Hereditary paraganglioma : genetics and tumor biology

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CHAPTER 6

Summary and Discussion

TCA cycle defects

Paraganglioma and pheochromocytoma are associated with more than 20 genes to date, in which germline and/or somatic mutations have been identified. A subgroup of these genes is associated with hereditary paraganglioma–pheochromocytoma, and encode the subunits forming complex II embedded in the mitochondrial inner membrane. Complex II, also termed succinate dehydrogenase (SDH) consists of *SDHA*, *SDHB*, *SDHC*, *SDHD* and the SDH-assembly factor *SDHAF2*. Tumors caused by mutations in these genes can be grouped together in a cluster (cluster 1) on the basis of their transcriptional profile, enriched for genes that are associated with angiogenesis and the hypoxic response (1). The SDH complex resides in the inner mitochondrial membrane and serves as a core component of both the tricarboxylic acid (TCA) cycle and the electron transport chain coupling the conversion of succinate to fumarate with the reduction of ubiquinone. Another component of the TCA cycle is fumarate hydratase (FH), responsible for the hydroxylation of fumarate to L-malate. *FH*-deficient tumors show gene expression profiles that are very similar of *SDH* mutant tumors, suggesting similarities in the mechanism of tumorigenesis (2). The link between *SDH* and *FH* inactivation was further strengthened by the identification of *FH* gene mutations in PCCs that displayed similar transcriptional and methylation profiles to those of *SDH* mutant tumors (3). Inactivation of either *SDH* or *FH* leads to accumulation of the respective substrates, succinate and fumarate, which act as oncometabolites. 'Oncometabolite' is a term coined to describe existing or de novo compounds that accumulate due to the action of a cancer-associated protein and which influence important cellular processes. In the case of succinate and fumarate this involves inhibition of α-ketoglutarate dependent hydroxylases, including prolyl hydroxylases (PHD), histone demethylases and the TET (ten-eleven translocation) family of DNA hydroxylases (Figure 1). TET is responsible for the oxidation of 5-methylcytosine to 5-hydroxymethylcytosine (5hmC).

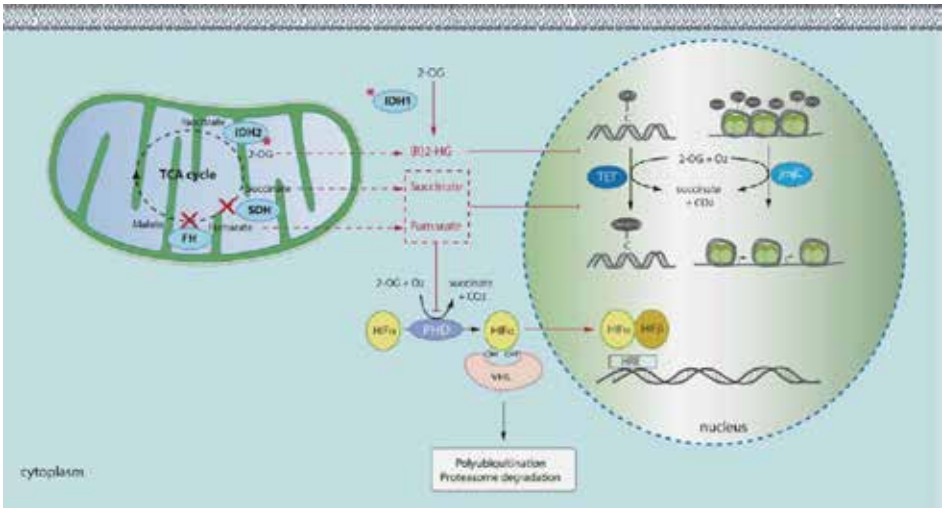

Figure 1. Inactivating mutations (red crosses) in genes encoding succinate dehydrogenase (SDH), fumarate hydratase (FH), and oncogenic mutations (red star) in isocitrate dehydrogenase (IDH1 and IDH2) lead to the accumulation of succinate, fumarate and (R)-2 hydroxyglutarate ((R)2-HG), respectively. These oncometabolites inhibit α-ketoglutarate (2-OG) dependent hydroxylases, including prolyl hydroxylases (PHD), jumonji C-domain-containing (JmjC) histone demethylases and the TET (ten-eleven translocation) family of DNA hydroxylases, respectively leading to pseudohypoxia, histone and DNA methylation. (Adapted from (4)).

