REVIEW

Epigenetic and metabolic reprogramming of SDH-deficient paragangliomas

Sophie Moog^{1,2}, Charlotte Lussey-Lepoutre^{1,3} and Judith Favier^{1,2}

¹PARCC, INSERM UMR970, Equipe Labellisée par la Ligue contre le Cancer, Paris, France

²Université de Paris, Paris, France

³Department of Nuclear Medicine, Sorbonne University, Pitie-Salpetriere Hospital, Paris, France

Correspondence should be addressed to J Favier: judith.favier@inserm.fr

Abstract

Pheochromocytomas and paragangliomas (PPGL) are rare neuroendocrine tumors arising from the adrenal medulla or extra-adrenal paraganglia. Around 40% of all cases are caused by a germline mutation in a susceptibility gene, half of which being found in an *SDHx* gene (*SDHA*, *SDHB*, *SDHC*, *SDHD* or *SDHAF2*). They encode the four subunits and assembly factor of succinate dehydrogenase (SDH), a mitochondrial enzyme involved both in the tricarboxylic acid cycle and electron transport chain. *SDHx* mutations lead to the accumulation of succinate, which acts as an oncometabolite by inhibiting iron(II) and alpha-ketoglutarate-dependent dioxygenases thereby regulating the cell's hypoxic response and epigenetic processes. Moreover, *SDHx* mutations induce cell metabolic reprogramming and redox imbalance. Major discoveries in PPGL pathophysiology have been made since the initial discovery of *SDHD* gene mutations in 2000, improving the understanding of their biology and patient management. It indeed provides new opportunities for diagnostic tools and innovative therapeutic targets in order to improve the prognosis of patients affected by these rare tumors, in particular in the context of metastatic diseases associated with *SDHB* mutations. This review first describes an overview of the pathophysiology and then focuses on clinical implications of the epigenetic and metabolic reprogramming of SDH-deficient PPGL.

Key Words

- ▶ pheochromocytoma
- ▶ paraganglioma
- ▶ succinate dehydrogenase
- ▶ epigenetics
- ▶ metabolism
- ▶ pseudohypoxia
- ▶ biomarkers
- ▶ targeted therapies

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Introduction

Pheochromocytomas and paragangliomas (PPGL) are rare neuroendocrine tumors arising from the adrenal medulla or extra-adrenal paraganglia, respectively. Paragangliomas can be found in the sympathetic nervous system of the thorax, abdomen, or pelvis or in the parasympathetic ganglia of the head or neck region. Pheochromocytomas and sympathetic paragangliomas commonly release catecholamines such as epinephrine, norepinephrine or dopamine, which can lead to cardio- or cerebro-vascular complications, whereas head and neck paragangliomas do not (Lenders *et al.* 2020). Most PPGL are curable by surgery

when they are localized, with a recurrence rate estimated at 1% per year per patient (Amar *et al.* 2016). However, 10–15% of PPGL are metastatic, defined by the occurrence of metastatic lesions either in lymph nodes, bones, lungs or liver (Lam 2017), with an overall survival decreasing to 62% at 5 years, and treatments remaining mainly palliative with a limited efficacy (Hescot *et al.* 2019). Approximately 70% of PPGL have a genetic determinism with more than 20 susceptibility genes discovered so far, including germline mutations in about 40% of cases and somatic mutations in 30% of cases (Buffet *et al.* 2020).

Mutations in genes encoding the succinate dehydrogenase (SDH) complex are the most prevalent, occurring in *SDHA*, *SDHB*, *SDHC*, *SDHD* or *SDHAF2* genes. Tumors carrying such mutations are classified in the pseudo-hypoxic expression cluster 1, together with tumors carrying mutations in *VHL* (von Hippel-Lindau), *FH* (fumarate hydratase), *DLST* (dihydrolipoamide S-succinyltransferase) genes and three genes encoding the malate-aspartate shuttle: *GOT2* (glutamic-oxaloacetic transaminase), *SLC25A11* (oxoglutarate carrier) and *MDH2* (malate deshydrogenase) (Favier *et al.* 2015, Buffet *et al.* 2020). Cluster 2 tumors are associated with abnormal kinase signaling and activation of the mTOR pathway and include mutations in *RET* (multiple endocrine neoplasia type 2), *NF1* (neurofibrosis type 1), *TMEM127*, *MAX*, *MET* and *HRAS*. Cluster 3 is characterized by the activation of the Wnt-signaling pathway with *MAML3* (mastermind-like transcriptional coactivator 3) anomalies (Fishbein *et al.* 2017).

