15 YEARS OF PARAGANGLIOMA

Metabolism and pheochromocytoma/ paraganglioma

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Abstract

The discovery of *SDHD* as a pheochromocytoma/paraganglioma susceptibility gene was the prismatic event that led to all of the subsequent work highlighting the key roles played by mitochondria in the pathogenesis of these tumors and other solid cancers. Alterations in the function of tricarboxylic acid cycle enzymes can cause accumulation of intermediate substrates and subsequent changes in cell metabolism, activation of the angiogenic pathway, increased reactive oxygen species production, DNA hypermethylation, and modification of the tumor microenvironment favoring tumor growth and aggressiveness. The elucidation of these tumorigenic mechanisms should lead to novel therapeutic targets for the treatment of the most aggressive forms of pheochromocytoma/paraganglioma.

Key Words

- ▶ pheochromocytoma/
paraganglioma
- ▶ succinate dehydrogenase
- ▶ mitochondria
- ▶ angiogenesis
- ▶ oncometabolites

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Introduction

Before the year 2000, there were three forms of syndromic familial pheochromocytoma: von Hippel-Lindau (VHL) (Latif *et al.* 1993); multiple endocrine neoplasia type 2 (MEN2) (Mulligan *et al.* 1993); and neurofibromatosis type 1 (NF1) (Viskochil *et al.* 1990). The mutated genes, responsible for these syndromes are *VHL*, *RET* and *NF1* respectively. Common features of these syndromes are tumor occurrence in the adrenal medulla (pheochromocytoma) and the very frequent involvement of both adrenal glands with either synchronous or metachronous presentations. The biochemical profiles were shown to be adrenergic in MEN2 and NF1 and noradrenergic in VHL (Eisenhofer *et al.* 2001).

Previously to 2000, the extra-adrenal form of pheochromocytoma, termed 'paraganglioma', was also not linked to any germline mutations and paragangliomas located in the head and neck region (HN-paragangliomas)

were considered as separate entities from those located in the abdomen, chest, and pelvis. In 2000, this scenario changed abruptly when Bora Baysal (Baysal *et al.* 2000) demonstrated that germline mutations in the gene encoding the D subunit of the succinate dehydrogenase (SDH) (mitochondrial complex II, an enzyme in the tricarboxylic acid (TCA) cycle) was responsible for a familial form of paragangliomatosis (PGL1) characterized by the occurrence of multiple HN-paragangliomas and occasionally associated with abdominal catecholamine-secreting paragangliomas. In the same year Niemann & Müller (2000) reported that germline mutations in the C subunit of the SDH were responsible for the occurrence of familial HN-paragangliomas. A year later, Astuti *et al.* (2001) demonstrated that germline mutations in the B catalytic subunit of the SDH caused another familial syndrome, termed PGL4, which was characterized by the occurrence of

primarily abdominal catecholamine-secreting paragangliomas. Subsequently it was recognized that *SDHB* mutations were associated with a higher risk of malignant paraganglioma (Gimenez-Roqueplo *et al.* 2003).

These discoveries initiated the 'SDH Era' of the pheochromocytoma/paraganglioma story, introducing new susceptibility genes – mutations in a mitochondrial complex linked changes in cell metabolism and tumorigenesis.