In **chapter two**, we demonstrated that *SDH* and *FH* mutations are associated with inhibition of DNA and histone demethylases, leading to loss of 5hmC and increased H3K9me3 levels in the tumor cells of *SDH*-deficient paragangliomas and *FH*-deficient smooth muscle tumors. Both DNA methylation and H3K9me3 are often associated with regulatory elements of transcriptionally repressed genes and constitutive heterochromatic regions of the genome, resulting in an altered transcriptome. The relationship between methylation and expression changes in *SDH*x and *FH*-related paragangliomas has been examined by Letouzé *et al*. (2), who revealed 191 genes showing both significant CpG island hypermethylation and significant downregulation in these tumors (2). Gene ontology analysis of this set of genes showed a significant enrichment in terms associated with neuroendocrine differentiation, indicating that methylome remodeling results in transcriptional abnormalities in *SDH*x and *FH*-related paragangliomas, directly associated with their phenotypic characteristics. *VHL*, *NF1*, and *RET*-mutated paragangliomas/pheochromocytomas did not display a hypermethylator phenotype (2), while those caused by defects in other genes of the TCA cycle, such as isocitrate dehydrogenase and malate dehydrogenase, did (5;6). These findings suggest clinical opportunities for epigenetic targeting in tumors caused by TCA cycle defects. The DNA methyltransferase inhibitors 5-azacytidine and decitabine are of interest, as 5-azacytidine has been shown to reduce the proliferative index in an *in vivo* IDH1 glioma model (7) and decitabine repressed the migration capacities of $Sdhb^{-/-}$ cells (2). Moreover, temozolomide, an alkylating agent, has been shown to be effective in the treatment for glioblastoma and neuroendocrine tumors (8;9). The cytotoxic effect of temozolomide has been attributed to its ability to induce DNA methylation at the O^6 position of guanine. Methylation of guanine results in DNA mismatch, ultimately resulting in apoptosis and tumor cell death. Efficacy of temozolomide was correlated with loss of the expression of the DNA repair enzyme O(6)-methylguanine-DNA methyltransferase (MGMT) and/or MGMT promoter methylation in glioblastoma and neuroendocrine tumors (8;9). In a limited cohort, temozolomide appeared to be more effective in patients with *SDHB* malignant paragangliomas/pheochromocytomas compared to non-*SDHB* mutant tumors. This increased response is probably explained by the loss of MGMT as its promoter was highly methylated in this subgroup of tumors (10).

The Hensen model

Mutations in *SDHD* or *SDHAF2*, unlike mutations of the other SDH subunit genes, show a remarkable parent-of-origin effect in which carriers develop tumors only when the mutation is inherited from the father. *SDHD* and *SDHAF2* share the same location, chromosome 11, in contrast to the *SDHA, SDHB* and *SDHC* genes, which are located on chromosome 5 (*SDHA*) and chromosome 1 (*SDHB* and *SDHC*), and in which germline mutations do not give rise to this parent-of-origin effect. Chromosome 11 harbors the main concentration of imprinted genes in the human genome in the 11p15 region, with 8 genes expressed exclusively from the maternal allele while the opposite allele is silenced by epigenetic mechanisms. Loss of the entire maternal copy of chromosome 11 is a frequent event in *SDHD*-linked paragangliomas (11). Hensen and colleagues (11) have proposed that the selective loss of the maternal copy of chromosome 11 results in the simultaneous deletion of the wild type copy of the *SDHD* gene and an exclusively maternally expressed gene, producing a parent-of-origin inheritance pattern (Figure 2). This second locus has remained elusive to date. In **chapter three** we present experiments to identify this second locus/loci. We hypothesized that in a human neuronal

cell line with two parental copies of chromosome 11 in which *SDHD* had been inactivated, the knockdown of candidate genes from the imprinted gene cluster on 11p15 might lead to a cellular phenotype resembling that of primary paragangliomas if the correct combination of genes were targeted. We studied cell proliferation, apoptosis, gene expression profiles and TCA cycle metabolites in these cells and identified two potential tumor modifier genes, *SLC22A18* and *CDKN1C*.