SDHx genes encode the four subunits of SDH in the Tricarboxylic acid (TCA) cycle that also constitutes the mitochondrial complex II of the Electron transfer chain (ETC). It catalyses the oxidation of succinate to fumarate in the former and the transfer of electrons to the ubiquinone pool in the latter. *SDHx* genes are tumor suppressor genes and follow Knudson's two-hit' model (Knudson 1971), with a germline heterozygous mutation associated with somatic loss of heterozygosity (LOH), leading to a completely abolished (Gimenez-Roqueplo *et al.* 2001) or highly impaired (Kim *et al.* 2015) SDH activity. These mutations are transmitted in an autosomal dominant manner, with the exception of *SDHD* and *SDHAF2*, which are submitted to maternal imprinting, with only few cases of maternal transmission described so far for *SDHD* (Burnichon *et al.* 2017). *SDHx* mutations are associated with different phenotypes regarding penetrance, manifestations and rate of malignancy. *SDHB* mutations lead to metastatic evolution in 50% of cases, with a penetrance of 50% at age 50 while *SDHD* mutations mainly lead to multiple cervical paragangliomas with a greater penetrance of up to 80% (Benn *et al.* 2006, Amar *et al.* 2007, Burnichon *et al.* 2009, Buffet *et al.* 2020). *SDHA*, *SDHC* and *SDHAF2* are scarcer. Around 40% of all PPGL patients carry a germline mutation in a susceptibility gene, half of which being found in an *SDHx* gene. Genetic counselling and testing are therefore recommended for all PPGL patients (Lenders *et al.* 2020), promoting the improvement of clinical outcome in patients carrying an *SDHx* or a *VHL* mutation (Buffet *et al.* 2019). Moreover, identification of one of these germline mutations allows

to propose a genetic counselling and a pre-symptomatic screening to relatives, in order to include them in an appropriate follow-up protocol (Buffet *et al.* 2020).

Metabolic reprogramming is one of the eight hallmarks of cancers defined by Weinberg and Hanahan (Hanahan & Weinberg 2011), as first suggested by Otto Warburg in 1926 with his hypothesis that a direct link existed between mitochondrial dysfunction and cancer (Warburg 1956). Since the initial discovery of *SDHD* gene mutations in 2000 (Baysal *et al.* 2000), studies on the pathophysiology of SDH-related PPGL have led to major discoveries, improving the understanding of their biology and patient management and contributing to the extension of the field of oncometabolism. Comprehension of these pathophysiological characteristics provides new opportunities for diagnostic and innovative therapeutic strategies in order to improve the prognosis of patients affected by these rare diseases, in particular in the context of metastatic forms associated with *SDHB* mutations. This review provides an overview of the pathophysiology and then focuses on clinical implications of the epigenetic and metabolic reprogramming of SDH-deficient PPGL.

Metabolic consequences of SDH deficiency and oncogenesis

Mutations in genes encoding SDH lead to the loss of SDH enzymatic activity and therefore to the interruption of the TCA cycle. To sustain cell proliferation, in this highly compromised metabolic context, SDH-deficient cells consume extracellular pyruvate and activate pyruvate carboxylation to re-supply the depleted pool of aspartate that is further used to provide amino and nucleic acids (Cardaci *et al.* 2015, Lussey-Lepoutre *et al.* 2015). Recently, several studies evaluating the respiration and mitochondrial complexes activity of different SDH-deficient cell types revealed some intriguing data that might be a first step in the understanding of the tissue specificity of tumor development in SDH-mutated patients. Indeed, while both respiration of intact cells and complex I-specific activity were found to be lower in SDH-deficient cells compared with WT adrenal fibroblast (Křůčková *et al.* 2020), renal (Cardaci *et al.* 2015, Lorendeau *et al.* 2017) and breast epithelial cells (Bezawork-Geleta *et al.* 2018), it is not the case in a chromaffin cell-derived model. This model retains complex I function and a respiratory activity comparable to WT cells, thereby maintaining the ability to oxidise NADH within the ETC (Křůčková *et al.* 2020). These data showed that in contrast to models of

Figure 1

Epigenetic and metabolic reprogramming of SDH-deficient pheochromocytomas and paragangliomas and their potential treatments. AA6, alpha-ketoglutarate-dependent dehydrogenase inhibitor; 2-OG, alpha-ketoglutarate; c-MET, hepatocyte growth factor receptor; CVD, cyclophosphamide-dacarbazine-vincristine; FGFR, Fibroblast growth factor receptor; HDAC, histone deacetylase; HIF, hypoxia-inducible factors; HIF-PHD, hypoxia-inducible factors prolyl hydroxylase; HRE, hypoxia-inducible factors response elements; MGMT, O(6)-methylguanine-DNA-methyltransferase; OXPHOS, oxidative phosphorylation; PARP, Poly(ADP)-ribose polymerase; PDGFR, platelet-derived growth factor receptor; RET, rearranged during transfection; ROS, reactive oxygen species; SDH, succinate dehydrogenase; TET, ten-eleven translocation enzyme; VEGFR, vascular endothelial growth factor receptor.

SDH deficiency based on epithelial cells, a chromaffin cell model preserves some aspects of metabolic 'health', which could form the basis of cell specificity of this rare tumor type.

The main consequence of the truncated TCA cycle is the accumulation of SDH substrate, succinate (Pollard *et al.* 2005), which is thought to be the major actor of SDH-related oncogenic processes (Fig. 1). Indeed, it has been demonstrated that succinate can act as an oncometabolite by inhibiting iron(II) and alpha-ketoglutarate-dependent (α -KG) dioxygenases, a large family of enzymes that catalyze the hydroxylation of a wide range of organic targets (Islam *et al.* 2018). Although it is still debated, ROS accumulation together with iron overload is also suspected to participate to this tumorigenic pathway and further inhibit α -KG-dioxygenases. Discrepant data have been published with some studies showing ROS increase (Guzy *et al.* 2008, Liu *et al.* 2020) and others not (Selak *et al.* 2006, Guzy *et al.* 2008). One hypothesis is that such differences may actually reflect the heterogeneity of the phenotype linked to the different subtypes of SDH subunits, with a general consensus that SDHB-mutated cells do show increased oxidative stress. Members of the α -KG-dioxygenases family that regulate the cell's hypoxic response and epigenetic processes, particularly the demethylation of histones and DNA, seem to play a particularly important role in SDH-related tumorigenesis. These enzymes are dependent on molecular oxygen and α -KG as necessary substrates, and use iron and ascorbate

as cofactors, making them critical in the processes of metabolic sensing.