Pheochromocytoma/paraganglioma susceptibility genes

Currently, there are 13 main pheochromocytoma/paraganglioma susceptibility genes, which belong to a wide range of functional categories (Dahia 2014, Martucci & Pacak 2014). These susceptibility genes include: the tumor-suppressor gene *VHL* (Latif *et al.* 1993); the proto-oncogene *RET* (Neumann *et al.* 1993); the tumor-suppressor gene *NF1* (White & O'Connell 1991); *TMEM127* (Qin *et al.* 2010); *MAX* (Comino-Méndez *et al.* 2011); hypoxia inducible factor 2 alpha (*HIF2α*) (Favier *et al.* 2012, Zhuang *et al.* 2012); the genes encoding the four subunits of the SDH (Baysal *et al.* 2000, Niemann & Müller 2000, Astuti *et al.* 2001, Burnichon *et al.* 2010); the *SDHAF2/SDH5* gene that is responsible for the flavination of the SDHA subunit (Hao *et al.* 2009); the gene encoding the fumarate-hydratase (FH) (Castro-Vega *et al.* 2014); and, the gene encoding the TCA cycle enzyme malate-dehydrogenase type 2 (*MDH2*) (Cascón *et al.* 2015). Mutations in these susceptibility genes are responsible for the occurrence of different clinical and biochemical phenotypes of pheochromocytoma/paraganglioma (Eng *et al.* 1994, Eng 1996, Walther *et al.* 1999, Neumann *et al.* 2002, 2004, 2011, Schiavi *et al.* 2005, Simi *et al.* 2005, Mannelli *et al.* 2007, 2009, Yao *et al.* 2010, Burnichon *et al.* 2012a,b, Welander *et al.* 2012, Comino-Méndez *et al.* 2013, Castinetti *et al.* 2014).

Based on gene profiling studies (Dahia *et al.* 2005, Favier *et al.* 2009), mutated as well as WT pheochromocytoma/paraganglioma can be assigned to two different 'clusters'. Cluster 1 includes *VHL*, *SDHx* and *FH* mutated tumors and is characterized by hypoxic signaling, while cluster 2 includes: *RET*, *NF1*, *TMEM127* and *MAX* mutated tumors and is characterized by an increased kinase signaling.

Although the association of pheochromocytoma/paraganglioma with these germline mutations and the corresponding altered intracellular pathways has been very well established, the molecular process leading from

gene mutations to tumor occurrence is still mostly unknown. In this context, the *SDHx* and *FH* mutated pheochromocytoma/paraganglioma represent an interesting subtype of tumor that links the hypoxia-related signals to mitochondria and to impairment and reprogramming of cell metabolism.

Cell metabolism and cancer: the Warburg hypothesis

The Warburg hypothesis, that cancer is caused by cell respiratory impairment and that enhanced glycolysis is the consequence of the dysregulation that underlies carcinogenesis, has been debated for at least two decades. Indeed, several observations have demonstrated that tumor mitochondria show an unaltered efficiency in oxidizing respiratory substrates (reviewed in Koppenol *et al.* (2011) and Wallace (2012)). However, according to the recent findings, in some tumors, such as pheochromocytoma and paraganglioma, mutations in some of the genes encoding the TCA enzymes, such as SDH and FH, cause cell metabolism impairment and reprogramming. These discoveries have not only renewed and revived interest in cancer metabolism, but also provide evidence that the Warburg hypothesis may have been correct, at least in some specific tumors. Thus, the reprogramming of energy metabolism has been added as the eighth hallmark of cancer (Hanahan & Weinberg 2011).

Mitochondria impairment and pheochromocytoma/paraganglioma

Mitochondrial SDH is an enzyme involved in the TCA cycle, where it catalyzes the oxidation of succinate to fumarate, and in the mitochondrial electron transport chain, where it serves as an electron donor, reducing ubiquinone to ubiquinol, which is subsequently used by complex III. SDHA and SDHB are the two catalytic subunits, while SDHC and SDHD are the structural subunits anchoring the complex to the inner mitochondrial membrane. Importantly, it has also been shown that mutations in the SDHB subunit, but not mutations in the other SDH subunits, are associated with a high incidence of malignant pheochromocytoma/paraganglioma (Gimenez-Roqueplo *et al.* 2003).

In addition, germline mutations of *FH* can cause the occurrence of solid tumors including pheochromocytoma or paraganglioma (Castro-Vega *et al.* 2014, Clark *et al.* 2014). FH follows SDH in the Krebs cycle, converting fumarate to L-malate. Although limited to a small number

of patients reported to date, these FH-mutated pheochromocytoma/paraganglioma, as seen in patients with *SDHB* germline mutations, appear to be associated with a high rate of malignancy.