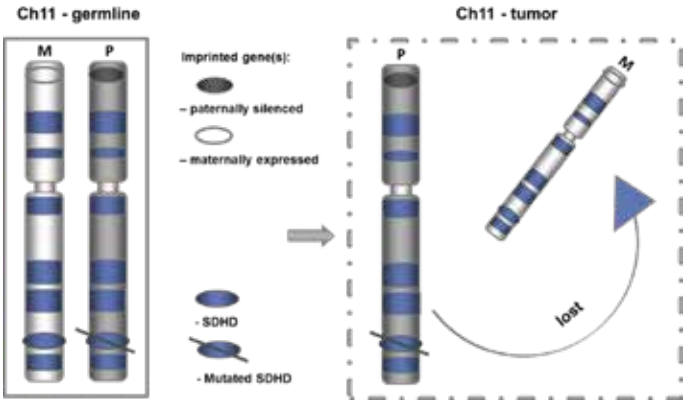

Figure 2. The 'Hensen' hypothesis in outline. Loss of a maternal expressed tumor suppressor gene(s) in the tumor, together with the normal gene copy of *SDHD*, leads to tumor formation. (m – maternal, p – paternal)

Compared to single knockdown of *SDHD*, knockdown of *SDHD* together with *SLC22A18* or with *CDKN1C* led to small but significant increases in cell proliferation and resistance to apoptosis and resulted in a gene expression profile closely related to the known transcriptional profile of *SDH*-deficient tumors. While the results of our cell line-based functional assays supported a role of *SLC22A18* and/or *CDKN1C* in tumor formation, further genetic and protein analyses of SLC22A18 and CDKN1C was performed in 60 *SDHD*-mutated tumors. Of the 60 *SDHD* mutant tumors investigated, 4 tumors showed retention of chromosome 11, suggesting that SLC22A18 and/or CDKN1C might be functionally deleted in these tumors. Indeed, we observed that SLC22A18 and CDKN1C protein expression levels in *SDHD* mutant tumors with retention of chromosome 11 were comparable to levels in tumors showing loss of chromosome 11 by immunohistochemistry and were significantly decreased compared to controls and/or non-*SDH* mutant tumors. In chapter two, we have observed the association of *SDHD*-related tumors with DNA hypermethylation and histone methylation; it is possible that this leads to repressed transcription of *SLC22A18* and *CDKN1C* and might explain the lowered expression of these genes in *SDHD* mutant tumors with retention of chromosome 11. Histone modifications leading to repression of *CDKN1C* transcription has been shown to contribute to breast cancer (12), rhabdoid tumors (13), and gastric cancer (14). Moreover, *SLC22A18* promoter methylation and downregulation is associated with the development and progression of glioma (15;16). Future studies should address whether *SLC22A18* and *CDKN1C* are methylated in this subgroup of *SDHD*-related tumors with retention of chromosome 11. Overall, our data strongly suggest that *SLC22A18* and *CDKN1C* are genes involved in the tumorigenesis of *SDHD*-linked paragangliomas.

CDKN1C, encoding the cyclin-dependent kinase inhibitor 1C, negatively regulates cell proliferation by inhibiting cyclin/CdK complexes during the G1 phase of the cell cycle and blocks cancer cell growth (17). It is likely that loss of CDKN1C expression in *SDHD* mutant paragangliomas confers further growth advantage to the tumors besides the inactivation of the *SDHD* gene. In agreement with this, our data showed that the double knockdown of *SDHD* and *CDKN1C* increased cell proliferation of neuronal cells and cell proliferation-related gene expression compared to single knockdown of *SDHD*.

SLC22A18 is a member of a family of polyspecific transporters and multidrug resistance genes, but the identity of its physiological substrates is presently unknown. As such, there is no information on molecular pathways that could explain a tumor suppressive function of this transporter. It has been shown that *SLC22A18* has a pro-apoptotic function in glioma cells and confers drug resistance, since the elevated expression of *SLC22A18* increased the expression of caspase-3 and the sensitivity of glioma cells to the anticancer drug BCNU (18). In agreement with this, our findings showed downregulation of apoptosis and cell death-related gene expression in neuronal cells by the knockdown of *SLC22A18*. No significant apoptotic activity has been detected in head and neck paragangliomas (19), and our results showed that by combined knockdown of *SLC22A18* and *SDHD*, cells became more resistant to apoptosis. This suggests that the joint loss of *SLC22A18* and *SDHD* might create a cellular condition that is favourable for tumor progression, i.e., a combination of metabolic and epigenetic changes induced by succinate accumulation, and increased resistance to apoptotic mechanisms. Via which mechanism *SDHD* and *SLC22A18* regulate apoptosis is still unknown. A possible explanation could be through the activation of hypoxia inducible factor 1 (HIF-1). *SDHD* mutations leads to a cellular pseudohypoxic state through the stabilization of HIF-1 caused by the accumulation of succinate (Figure 1). Activated HIF-1 can act as a transcription factor in the nucleus and activates the transcription of a large number of genes, resulting in increased cellular proliferation and reduced apoptosis (1).