Pseudohypoxia

Their role as oxygen sensor is mediated by the hypoxia-inducible factors (HIF) prolyl hydroxylases (PHD 1–3) that regulate HIFs stability. In normoxic conditions, PHD enzymes hydroxylate the α subunits of hypoxia-inducible factors (HIF1 and HIF2) on two highly conserved proline residues, making their recognition by von Hippel Lindau protein (pVHL) possible. pVHL is a E3 ubiquitin ligase that will then promote their ubiquitination and subsequent degradation by the proteasome. Being dependent on oxygen to be fully active, PHDs are inhibited when oxygen concentrations fall in hypoxic conditions; HIFs can therefore escape from their proteasome destiny and translocate to the nucleus where they form an active transcription factor following their heterodimerization with their β counterpart, namely the aryl hydrocarbon receptor nuclear translocator (ARNT or HIF1 β). In hereditary PPGL, as in clear cell renal cell carcinoma (ccRCC), activation of HIFs occurs even in the presence of oxygen, through the process of pseudohypoxia. This molecular or metabolic activation of the hypoxic response can be mediated either directly by loss-of-function mutations (such as tumors caused by germline or somatic inactivating mutations of the *VHL* gene) or through their metabolic consequences. In *SDH*- and *FH*-mutated PPGL,

the accumulation of succinate and fumarate respectively promotes the inhibition of PHDs and a pseudo-hypoxic signaling (Briere *et al.* 2005, Pollard *et al.* 2005, Selak *et al.* 2005, Letouze *et al.* 2013). The wide range of HIF target genes make this response critical for many cellular functions and participate to the occurrence of tumors through many aspects such as metabolic adaptation (glycolytic switch), proliferation, regulation of cell death, angiogenesis, modification of the extracellular matrix components, acquisition of mesenchymal hallmarks (Favier & Gimenez-Roqueplo 2010).

Epigenetic reprogramming

Another class of α -KG-dependent dioxygenases that were demonstrated to be inhibited by oncometabolites are the DNA and histone demethylases: TET dioxygenases that hydroxylate DNA methylated cytosines (5mC) into 5hmC and JmjC-domain containing histone lysine demethylases (KDM) (Cervera *et al.* 2009). In PPGL as in GIST, *SDHx* mutations were shown to promote a massive hypermethylator phenotype. Several groups, including ours, studied the methylome of these oncometabolite-driven tumors and reported enhanced DNA methylation in and outside CpG islands (Killian *et al.* 2013, Letouze *et al.* 2013). Interestingly, *SDHB*-deficient PPGL displayed higher global methylation levels than other subtypes of *SDH*-deficient tumors (with mutations in *SDHA*, *C* or *D*), suggesting that epigenetic reprogramming may participate to the increased metastatic capacities of *SDHB*-mutated tumors. Recently, we were able, using oxidative reduced representation bisulfite sequencing (oxRRBS), to demonstrate that *SDHB*-mutated PPGL did display lower levels of 5hmC than NF1-mutated tumors (Morin *et al.* 2020). Moreover, we showed that hypermethylated DNA sequences were enriched in polycomb repressor complex 2 (PRC2) target genes, albeit independently of PRC2 activity. These observations revealed a tropism of TET enzymes for PRC2 targeted chromatin regions, thereby linking the hypermethylator phenotype of a de-differentiation process. Using functional assays in an *Sdhb*-deficient immortalized mouse chromaffin cell line, this study provided the first demonstration that TET-mediated DNA hypermethylation indeed promoted a massive transcriptomic reprogramming, but that the synergy between these epigenetic modifications and a HIF2 α -mediated pseudo-hypoxic response was required to promote the invasive and the mesenchymal phenotypes of *Sdhb*-deficient cells.

Metabolic and epigenetic biomarkers

Rapid identification of *SDH* deficiency is important at various levels, by accelerating genetic testing or facilitating the interpretation of genetic variations in *SDHx* genes provided by next-generation sequencing (NGS) (Toledo & Dahia 2015). Moreover, it can impact patient management by considering the higher risk of metastasis of *SDHB*-related tumors, the high rate of multiple cervical PGL for *SDHD*-mutated patients and driving therapeutic choices for inoperable and metastatic patients. Thus, several biomarkers have been developed based on assessment of protein expression, functionality and its metabolic and/or epigenetic consequences (Table 1).