The *SDHx* and *FH* genes are tumor suppressor genes that follow the Knudson's two-hit hypothesis. A heterozygous germline mutation in one of these genes is very frequently associated with loss of heterozygosity (LOH) leading to a complete loss-of-function. However, there must be other mechanisms by which the WT allele can be silenced because, in some patients, mutations in one of the *SDHx* genes can cause an almost complete loss of the enzymatic activity, even when LOH is not present (Rapizzi et al. 2012). The enzymatic deficiency is due to the decreased expression of the SDH complex that, when altered and unstable, is more susceptible to degradation (Gimenez-Roqueplo et al. 2001, 2002, Douwes Dekker et al. 2003, Burnichon et al. 2010, Yang et al. 2012). A consequence of the enzymatic loss of function is the accumulation in the cytoplasm of the corresponding substrate. Two different groups demonstrated that in *SDHx* mutated pheochromocytoma/paraganglioma tumor tissues succinate levels are significantly higher than in WT tumors (Pollard et al. 2005, Lendvai et al. 2014, Richter et al. 2014, Imperiale et al. 2015). The defect of enzymatic activity is associated not only with an impairment of the TCA cycle but also to changes in the structure of mitochondria that appear increased in number, swollen, and with a significant reduction of the internal cristae (Fig. 1). Similarly, FH impairment leads to intracellular fumarate accumulation (Pollard et al. 2005, Letouzé et al. 2013). Accumulation of these 'oncometabolites' (succinate or fumarate) impacts a wide spectrum of

cell functions ranging from the activation of a pseudo-hypoxia response to epigenetic reprogramming (for recent reviews, see Kaelin & McKnight (2013), Adam et al. (2014) and Morin et al. (2014)). Remarkably, FH-deficient pheochromocytoma/paraganglioma display the same pattern of epigenetic deregulation as *SDHB*-mutated malignant pheochromocytoma/paraganglioma.

SDH deficiency and pseudo-hypoxia response

Increased levels of succinate inhibit prolyl hydroxylases (PHDs) in the cytosol, leading to stabilization and activation of HIF1 α and HIF2 α (Dahia et al. 2005, Pollard et al. 2005, Brière et al. 2005, Selak et al. 2005, Koivunen et al. 2007). The expression of HIFs result in an activation of angiogenesis, which induces adaptive changes in cell metabolism (for a recent review, see Semenza (2013)). However, it is not clear which HIF isoform is dominant in this process. It has been shown that many human cancers express HIFs and thus, it has been hypothesized that there is a central role for both HIF1 α and HIF2 α in tumor progression through overlapping functions. Nevertheless, it is still not completely clear how HIF1 α and HIF2 α modulate tumorigenesis. Indeed, there is mounting evidence that HIF1 α and HIF2 α may induce highly divergent and even opposing effects. For example, in some tumors, HIF expression correlates with poor prognosis, but often, this correlation is valid only for one of HIF α subunits, suggesting a predominant role of that particular subunit in that specific cancer (Keith et al. 2011, Young & Simon 2012).

With regard to chromaffin tumors, it has been reported that in *SDHx*-mutated pheochromocytoma/paraganglioma, HIF2 α is significantly more expressed than in sporadic

Figure 1

Analysis of mitochondria by electron microscopy. Representative images of WT, SDH-, and NF1-mutated pheochromocytoma. Only in the SDH-mutated

pheochromocytoma are the mitochondria cristae clearly impaired compared to those found in the other tumors.

tumors (Gimenez-Roqueplo *et al.* 2001, 2002). The same research group (Favier *et al.* 2009) also found that HIF2 α mRNA is overexpressed in both *VHL* and *SDH*-mutated tumors. In contrast, Pollard *et al.* (2006) reported that HIF2 α is relatively more overexpressed in *VHL*-related pheochromocytoma/paraganglioma than in *SDHx*-related pheochromocytoma/paraganglioma, where HIF1 α is predominately expressed. In a recent report, López-Jiménez *et al.* (2010) found that both canonical HIF1 α and HIF2 α target genes are overexpressed in the *SDH/VHL* cluster. However, when *VHL* tumors were compared with *SDH* tumors, HIF1 α target genes showed a predominant activation in the *VHL* paragangliomas (López-Jiménez *et al.* 2010).

