Parent-of-origin tumourigenesis is mediated by an essential imprinted modifier in SDHD-linked paragangliomas: SLC22A18 and CDKN1C are candidate tumour modifiers

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Abstract

Mutations in SDHD and SDHAF2 (both located on chromosome 11) give rise to hereditary paraganglioma almost exclusively after paternal transmission of the mutation, and tumours often show loss of the entire maternal copy of chromosome 11. The ‘Hensen’ model postulates that a tumour modifier gene located on chromosome 11p15, a region known to harbour a cluster of imprinted genes, is essential to tumour formation. We observed decreased protein expression of the 11p15 candidate genes CDKN1C, SLC22A18 and ZNF215 evaluated in 60 SDHD-mutated tumours compared to normal carotid body tissue and non-SDH mutant tumours. We then created stable knockdown in vitro models, reasoning that the simultaneous knockdown of SDHD and a maternally expressed 11p15 modifier gene would enhance paraganglioma-related cellular characteristics compared to SDHD knockdown alone. Knockdown of SDHD in SNB19 and SHSY5Y cells resulted in the accumulation of succinate, the stabilization of HIF1 protein and a reduction in cell proliferation. 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 to a gene expression profile closely related to the known transcriptional profile of SDH-deficient tumours. Of the 60 SDHD tumours investigated, four tumours showing retention of chromosome 11 showed SLC22A18 and CDKN1C expression levels comparable to levels in tumours showing loss of chromosome 11, suggesting loss of protein expression despite chromosomal retention.

†The authors wish it to be known that, in their opinion, the last two authors should be regarded as joint Senior Authors, both authors contributed equally to this paper.

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Our data strongly suggest that SLC22A18 and/or CDKN1C are tumour modifier genes involved in the tumourigenesis of SDHD-linked paraganglioma.

Introduction

Hereditary paraganglioma-pheochromocytoma syndrome is characterized by neuroendocrine tumours that originate from both the sympathetic and parasympathetic branches of the autonomic nervous system. Pheochromocytomas (PCC) are generally benign catecholamine-secreting tumours of the adrenal medulla (1), whereas extra-adrenal paragangliomas (EA-PGL) are frequently aggressive tumours that arise in the thorax and abdomen. Paragangliomas of the head and neck (HNPGGL) arise most commonly in the carotid body, the main sensor of blood oxygenation, and these highly vascular tumours are often characterized by an indolent, non-invasive growth pattern (2).

Although more than 14 different genes have been linked to PGL/PCC, a subgroup of these genes is associated with hereditary PGL/PCC, including SDHA (3), SDHB (4), SDHC (5), SDHD (6), and SDHAF2 (7). These genes encode subunits of the mitochondrial succinate dehydrogenase (SDH) complex, which plays a central role in the citric acid cycle and the electron transport chain. In the TCA cycle, SDH converts succinate to fumarate while providing electrons for oxidative phosphorylation in the inner mitochondrial membrane. SDH inactivation results in accumulation of its substrate succinate, which can function as a competitor of α-ketoglutarate (α-KG) to broadly inhibit α-KG-dependent dioxygenases leading to HIF activation (8–11). Expression profiling of PGL and PCC shows increased hypoxic-angiogenic expression features and reduced oxidoreductase profiles in SDH-deficient tumours compared to non-SDH mutant tumours (12,13).

Germline mutations of the SDHD and SDHAF2 genes, unlike mutations of the other SDH subunit genes, show a parent-of-origin expression phenotype, with tumour development occurring almost exclusively due to mutations inherited via the paternal line (14,15). Carriers of maternally-inherited mutations develop tumours only very rarely. SDHD and SDHAF2 are both located on the long arm of chromosome 11, whereas the SDHA, SDHB and SDHC subunit genes are located on chromosome 5 (SDHA) or chromosome 1 (SDHB and SDHC). The 11p15 region of chromosome 11 harbours the main concentration of imprinted genes in the human genome, with eight genes (Table 1) expressed exclusively from the maternal allele on 11p15. Carriers of maternally-inherited mutations are potential tumour modifier genes involved in tumour formation of SDHD-linked paraganglioma (16–18). In addition, although more than 14 different genes have been linked to PGL/PCC, a subgroup of these genes is associated with hereditary PGL/PCC, including SDHA (3), SDHB (4), SDHC (5), SDHD (6), and SDHAF2 (7).