Enzyme functionality and metabolomics

For *SDH* expression, the routinely method employed is *SDHB* immunohistochemistry (IHC) performed on formalin-fixed paraffin-embedded (FFPE) tumors that predicts *SDHx* mutations with a good sensitivity, specificity and reproducibility among expert endocrine pathologists in PPGL (van Nederveen *et al.* 2009, Gill *et al.* 2010, Papathomas *et al.* 2015) and in other tumors as GIST (Gaal *et al.* 2011, Oudijk *et al.* 2013) and kidney cancers (Gill *et al.* 2011). The mitochondrial-specific granular staining is lost in tumor cells for all subtypes of *SDHx* mutations (*SDHA*, *SDHB*, *SDHC*, *SDHD* and *SDHAF2*) while it is maintained in stromal cells such as endothelial cells, adipocytes or sustentacular cells, providing internal positive controls (van Nederveen *et al.* 2009, Gill *et al.* 2010). *SDHA* IHC is also a valuable method in tumors with a negative *SDHB* immunostaining to specifically identify *SDHA*-related tumors, as it is negative for *SDHA* mutations but positive for all other *SDH*-related tumors (Korpershoek *et al.* 2011). In some cases, especially *SDHD*-related PGL, *SDHB* IHC may fail because of a weak diffuse cytoplasmic blush that should be considered as negative but can be misinterpreted (Gill *et al.* 2010, Papathomas *et al.* 2015). In that context, *SDHD* IHC can be useful, with a mirror result, that is negative in non-*SDHx* PGL and a positive labeling characterized by a cytoplasmic diffuse signal with patchy accumulation in *SDH*-mutated PGL (Menara *et al.* 2015).

The direct method to assess *SDH* functionality is the quantification of *SDH* activity on fresh frozen tumor samples using a spectrophotometric assay (Rustin *et al.* 1994). Measurement of Krebs cycles metabolites by liquid chromatography-mass spectrometry (LC-MS/MS) is

Table 1 Metabolic and epigenetic biomarkers of SDH-deficient pheochromocytomas and paragangliomas.

Type	Interpretation	Sn/Sp	Current use	References
Immunohistochemistry SDHB	Mitochondrial-specific granular staining lost in tumor cells of non-SDHx mutated tumors. Negative in tumors cells with all types of SDHx mutations (SDHA, SDHB, SDHC, SDHD and SDHAF2). Weak diffuse cytosolic staining should be considered as negative.	94%/84% (Papathomas <i>et al.</i> 2015)	Clinical	van Nederveen <i>et al.</i> 2009, Gill <i>et al.</i> 2010, Papathomas <i>et al.</i> 2015
SDHA	Negative in SDHA-mutated tumors but positive in all other SDHx- or non-SDHx-mutated tumors	75%/93% (Papathomas <i>et al.</i> 2015)	Clinical	Korpershoek <i>et al.</i> 2011, Papathomas <i>et al.</i> 2015
SDHD	Used in case of SDHB IHC interpretation (such as weak diffuse staining); negative in non-n-SDHx PGL and positive labeling (cytoplasmic diffuse signal with patchy accumulation) in SDHx-mutated PGL	96%/97% (Menara <i>et al.</i> 2015)	Research	Menara <i>et al.</i> 2015
DNA methylation: 5hmc	5hmc staining inferior (or absent) in the nuclei of SDHx-mutated tumor cells compared with endothelial or sustentacular cells	Not available	Research	Letouze <i>et al.</i> 2013, Hoekstra <i>et al.</i> 2015
Histone methylation: H3K4me3, H3K9me3, H3K27me3	Increase in H3K9me3 in SDHx-related tumors (no change in H3K4me3 and H3K27me3)	Not available	Research	Letouze <i>et al.</i> 2013
Succinate <i>in vitro</i> (LC-MS/MS)	Accumulation of succinate in case of an SDHx mutation with very low levels of fumarate. (high succinate to fumarate ratio)	88%/99.2% (Wallace <i>et al.</i> 2020)	Research	Pollard <i>et al.</i> 2005, Rao <i>et al.</i> 2013, Richter <i>et al.</i> 2014, Imperiale <i>et al.</i> 2015, Wallace <i>et al.</i> 2020
Succinate <i>in vivo</i> (¹ H-MRS)	Accumulation of succinate in tumor in case of an SDHx mutation	87%/100% (Lussey-Lepoutre <i>et al.</i> 2020)	Research/clinical	Varoquaux <i>et al.</i> 2015, Lussey-Lepoutre <i>et al.</i> 2016, 2020, Casey <i>et al.</i> 2018

5hmc, 5-methylcytosine; ¹H-MRS, proton magnetic resonance spectroscopy; IHC, immunohistochemistry; LC-MS/MS, liquid chromatography with mass spectrometry; SDH, succinate dehydrogenase; Sn, sensitivity; Sp, specificity.

another valuable method (Pollard *et al.* 2005, Rao *et al.* 2013, Richter *et al.* 2014, Imperiale *et al.* 2015) to assess the consequences of loss of enzymatic activity. Metabolic profiling of SDH-mutated PGL show important accumulation of succinate (SDH substrate), with concentrations up to 100-fold higher than tumors without SDHx mutations and very low levels of its product, fumarate. Thus, the tissue-specific succinate to fumarate ratio (SFR) has a good sensitivity and a recent study showed a higher specificity than SDHB IHC to detect SDHx mutations on fresh frozen tumors and FFPE (Wallace *et al.* 2020). Interestingly, SFR appeared particularly high in SDHB-mutated and metastatic PGL but lower in head and neck PGL (Richter *et al.* 2014). Other TCA cycle metabolites are also significantly impacted in SDH-mutated PGL: a decrease in isocitrate, cis-aconate (Richter *et al.* 2014), aspartate and glutamate (Imperiale *et al.* 2015)

may also be used as biomarkers but above all can improve understanding of metabolic reprogramming. Indeed, decrease of aspartate has been elucidated by *in vitro* ¹³C-glucose fluxes experiments as a consequence of the high dependence of SDH-deficient cells upon mitochondrial aspartate for cellular anabolism, suggesting novel therapeutic perspectives (Lussey-Lepoutre *et al.* 2015).