Somatic genetic changes

If the loss of chromosome 11 is important for the development of all *SDH*-related tumors, one prediction of the Hensen model is that mutations in *SDHD* and *SDHAF2* will display higher penetrance than mutations in *SDHA*, *SDHB*, or *SDHC*. Tumorigenesis in *SDHD* and *SDHAF2* mutation carriers requires only a single somatic genetic event (chromosome 11 loss), as opposed to the two events required in *SDHA*, *SDHB*, and *SDHC* mutation carriers (loss of the respective wild type allele, together with independent loss of chromosome 11). This prediction appears to be borne out by the wide difference in lifetime penetrance between mutations in *SDHD* (~90%) (20) and *SDHAF2* (>95%) (21), compared to *SDHB* (~30%) (22-24) and *SDHA* and *SDHC* (both with unknown, but probably very low, penetrance). In **chapter four** we studied whether loss of the maternal copy of chromosome 11p is an important feature for the development of *SDHAF2*, *SDHD*, *SDHB*, and *VHL*-linked tumors. We demonstrated in 89% of *SDHAF2*, 85% of *SDHD*, and 75% of *VHL*-related tumors loss of maternal chromosome 11p, using highly polymorphic microsatellite markers. No paternal chromosome 11 loss was found in these tumors. By contrast, both copies of chromosome 11 were found to be retained in 62% of *SDHB*-mutated PGLs/PCCs, while only 31% showed loss maternal chromosome 11p15,

suggesting a different genetic mechanism for tumor development in a substantial group of *SDHB*-related tumors.

SNP array analysis revealed a strikingly simple pattern of chromosome involvement in *SDHAF2* and *SDHD*-related tumors, in which copy number loss/LOH primarily affects chromosome 11 and not much else. *VHL* and *SDHB*-related tumors show a much more complex pattern, involving several other chromosomes beyond those containing *VHL* and *SDHB* (chromosome 3 and 1, respectively). Moreover, *SDHB* mutant tumors have a greater degree of genome instability (mean 12%) compared to *SDHD* (mean 4%) and *SDHAF2* (mean 4,5%) mutant tumors. The most commonly affected chromosomal regions in *SDHB*-related tumors were gain of 1q (57%), chromosome 7 (28%) and 17q (28%), and loss of 1p (100%) and 17p (57%). These regions have also been shown to be similarly affected in *RET*, *NF1* and sporadic paragangliomas/pheochromocytomas (25), indicating the potential presence of modifier genes on these autosomes. Many chromosomal changes are recurrent and often non-overlapping, suggesting a potential redundancy in modifiers. As such, altered expression of different groups of modifier genes might be involved in *SDHB* tumorigenesis. Analysis of a much larger number of *SDHB* tumors will be required to resolve this question.

Interestingly, recent work showed that somatic copy number alterations impact cancer by the concomitant loss of multiple genes, leading to an altered metabolism (26;27). This mechanism might also be at work in *SDH*x-related tumors, with chromosome 11p loss necessary and sufficient to trigger *SDHD* and *SDHAF2* tumorigenesis, while *SDHB* tumors can develop upon amplification or deletion of multiple modifier genes located on different chromosomes. As shown in chapter three, *SLC22A18* and *CDKN1C* could be the genes involved in the tumorigenesis of *SDHD*-linked paragangliomas and might also be involved in *SDHAF2* and *VHL*-related tumors, but this has yet to be determined. In conclusion, our results clearly show that loss of maternal chromosome 11 is a signature event in *SDHAF2*, *SDHD*, and *VHL*-related paragangliomas, while *SDHB* tumors follow a more complex and possibly different path to tumorigenesis, involving loss or gain of a greater proportion of the genome.

