster 1 tumors and the other half, the cluster 2 tumors, present with variable amounts of both norepinephrine and epinephrine [29, 37]. Sympathetic paragangliomas usually exclusively produce norepinephrine, in some cases additional variable amounts of dopamine, and in isolated cases exclusively dopamine [29]. Head and neck paragangliomas, which are derived from parasympathetic ganglia, show only limited expression of TH [38, 39]; thus, most are biochemically non-functional, though about 30% produce dopamine as manifested by the increase in the dopamine metabolite, methoxytyramine [40].

A recent study focusing on Sino-European differences in the genetic landscape of patients with PPGLs initiated a paradigm shift in the understanding of genotype-phenotype relationships of these genetically heterogeneous tumors [41]. Epinephrine production in the adrenal medulla depends on the expression of PNMT, which is regulated by glucocorticoids produced in the surrounding adrenal cortex [42]. According to previous understanding, mainly based on findings in Caucasian populations, this explains why epinephrine production is confined to adrenal pheochromocytoma [37, 43, 44]. Among the Chinese population, a substantial proportion of extra-adrenal PGLs produce epinephrine mainly associated with somatic mutations in HRAS and FGFR1. These findings further clarify that the adrenergic phenotype of PPGLs primarily depends on the underlying genetic mutation rather than tumor location [41]. Nevertheless, the aforementioned revised understanding does not mean that glucocorticoids are not relevant to induction of PNMT, but only that they may act on chromaffin cells to impact phenotypic features more distantly than previously believed. As outlined earlier, failure to induce PNMT, whether at adrenal or extra-adrenal locations, is related to the activity of HIF2α to block glucocorticoid-mediated induction of the enzyme. As also clarified by Qin et al. [26], these activities possibly involve interactions of HIF2α with the MYC/MAX complex, actions that are independent of binding of HIF2α to ARNT/HIFβ.

HIF2α mutations in PPGL tumorigenesis

Gain-of-function mutations of HIF2α are associated with pheochromocytomas and extra-adrenal paragangliomas and are often multifocal and recurrent. HIF2α-mutant PPGLs are more frequent in females than in males and are characterized by the production of norepinephrine [23]. Mutations in HIF2α are predominantly somatic and postzygotic, and are closely located on the oxygen-dependent degradation domain of HIF2α; this hinders hydroxylation by PHDs and degradation by VHL [45, 46]. Germine mutations (germline variant F374Y) are rare, but mosaicism is more common [47, 48]. Such patients present with PPGL–somatostatinoma–polycythemia syndrome (Pačak–Zhuang syndrome), which occurs exclusively in females [46, 49, 50].

In Caucasian cohorts, frequency of HIF2α mutations in PPGLs varies between 1.6–4.6% [8, 17, 45, 51], which is lower than in a Chinese cohort at 6.2% [41]. In our cohort, one-third of patients bearing HIF2α mutations were diagnosed with metastatic disease [8]. This confirmed a previous study in which 29% of HIF2α mutant PPGLs showed metastatic disease [52]. The comparatively high metastatic tendency of HIF2α mutant PPGLs together with the generally increased metastatic risk of cluster 1 PPGLs, characterized by stabilization of HIF2α, further supports the potential role of HIF2α as a pro-metastatic factor in PPGLs.

HIF2α-driven mesenchymal transition promotes a pro-metastatic phenotype in PPGLs

During the invasion-metastasis cascade, tumor cells must pass multiple steps to reach distant organs. Acquisition of a motile and in-
vasive phenotype (epithelial-mesenchymal transition, EMT) is the initiation step of this cascade and involves various changes in gene expression, including genes encoding proteins involved in cell adhesion and extracellular matrix interactions [53]. Due to the non-epithelial origin of chromaffin cells, PPGs are assumed to undergo a neuroendocrine-to-mesenchymal transition (neuroendoMT) associated with activation of specific signaling pathways [54–56]. In pheochromocytoma cells, SDHB mutation-induced neuroendoMT is associated with a pro-metastatic phenotype [54]. Our data also confirmed the involvement of HIF2α in this transition. The expression of HIF2α leads to changes in focal adhesion and extracellular matrix-receptor interaction pathways [55].