Altogether, metabolomic profiling and IHC provide valuable biomarkers of SDHx mutation but rely on the availability of tissue from the resected tumor. Because of the high vascularization of these tumors (Favier *et al.* 2009) and the secretion of catecholamines, the recourse to a biopsy is a contraindication and the surgical removal of the lesion is not always feasible.

Data on whether circulating or urinary levels of succinate are efficiently measurable in patients with

germline *SDHx* variants are still scarce. Using gas chromatography-mass spectrometry (GC-MS), Hobert *et al.* reported high succinate levels in the plasma of patients with *SDHB*- (1/1) and *SDHD*-(2/5) associated PPGL (Hobert *et al.* 2012). Assessment of SFR in plasma samples of patients with PPGL was then reported by Lendvai *et al.* but again not fully conclusive (Lendvai *et al.* 2014). Therefore, there was a need for a specific tumor biomarker allowing the characterization of inoperable tumors and suspicious lesions.

Succinate shows a characteristic peak at 2.44 ppm in spectral analysis that can be detected noninvasively by *in vivo* proton magnetic resonance spectroscopy (^1H -MRS). ^1H -MRS is a MRI sequence specific for the observation of tissue compounds after elimination of water protons signal. ^1H -MRS has been initially developed in the field of cerebral tumors, especially gliomas, that have recurrent somatic mutations in one of the isocitrate dehydrogenase genes (*IDH1* and *IDH2*) leading to the overproduction of 2-hydroxyglutarate (2-HG), detectable *in vivo* by ^1H -MRS (Andronesi *et al.* 2012, Choi *et al.* 2012). Detection of succinate accumulation *in vivo* as a biomarker for the presence of an *SDHx* mutation is also feasible and has been developed in recent years (Varoquaux *et al.* 2015, Lussey-Lepoutre *et al.* 2016, 2020, Casey *et al.* 2018).

We previously developed a pulse ^1H -MRS sequence to measure succinate in an allografted mouse model of *Sdhb*-deficient tumors and in a pilot study performed in nine patients with PPGL (five with *SDHx* mutations and four sporadic cases). We demonstrated the feasibility of detecting succinate *in vivo* by ^1H -MRS as a very specific biomarker of *SDHx* mutations (Lussey-Lepoutre *et al.* 2016). The diagnostic performance of ^1H -MRS was subsequently validated on a prospective cohort of patients carrying 50 PPGL (Lussey-Lepoutre *et al.* 2020), validating the good sensitivity (87%) and the perfect specificity (100%) of this technique, in particular for PGL of the head and neck. The limits of ^1H -MRS are mainly the size of the tumor (which determines the size of the voxel) and the presence of necrosis in the tumor. On the other hand, sensitivity and specificity remain identical whatever the subtype of mutation (*SDHA*, *B*, *C* or *D*). ^1H -MRS can also be applied to other tumors with *SDHx* deficiency such as GIST, as well as metastases of PPGL (Casey *et al.* 2018). Moreover, animal experiments demonstrated that the area under the succinate peak of the ^1H -MRS spectra *in vivo* was correlated with the concentrations of succinate measured in the resected tumors by GC-MS *in vitro*, allowing quantifying succinate levels *in vivo* (Lussey-Lepoutre *et al.* 2016). Future studies are needed to show whether this correlation holds

also in patients. If it turned out to be the case, it could provide a quantifiable surrogate marker of early response to treatment in *SDHx*-related PPGL patients.

Molecular biomarkers: when epigenetics enters the game

Epigenetic reprogramming being a major consequence of *SDH* deficiency, it rapidly emerged as a hallmark of choice to be used to develop specific biomarkers, either of *SDHx* mutations or of metastatic disease. Specific antibodies have been developed to detect the different states of DNA cytosines (5mC or 5hmC) or the methylation marks of histones (H3K9me2, H3K9me3, H3K27me2, H3K27me3, H3K4me3). These antibodies have been used on paraffin embedded PPGL (Letouze *et al.* 2013, Hoekstra *et al.* 2015) or GIST (Killian *et al.* 2013) and correlated with the genetic status of the tumors. In PPGL tumors, we first evaluated the levels of 5mC, 5hmC, H3K9me3 and H3K27me3 in 40 tumor samples including 16 *SDH*-mutated. We showed that 5mC staining was not different between the different tumors, while histone methylation was indeed increased in *SDH*-related tumors. However, such immunostainings were neither specific nor sensitive enough to be used as biomarkers. In contrast, low 5hmC levels in the nuclei of tumor cells were detected in all *SDH*-mutated and in only 6/24 non-*SDH* samples. Following these initial observations, Hoekstra *et al.* evaluated these markers in a large collection of 134 PPGL (including 75 *SDHx*-mutated) and also concluded that 5hmC was significantly lower or even absent in the case of an *SDHx* gene mutation (Hoekstra *et al.* 2015). Interestingly, it is worth noting that low 5hmC is detected not only in *SDH*-deficient tumors but also in PPGL carrying other types of mutations affecting mitochondrial proteins: *FH* (Castro-Vega *et al.* 2014, Hoekstra *et al.* 2015), *SLC25A11* (Buffet *et al.* 2018), and *DLST* (Buffet *et al.* personal communication).