The HIF pathway schema has been made even more complex by unexpected findings that show tumor suppressive activity of HIF1 α in *VHL*-mutated clear cell renal carcinoma and isocitrate dehydrogenase 1/2 mutated (IDH1/2) gliomas (Shen *et al.* 2011, Koivunen *et al.* 2012). Furthermore, it has recently been demonstrated that in chromaffin cells, HIF1 α has opposing actions to HIF2 α , suggesting differential actions on tumorigenic processes via a MYC/MAX-related pathway (Qin *et al.* 2014). HIF signaling pathways appear to be crucial not only in pheochromocytoma, but also in other neuroendocrine tumors, and HIF2 α signaling has particularly prominent functions in regulating developmental processes of growth and differentiation in cells of the sympathoadrenal lineage (for reviews, see Richter *et al.* (2013) and Jochmanová *et al.* (2014)).

SDH deficiency and reactive oxygen species production

There is evidence that reactive oxygen species (ROS) may play a pathogenic role in the formation of pheochromocytoma/paraganglioma in *SDHx* mutation carriers. The electron transport system is the major endogenous source of ROS, which in turn can readily alter a wide variety of cellular components leading to potential cellular damage. Consistent with this model, Ishii *et al.* (2007) detected an increase in superoxide production, along with an increase in tumorigenesis in cells with *SDHC* mutations. In addition, Guzy *et al.* (2008) showed that inhibition of *SDHB* leads to ROS production, induces HIF1 α stabilization in a ROS-dependent manner, and increases growth rate, suggesting a contribution by these factors in tumorigenesis. However, Selak *et al.* (2006) demonstrated that pseudo-hypoxia can be observed in *SDH*-suppressed cells in the absence of oxidative stress. Lastly, using the yeast model, we demonstrated that a *SDHB* mutation causes

a significant increase in ROS production and increased mitochondrial DNA mutability (Goffrini *et al.* 2009).

SDH deficiency and DNA methylation

Many tumors show abnormal levels of histone methylation due to a deregulation of several histone methyltransferases as well as demethylases. Methylation usually represses gene transcription, thus hypermethylation of tumor suppressor gene promoters plays an extremely important role in tumorigenesis. It has been recently demonstrated that oncometabolites play a pivotal role in this epigenetic regulation. For example, Cervera *et al.* (2009) reported that the oncometabolite succinate increases histone methylation. Subsequently, it has been demonstrated that this increase is due to the inhibition of the jumoni-domain histone demethylases (Xiao *et al.* 2012). Ten-eleven translocation (TET) demethylases are enzymes belonging to a large family of 5-methylcytosine hydroxylases that convert 5-methylcytosine to 5-hydroxymethylcytosine. Inhibition of these enzymes in *SDH*- or *FH*-mutated pheochromocytoma/paraganglioma leads not only to an increase of DNA methylation with a decrease of DNA hydroxymethylation (Castro-Vega *et al.* 2014), but also to changes in tumor phenotype which becomes noradrenergic because of the loss of phenylethanolamine N-methyltransferase activity (Letouzé *et al.* 2013). In addition, DNA hypermethylation has been observed in *SDH* and *FH* mutated gastrointestinal stromal tumors (Killian *et al.* 2013). Thus, it is reasonable to hypothesize that the metabolic reprogramming that occurs in cancers might be modulated by oncometabolites through their epigenetic actions.

SDH deficiency and cell metabolism

It has been demonstrated that in *SDHx*-related pheochromocytoma/paraganglioma the remarkable decrease in *SDH* activity is concomitant to a relevant, probably compensatory, increase in complex I, III, IV, and citrate synthase enzyme activities as well as to increased levels of succinate – changes that are coupled to decreased levels of ATP, ADP, and AMP. This phenomenon is not observed in sporadic or other genetically-determined forms of pheochromocytoma/paraganglioma (Rao *et al.* 2015).