In relation to the above-mentioned findings, a study from Morin et al. showed that the neuroendoMT and pro-metastatic phenotype of SDHB-deficient cells results from synergistic effects of HIF2α and ten-ten translocation (TET) dioxygenase-mediated demethylation [27]. The 2-oxoglutarate-dependent TET hydroxylates DNA-methylated cytosine to form 5-hydroxymethylcytosine and is thereby directly involved in epigenetic regulation. Especially oncometabolite-driven tumors, such as PPGs of the TCA cycle-related cluster 1, and here in particular tumors with SDHB mutations show hypermethylation [57, 58]. A genome-wide DNA methylation analysis in metastatic PPGs identified hypermethylation of negative elongation factor complex member E (RDBP) as a prognostic marker for stratifying patients according to the risk to develop metastatic disease [59]. This, therefore, provides a potential explanation for the much higher metastatic risk in patients with PPGs due to mutations of SDHB compared to other cluster 1 genes. Thus, although HIF2α may be pro-metastatic, other factors clearly play important roles in the development of metastatic disease. These factors can include secondary mutations, such as those in ATRX, TERT, and p53, which may occur after initiation of tumorigenesis and lead to an event that further contributes to the development and progression of metastatic disease [7, 51].

In addition to its participation in neuroendoMT and thus the initiation of tumor cell migration from a primary tumor, HIF2α is also involved in the formation of pseudopodia in PPGs [8]. This critical step of mesenchymal cell migration enables invasiveness; this involves penetration by tumor cells of the endothelial basement membrane, thereby facilitating their entry into adjacent blood or lymph vessels [61]. The increased ability of HIF2α-expressing cells to attach to extracellular matrices, such as laminin, facilitates the

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**Fig. 2** Involvement of HIF2α in tumorigenesis and metastasis of cluster 1 pheochromocytomas and paragangliomas (PPGs). During embryogenesis, HIF2α is involved in the migration of trunk neural crest cells to sympathetic paraganglia or adrenal medulla. Mutations in HIF2α predispose to the development of pheochromocytomas (PCCs) and paragangliomas (PGs). Moreover, mutations in genes related to activation of hypoxia pathways (pseudohypoxic cluster 1 PPGs) are characterized by an increased expression and stabilization of HIF2α. Patients with pseudohypoxic cluster 1 PPGs bear a higher metastatic risk. HIF2α supports, among other factors, the pro-metastatic behavior of these tumors by the acquisition of a motile and invasive phenotype (neuroendocrine-to-mesenchymal transition, neuroendoMT). HIF2α blocks the glucocorticoid-mediated induction of phenylethanolamine N-methyltransferase (PNMT) and is thereby directly linked to the immature catecholamine phenotype of cluster 1 PPGs. The versatile involvement of HIF2α during tumorigenesis and metastasis in PPGs makes it an ideal target for therapeutic interventions. Available HIF2α inhibitors block dimerization with ARNT, but some tumors do not seem to respond to these kinds of inhibitors. Addressing ARNT-independent mechanisms of HIF2α, such as interaction with the MYC/MAX complex, maybe an alternative strategy for these resistant tumors. Genes reported as altered in PPGs are highlighted by stars (yellow: loss-of-function mutation, red: gain-of-function mutation). ARNT: aryl hydrocarbon receptor nuclear translocator; MAX: MYC associated factor X; PHDs: prolyl hydroxylases; VHL: von Hippel-Lindau tumor suppressor.
adhesion to the endothelial cell layer of the blood vessel, thereby encouraging subsequent extravasation [8]. The expression of genes involved in focal adhesion and the interaction of extracellular matrix-receptor promotes settlement of tumor cells in the pro-metastatic niche; the enhanced proliferation ability of HIF2α-expressing cells allows further metastatic colonization [8]. The multifaceted involvement of HIF2α in the invasion-metastasis cascade emphasizes the importance of this factor for potential therapeutic approaches in metastatic PPGLs.