Global DNA methylation was also evaluated in large collections of tumors to identify hypermethylated CpG that may be exploitable as predictors of genetic status or of the metastatic potential of a primary PPGL (Letouze *et al.* 2013, de Cubas *et al.* 2015). Hypermethylation of *PNMT* was reported in *SDHB*-related PPGLs while *RET*- and *NF1*-mutated PPGL showed hypomethylation and *VHL*-related one, hemimethylation. These observations are of particular interest because of the role of *PNMT* in catecholamine biosynthesis and explain the neurochemical phenotypes of these different subsets of tumors, with *SDH* and *VHL*-deficient PPGL showing a noradrenergic secretion (Eisenhofer *et al.* 2017). Hypermethylation of *RDBP*

(negative elongation factor complex member E) was detected as a specific biomarker of metastatic tumors, independently of their genetic status (de Cubas *et al.* 2015).

Therapeutics perspectives

Around 10–15% of PPGLs will become metastatic and have a poor prognosis with a mortality rate of 37% at 5 years (Hamidi *et al.* 2017). Unfortunately, there are limited options for these cases, based on metabolic radiotherapy or chemotherapy, with imperfect efficacy (Baudin *et al.* 2014). Knowledge on PPGL biology has recently been turned upside down by genomics and metabolomics data, making it possible to envisage the use of targeted therapies and precision medicine (Favir *et al.* 2015) (Table 2). This review being focused on *SDH*-mutated PPGL based on new discoveries, especially those depending on epigenetic and metabolic reprogramming, we will not discuss several potentially promising therapies for PPGL such as radionuclide therapy, mTOR inhibitors, somatostatin analog or immunotherapy.

Targeting angiogenesis

SDH-mutated tumors being highly vascularized, the use of antiangiogenic therapies that block the VEGF pathway are an important hope for metastatic patients and in particular for the very aggressive forms linked to *SDHB*. In addition to

their antiangiogenic properties, tyrosine kinase inhibitor (TKI) can also inhibit other receptors such as fibroblast growth factors receptor (FGFR) or c-met receptors, which may prevent tumor invasiveness and metastases. A recent and complete overview of antiangiogenic therapies can be found in Jimenez *et al.* (2020). Several retrospective studies, mostly with sunitinib (TKI targeting VEGFR, PDGFR, RET and c-kit), seem promising and this drug is currently being evaluated in prospective trials. The phase II clinical SNIPP study evaluated sunitinib with 50 mg in 23 patients and found a disease control rate of 83% (95% CI: 56–93%), a progression-free survival (PFS) of 13.4 months (95% CI: 5.3–24.6 months) but with a very low objective response rate (O’Kane *et al.* 2019). The results of the first randomized trial of 78 metastatic PPGL patients with sunitinib 37.5 mg should be available soon (FIRSTMAPPP, NCT01371201). Others TKI have been or are currently evaluated such as axitinib (VEGFR), pazopanib (VEGFR, PDGFR, FGFR and the RET receptor) and lenvatinib (FGFR) but suffer from high toxicity, notably hypertension (Jimenez *et al.* 2020). The more promising one, cabozantinib (VEGFR and c-Met receptor), is currently evaluated in a phase II trial with encouraging preliminary results and unexpectedly, no severe hypertension or cardiovascular events (NCT02302833) (Jimenez *et al.* 2020). However, knowledge acquired in other types of cancers show that although TKI allows stabilizing tumor growth, anti-angiogenic agents rarely achieve complete responses, and it is common that after

Table 2 Therapeutic approaches for the treatment of *SDH*-mutated metastatic PPGL.

Target	Drug	Type	Status	Ref or clinical trial identifier on clinicaltrial.gov
Tyrosine kinase receptors	Sunitinib	Phase II	Recruiting	NCT01371201
	FIRSTMAPPP	Phase II	Published	NCT00843037, O’Kane <i>et al.</i> 2019
	Axitinib	Phase II	Recruiting and active, not recruiting	NCT03839498 and NCT01967576
	Pazopanib	Phase II	Terminated	NCT01340794
	Lenvatinib	Phase II	Active, not recruiting	NCT03008369
HIF2 α Hypermethylation	Cabozantinib	Phase II	Recruiting	NCT02302833
	PT2977	Phase I (one PGL)	Recruiting	NCT02974738
	Decitabine	Pre-clinical (<i>in vitro</i>)	Published	Letouze <i>et al.</i> 2013
	HDAC	Pre-clinical (<i>in vitro</i>)	Published	Martiniova <i>et al.</i> 2011
	Guadecitabine (SGI-110)	Phase II	Completed, not yet published	NCT03165721
Metabolomic reprogramming	Metformin	Pre-clinical (<i>in vitro</i>)	Published	Thakur <i>et al.</i> 2019
	Dichloroacétate + Metformin	Pre-clinical (<i>in vitro</i>)	Published	Florio <i>et al.</i> 2018
	PARPi: Talazoparib	Pre-clinical (<i>in vitro</i> and <i>in vivo</i>)	Published	Pang <i>et al.</i> 2018, Sulkowski <i>et al.</i> 2018
	Olaparib + Temozolomide	Phase II	Not yet recruiting	NCT04394858
Redox imbalance	Ascorbate high dose	Pre-clinical (<i>in vitro</i>)	Personal communication	Liu <i>et al.</i> 2020
		Pre-clinical (<i>in vitro</i> and <i>in vivo</i>)	Published	

6–12 months of treatment, patients develop resistance. It requires the development of others alternative therapeutic strategies or the combination of antiangiogenic therapies with new treatments.