Recently, our group investigated the role of *SDHB* mutations in modulating cell metabolism and function. We used the neuroblastoma cell line (SK-N-AS) stably transfected with the WT human *SDHB*, or different *SDHB* mutated constructs carrying some disease-causing

mutations found in our patients affected by pheochromocytoma/paraganglioma. Similar to SDH mutated tumor cells, mutated SK-N-AS clones showed reduced SDH enzyme activity, reduced oxygen consumption and reduced carbonic anhydride production, and thus demonstrating a decrease in mitochondrial metabolism. Surprisingly and unexpectedly, in all the *SDHB*-mutated clones we found a significant decrease in glucose uptake and in lactate culture medium concentration associated with an increase in cell proliferation and migration (Rapizzi et al. 2014).

Cancer cells are only one component of the solid tumors. Within tumors there are non-malignant stromal cells such as endothelial cells, fibroblasts, immune cells and extracellular matrix, forming the 'tumor microenvironment'. The tumor microenvironment is proving to have a pivotal role in modulating cancer progression and metastasis and has become a potential therapeutic target. The continuous interplay between cancer cells and the microenvironment generates favorable conditions that lead cancer cells to survive, grow, and spread metastases to healthy tissues (for recent reviews, see Hanahan & Weinberg (2011), Hanahan & Coussens (2012), Quail & Joyce (2013), Sounni & Noel (2013) and Klemm & Joyce (2015)).

Very recently, we reported the effects of *SDHB* silencing on cellular metabolism, confirming succinate as an oncometabolite, and linking impaired cell metabolism to cancer (Rapizzi et al. 2015). In addition, we confirmed the influence and the role of tumor microenvironment on tumor progression. The results obtained in the previous work on *SDHB*-mutated cells (Rapizzi et al. 2014), were confirmed in this study, where *SDHB* was silenced (Rapizzi et al. 2015). Consistent with a reduction of SDH activity in the TCA cycle in *SDHB* silenced cells, we observed a decrease in reducing equivalent production, a significant decrease in oxygen consumption, reduced glucose uptake, and an increased lactate uptake – findings that proved that SDH impairment itself induces a metabolic reprogramming and an associated increase in cell proliferation and invasion.

Consistent with the findings from Fiaschi et al. (2012), we also demonstrated that microenvironment, represented by fibroblasts, strongly affects tumor metabolism and growth capacity. In particular, we demonstrated that primary fibroblasts and tumor cells establish reciprocal metabolic changes. Control tumor cells, when co-cultured with human fibroblasts, showed a significant decrease in glucose uptake and a significant increase in cell proliferation vs their mono-cultured counterparts. *SDHB* silencing made these effects significantly more evident. On the other hand, co-cultured fibroblasts increase glucose uptake and

Figure 2

Schematic demonstrating pathways of pheochromocytoma/paraganglioma tumorigenesis. Abbreviations used: HIFs, hypoxia inducible factors; JMJD, jumonji-domain histone demethylases; PHD, prolyl hydroxylases; ROS, reactive oxygen species; TCA, tricarboxylic acid cycle; TETs, ten-eleven translocation (TET) demethylases.

its conversion into lactate, thus shifting to a Warburg-like glycolytic metabolism. Subsequently, lactate extruded by fibroblasts, is uploaded by tumor cells. Through these metabolic changes, cancer cell increase anabolic processes, proliferation, and metalloproteinase activation.

Conclusions

In the last 15 years, important achievements in genetic research have deeply impacted our knowledge and clinical approach to pheochromocytoma/paraganglioma. The field was founded by the seminal work of Baysal et al. (2000) on the role played by SDH mutations in pheochromocytoma/paraganglioma tumorigenesis (Fig. 2). Subsequently, other susceptibility genes were progressively discovered and led to the recognition of two different genetic clusters characterized by the activation of different intracellular pathways. Alterations in SDH activity and thus in mitochondrial function have strengthened the interest in the role played by changes in cell metabolism and cancer development. SDH-mutated tumors have become an interesting model to expand our knowledge on the complex interrelationships that link solid cancer growth to mitochondria, cell metabolism, and the microenvironment.

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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