Clinical presentation

Despite the fact that the SDH proteins are all associated with the same protein complex, mutations in individual subunit genes lead to clear differences in clinical phenotype (Table 1). The molecular basis for this clinical divergence is as yet unknown. *SDHD* mutations are generally associated with a higher risk of head and neck paragangliomas. For *SDHB* mutations, extra-adrenal and adrenal is more often the presenting feature, and *SDHB* mutation carriers have a higher risk of metastasis (31;32). Metastasis is thought to occur through epithelial–mesenchymal transition (EMT), while gene expression analysis of *SDHB*-related metastatic paragangliomas showed the activation of the EMT programme (26;33). This might confer cancer cells with stem cell-like properties including the ability to migrate to and invade distant anatomic sites.

Table 1. Clinical presentation of *SDH*-related paragangliomas/pheochromocytomas

Gene	Gene locus	Protein function of SDH complex	Inheri-tance	Pene-trance	Malignancy rate	PGL predeliction site
SDHA	5p15	Flavoprotein, catalytic subunit	AD	low	-	Extra-adrenal
SDHB	1p36	Iron-sulphur catalytic subunit	AD	20-30%	+++	Extra-adrenal, adrenal
SDHC	1q21	Anchoring subunit	AD	low	+	Head and neck
SDHD	11q23	Anchoring subunit	ADPI	88-100%	+	Head and neck
SDHAF2	11q13	Assembly factor	ADPI	87-100%	-	Head and neck

PGL: paraganglioma, AD: autosomal dominant, ADPI: autosomal dominant paternal imprinting.

It was recently proposed that a quantitative epigenetic switch, more pronounced in *SDHB* mutants than in other *SDH*x-related tumors, explains the increased malignancy risk conferred by *SDHB* mutations (2). Certain methylated genes have been linked to neuroendocrine differentiation (*PNMT*, *NPY* and *SLC6A2*) and EMT differentiation (*KRT19*). And as explained above, epigenetic targeting by the DNA methyltransferase inhibitor decitabine repressed the migration capacities of *SDHB*-deficient cells (2). Whether suppression of certain genes by CpG island or histone methylation provides an advantage in *SDHB*-mutated tumor progression, however, remains to be directly demonstrated.

Genetic heterogeneity of paragangliomas is further highlighted by the identification of the variety of *SDH* germline mutations, with the majority of mutations being point mutations and small deletions (30). In **chapter five**, we have molecularly characterized 16 germline deletions in *SDHB*, *SDHC*, *SDHD* and flanking genes of up to 104 kb in size using a simple and rapid long-range PCR method. This study increases the number of known *SDH* germline deletions by over 50%. It further underlines the fact that clinically relevant deletions may encompass neighboring genes, with the potential to modify phenotype. In 6 patients, we found a deletion affecting genes proximal to *SDHB*, *SDHC*, or *SDHD*, including *PADI2*, *MFAP2*, *ATP13A2* (*PARK9*), *CFAP126*, *TIMM8B* and *C11orf57*. These genes were either partially or completely deleted, but did not influence the phenotype of the patients. In addition, our results suggest that mechanisms underlying deletion such as *Alu* repeat-mediated recombination, which is known to play a major role in the germline deletion of *VHL* (31), does not play a significant role in the deletion of *SDH* genes. Since the *VHL* locus sequence shows a very high *Alu* density of 49%, this may predispose the *VHL* gene to a high frequency of *Alu*-mediated deletions, in contrast to the *SDH* genes, which show a lower *Alu* density (mean of 29%). Benefits of precise breakpoint mapping include exact identification of a variant, which facilitates family studies and the identification of founder mutations. Improved understanding of the function of deleted neighboring genes will bring new insights into subtle clinical effects.

Overall, we and others (25) identified recurrent copy number alterations as well as gene expression and methylation patterns in *SDH*-deficient tumors. However, the particular mechanisms in which these events are involved in paraganglioma tumorigenesis remain to be clarified. We still do not know if the primary link between loss of SDH and tumorigenesis is due to the activation of HIF-1 through the accumulation of succinate or due to inhibition of DNA and histone demethylases (Figure

1), leading in both cases to an altered transcriptome. Recently, a causal link between hypoxia and DNA hypermethylation has been identified, leading to altered gene expression (32). Since paragangliomas are hypoxic tumors and show a hypermethylator phenotype, this might suggest these mechanisms are related and both important for tumorigenesis. Whether changes in gene expression are a cause or a consequence of tumor formation needs to be addressed in the future. Progress will require the development of relevant animal and cell culture models that link SDH dysfunction to tumor formation.

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