Targeting pseudohypoxia

Over the past decade, numerous data have suggested and then confirmed the oncogenic role of HIF2 α in metastatic PPGLs (Toledo *et al.* 2013, Morin *et al.* 2020). HIF2 α , although a crucial target, was long considered to be pharmacologically inaccessible. Based on the structure of the HIF2 α -ARNT/HIF1 β dimer, two antagonists of HIF2 α (PT2385 and PT2977 (MK-6482)) were generated. These small molecules allosterically block the binding of HIF2 α to ARNT/HIF1 β , thereby inhibiting their transcriptional activity (Scheuermann *et al.* 2015).

Recent reports have shown very promising results of these two molecules in ccRCC, with a clear decrease in tumor growth *in vitro* and *in vivo* and a reduction in the expression of HIF2 α target genes (Chen *et al.* 2016, Cho *et al.* 2016, Xu *et al.* 2019). ccRCC is another example where tumorigenesis is mediated via a process of ‘pseudohypoxia’ through *VHL* mutations (Nickerson *et al.* 2008). These compounds were also found to be active in tumors progressing under sunitinib. A phase I study conducted with PT2385 in 51 patients with locally advanced or metastatic ccRCC previously treated with a TKI suggested its efficacy with a very good safety profile (Courtney *et al.* 2018). Several phase II studies are underway in ccRCC, alone (NCT03108066 or NCT03401788 only for *VHL* disease) or in combination with cabozantinib (NCT03634540) or everolimus (NCT04195750).

Given the very promising results of HIF2 α antagonists in ccRCC and the tumor mechanisms of PPGLs known to date, anti-HIF2 α appears to be good candidate for the treatment of metastatic PPGLs, in particular those mutated on the *SDHB* gene (Toledo 2017). In a currently recruiting basket trial of patients with advanced solid tumors treated with PT297, one patient with an *SDHB* mutant paraganglioma had stable disease >25 weeks with sustained decrease in normetanephrine levels (NCT02974738) (Papadopoulos *et al.* 2018). These data must however be counterbalanced by a recent *in vitro* study on a model of neuroblastoma that also shows overexpression of HIF2 α , which found no effects on HIF2 α target genes expression and no major impact on cell survival *in vitro* or tumor growth *in vivo* upon treatment with PT2385 (Persson *et al.* 2020). In any case, the data are currently insufficient and other preclinical and clinical studies are necessary to

evaluate this treatment, or the combination of the HIF2 α inhibitors with another, in metastatic PPGLs.

Targeting epigenetic alteration

Cyclophosphamide-dacarbazine-vincristine (CVD) regimen is recommended as the standard chemotherapy for metastatic PPGL, with a partial response concerning tumor volume that can be achieved in about 37% in a meta-analysis (Niemeijer *et al.* 2014). However, temozolomide, the oral form of dacarbazine, seems to have higher response rates in patients with *SDHB* mutations, with a longer PFS compared with patients without *SDHB* mutation (19.7 vs 2.9 months). The efficiency of temozolomide, a DNA alkylating agent, is in fact dependent of the O(6)-methylguanine-DNA-methyltransferase (MGMT) enzyme which repairs the DNA adducts induced by this treatment. In *SDHB*-mutated tumors, the silencing of *MGMT* promoter region by hypermethylation explains why *SDHB* patients have better responses (Letouze *et al.* 2013, Hadoux *et al.* 2014).

The description of the hypermethylated phenotype of *SDH*-deficient tumors revealed the possibility of innovative epigenetic therapies involving DNA or histone demethylating agents. Decitabine, which acts as a hypomethylating agent through the inhibition of DNA-methyltransferase (DNMT), has been tested in *Sdhb*^{−/−} mouse chromaffin cells and led to the reduction of their migratory capacities (Letouze *et al.* 2013). Histone Deacetylase (HDAC) inhibitors were found to enhance the amount of norepinephrine transporter and ¹²³I-MIBG uptake in a mouse model of metastatic pheochromocytoma, leading to a possible combination of MIBG therapy and HDAC inhibitors (Martiniova *et al.* 2011). Preclinical studies with any of these treatments are still missing. However, a second-generation of DNA methylation inhibitor (guadecitabine, SGI-110), tested in acute myeloid leukemia (AML) and myelodysplastic syndrome, has been very recently evaluated by the NIH in a phase II study involving nine patients (seven with *SDH*-deficient GIST, one with *SDH*-deficient paraganglioma and one *FH*-mutated RCC (NCT03165721)). Results presented at the 2020 ASCO meeting unfortunately showed no complete or partial responses (Wedekind *et al.* 2020).

Another tentative option would be to restore α -KG-dependent enzymes activity, by inducing an excess of α -KG. In a mouse model of breast cancer-associated lung metastasis, an α -KG-dependent dehydrogenase inhibitor (AA6) increased the intracellular level of α -KG, thereby increasing TETs activity and reducing metastasis formation

(Atlante *et al.* 2018). This promising treatment has never been tested in PPGLs so far.