Targeting HIF2α as a therapeutic option for metastatic PPGLs

For metastatic PPGLs, treatment options are limited and could benefit from personalized considerations according to the nature of underlying disease-causing mutations [62]. The enlarging understanding of the genetic background and associated molecular alterations in metastatic PPGLs offers for the first time a promising approach for individualized treatment of these patients. HIF2α appears to play an important role in tumorigenesis and metastatic spread of PPGLs (▶ Fig. 2), making it an ideal target for therapeutic approaches. Hypoxia/pseudohypoxia and derived signaling pathways are also associated with increased resistance to chemotherapy and radiotherapy [63, 64]. Therefore, targeting HIF2α may provide a potential chemo- and/or radiosensitizing approach to employ alongside other therapies.

Initially, HIF2α was considered to be undruggable, but the discovery of specific structural features led to the development of small molecule antagonists that can block the dimerization of HIF2α with ARNT/HIFβ [65, 66]. In clear cell renal cell carcinoma (ccRCC), which is frequently characterized by inactivation of VHL causing an enhanced stabilization of HIF2α, two specific HIF2α inhibitors, PT2385 and Beluzutin (PT2977) showed promise in phase I clinical trials [67, 68]. Phase II clinical trials in glioblastoma [69] and ccRCC (e.g., NCT03108066, NCT04489771, NCT03401788) are ongoing and a phase I clinical trial is also in planning stages for patients with PPGLs (personal communication). However, some VHL-mutant ccRCC cell lines showed resistance towards HIF2α inhibitors [70], which is also in line with recent clinical findings [71]. The ccRCC cell lines that were sensitive to HIF2α inhibitors displayed a distinct HIF2α-dependent gene signature and higher levels of HIF2α than other cell lines [70]. Resistance can be induced by prolonged treatment with HIF2α inhibitors [70, 71]. A gatekeeper mutation in HIF2α (G323E) that interferes with drug binding further promotes the acquisition of resistance [71]. Our data showed a lack of efficiency of PT2385 in HIF2α-dependent pheochromocytoma cell models [8]. Expression of Hif2α induced a pro-metastatic phenotype in these cells, which could not be reversed by treatment with PT2385 [8]. Similar results were also obtained in HIF2α-dependent neuroblastomas [72]. This raises the possibility of an ARNT/HIFβ-independent mechanism in these models, for example through interactions with the MYC/MAX complex [73, 74]. These ARNT/HIFβ-independent mechanisms of HIF2α, already mentioned earlier, may offer alternative therapeutic approaches for patients who show resistance to HIF2α inhibitors (▶ Fig. 2).

Further studies are needed to demonstrate the suitability of HIF2α inhibitors for the treatment of metastatic disease. Due to the described resistance mechanisms and the radio-and chemotherapeutic resistance associated with hypoxia/pseudohypoxia, suitable combination therapies should also be considered. There is also a need to identify markers that can predict sensitivity towards HIF2α inhibitors in suitable patients for such therapy.

Conclusion

Mutations in HIF2α provide a direct link of HIF2α to PPGL tumorigenesis. Increased expression and stabilization of HIF2α are also associated with increased aggressiveness and phenotypic characteristics of these tumors. The involvement of HIF2α in multiple steps of the invasion-metastasis cascade underscores the therapeutic relevance of targeting impaired HIF2α signaling pathways in PPGLs. However, because of resistance to current HIF2α inhibitors that has emerged for certain conditions, ARNT/HIFβ-independent mechanisms of HIF2α might also be considered for therapeutic intervention.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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