Targeting metabolic reprogramming

Reprogrammed metabolic activities of PPGLs can also be exploited to treat them. Recently, IACS-010759, a clinical-grade small molecule inhibitor of oxidative phosphorylation (OXPHOS) through inhibition of mitochondrial complex I, has been tested with promising results in mouse models of AML and brain cancer (Molina *et al.* 2018). It inhibits proliferation and induces apoptosis through a combination of energy depletion and reduced aspartate production, leading to impaired nucleotide biosynthesis. IACS-010759 is currently evaluated in a phase I trial in solid tumor and relapsed/refractory AML and early results indicate that this treatment appears safe (NCT02882321 and NCT03291938). As explained before, upregulation of complex I and mitochondrial aspartate synthesis by pyruvate carboxylation are essential for *Sdhb*^{−/−} chromaffin cells survival and proliferation, making IACS-010759 as potential drug for PPGL (Lussey-Lepoutre *et al.* 2015). Other OXPHOS inhibitors are under development, notably in glioblastoma (Shi *et al.* 2019).

Interestingly, metformin is another inhibitor of OXPHOS through complex I inhibition. Two *in vitro* studies performed on the cell lines derived from PPGL, mostly rat derived cells (PC12 cells), suggested an anti-proliferative potential of metformin (Li *et al.* 2017, Thakur *et al.* 2019). These effects have to be confirmed in animal models and human studies. In a model of head and neck PGL cells with *SDH* loss, dichloroacétate (DCA), a structural analog of pyruvate, reduced cell viability alone or in combination with metformin through mechanisms involving pyruvate dehydrogenase kinase inhibition. It results in reactivation of pyruvate dehydrogenase promoting oxidative metabolism, decreasing extracellular lactate and increasing intracellular ROS levels (Florio *et al.* 2018). These treatments promote both cell cycle arrest and apoptosis in these cells and have to be explored further in animal models.

Finally, alterations of complex I activity in *Sdhb*^{−/−} cells induce an upregulation of NADH dehydrogenase activity which alters NAD⁺/NADH balance resulting in increased NAD⁺ availability and enhanced Poly(ADP)-ribose polymerase (PARP) DNA repair (Pang *et al.* 2018). Similar to MGMT, PARP is a highly conserved enzyme which repairs single strand DNA breaks and stabilizes DNA replication. In parallel, succinate has been found to suppress the homologous recombination

DNA-repair pathway (that repairs double strand DNA breaks) through inhibition of the lysine demethylase KDM4B thus rendering renal cancer cells and tumor xenograft with *SDHB*-deficiency, all the more vulnerable to PARP inhibitors (Sulkowski *et al.* 2018, 2020). In mice with *Sdhb*^{−/−} PPGL allografts, PARP inhibitors potentiated DNA damaging effects of temozolomide (but showed no effect alone) and reduced metastatic lesions, with prolonged overall survival of mice (Pang *et al.* 2018). Thereby, a new phase II testing the addition of olaparib to temozolomide will start soon (NCT04394858).

Targeting redox imbalance

Sdhb^{−/−} cells exhibit dysregulation of oxygen metabolic pathways, leading to increased formation of ROS concomitantly with iron overload, suggesting that targeting the redox imbalance pathway could be a potential therapeutic approach. Very recently, we and others have demonstrated that pharmacologic ascorbate induces ROS overload in *Sdhb*^{−/−} cells leading to impaired cell survival. Moreover, ascorbate delays *Sdhb*-KD allograft *in vivo* (Liu *et al.* 2020). In addition to redox imbalance, ascorbate targets other vulnerabilities of *SDHB* tumors, that is the epigenetic reprogramming through TET activity and the oxygen-sensing regulation by inhibiting HIF activity (Ngo *et al.* 2019). Actually, several ongoing clinical trials are exploring the safety and efficacy of high-dose parenteral ascorbate in combination with chemotherapy or radiation, in various types of cancers such as glioblastoma, pancreatic, colorectal or non-small-cell lung cancers (Ngo *et al.* 2019). In all cases, high-dose i.v. ascorbate (generally >1g/kg) exhibits an excellent safety profile. Consequently, parenteral high-dose vitamin C, despite its controversial history, could to be a promising candidate for future treatment of SDH-related metastatic PPGL, probably in combination with others.

Conclusion

It has now been 20 years since the report by Baysal *et al.* of the first *SDHD* gene mutation in a family with hereditary paraganglioma (Baysal *et al.* 2000), immediately followed by the identification of *SDHC* (Niemann & Muller 2000) and *SDHB* (Astuti *et al.* 2001) gene mutations and later by the demonstration of the similar tumor suppressor roles of *SDHAF2* (Bayley *et al.* 2010) and *SDHA* (Burnichon *et al.* 2010). Twenty years of clinical and basic research

on SDH-related tumors have highlighted to complexity of the processes that link mitochondrial dysfunction and tumorigenesis. They have revealed a central role of oncometabolites in the biology of these tumors and improved our understanding of oxygen sensing and epigenetic reprogramming in cancer development. They have changed the management of patients with these rare tumors and the counseling and follow-up of their family members, thereby improving the prognosis of affected subjects (Buffet *et al.* 2019). They revealed many innovative therapeutic strategies that are or will soon be tested in clinical trials or in isolated individual and that will hopefully provide an increasing hope for the treatment of metastatic patients.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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