Table 1. Imprinted genes expressed exclusively from the maternal allele on 11p15

| Gene       | Chromosome location | Description                                      | Expressed allele | Imprinted allele |
|------------|---------------------|-------------------------------------------------|------------------|------------------|
| KCNQ1DN    | 11p15.4             | non-coding RNA                                  | Maternal         | Paternal         |
| ZNF215     | 11p15.4             | zinc finger protein 15                          | Maternal         | Paternal         |
| OSBPL5     | 11p15.4             | member oxysterol-binding protein family         | Maternal         | Paternal         |
| PHLDN2     | 11p15.5             | pleckstrin homology-like domain family A member 2| Maternal         | Paternal         |
| CDKN1C     | 11p15.5             | cyclin-dependent kinase inhibitor 1c             | Maternal         | Paternal         |
| H19        | 11p15.5             | non-coding RNA                                  | Maternal         | Paternal         |
| KCNQ1      | 11p15.5             | encoding voltage-gated potassium channel        | Maternal         | Paternal         |
| SLC22A18   | 11p15.5             | poly-specific organic cation transporter         | Maternal         | Paternal         |

Bold indicates the candidate genes investigated in this study.

Results

Protein expression of chromosome 11p15 candidate genes in SDHD mutant PGL

We selected imprinted 11p15 candidate modifier genes reasoning that a gene of interest would be expressed in normal carotid body tissue and lost in SDHD mutant tumours. Using immunohistochemistry (IHC), we analysed the protein expression of specific 11p15 genes (indicated in bold in Table 1) in normal human post-mortem carotid bodies, SDHD-related tumours and non-SDH mutant PCCs. Expression of SLC22A18 was significantly decreased in the chief cell, the neoplastic cell population, of SDHD mutant tumours, while remaining abundant in all normal carotid bodies and non-SDH mutant tumours (Fig. 2A–D).
SDHD knockdown leads to succinate accumulation, reduced ATP levels and HIF activation

In order to evaluate metabolic changes and HIF1 activation induced by loss of SDHD, we generated subclones of SNB19 and SHSY5Y cells with stable knockdown of SDHD. Knockdown of SDHD was confirmed by real-time analysis of RNA expression levels (Supplementary Material, Fig. S1) and by immunoblotting (Fig. 3A), with decreased SDHB protein levels taken as a marker for SDH deficiency (21). As expected (11), suppression of SDH resulted in the significant accumulation of succinate in both SNB19 and SHSY5Y cells (Fig. 3B). In addition, ATP levels were also significantly decreased (Fig. 3C). Suppression of SDH expression increased HIF1α protein levels (Fig. 3D) as well as mRNA expression of HIF1 target genes by at least two-fold, including glucose transporter 1 (GLUT1), Bcl-2-like 19kDa-interacting protein 3 (Bnip3), prolyl hydroxylase 3 (EGLN3), enolase 1 (ENO1) and vascular endothelial growth factor (VEGF) compared to scrambled control cells (Fig. 3E).

To study the effects of 11p15 candidate gene loss in SDHD knockdown cells, we carried out additional knockdown of OSBPL5, SLC22A18, CDKN1C or ZNF215 in both SNB19 and SHSY5Y cells. Stable knockdown of each gene was confirmed by real-time analysis of RNA expression levels (Supplementary Material, Fig. S2). Cells with double knockdown of SDH and either SLC22A18, CDKN1C or OSBPL5 exhibited succinate levels equivalent to single knockdowns of SDHD, while combined knockdown of SDHD and ZNF215 resulted in a small (non-significant) reduction in succinate levels (Fig. 4A and 4B). Consistent with these findings, the ratio of succinate to fumarate was increased in cells with single knockdown of SDHD compared to scrambled control cells and did not change significantly following additional knockdown of either SLC22A18, CDKN1C, OSBPL5 or ZNF215 (Fig. 4C and 4D). Furthermore, the elevated succinate/α-KG ratio following SDHD knockdown did not change significantly upon additional knockdown of any candidate gene (Fig. 4E and 4F).

Increased cell proliferation following SDHD and CDKN1C or SLC22A18 double knockdown

HNPG is characterized by unusually slow growth, with a reported doubling time of 4 years (2). Using a real-time cell proliferation system, SDHD knockdown in SHSY5Y resulted in reduced proliferation (Fig. 5A) and a lower S-phase fraction (Fig. 5B), relative to controls. However, reduced proliferation could not be attributed to cell cycle arrest at G2/M, since no changes were found in the G2/M fraction in SDHD knockdown compared to control cells (Fig. 5B).

However, a significantly increased rate of cell proliferation was seen following double knockdown of SDHD and CDKN1C (Fig. 5C), or of SDHD and SLC22A18 (Fig. 5D), compared to single knockdown of SDHD. By contrast, knockdown of SDHD together with OSBPL5 (Fig. 5E) or ZNF215 (Fig. 5F) did not result in enhanced proliferation compared to single knockdown of SDHD. Only very minor changes in cell proliferation were observed in SNB19 cells, perhaps because these cells show much faster intrinsic growth compared to SHSY5Y cells (Supplementary Material, Fig. S3).
cells showed similar results, but with lower overall sensitivity to apoptosis (Fig. 6B). SDHD knockdown also resulted in resistance to cisplatin-induced apoptosis, compared to control cells (Fig. 6C). Induction of apoptosis using staurosporine or cisplatin was also accompanied by decreased cell viability (Fig. 6D). Only one candidate gene showed significant differences in apoptosis resistance following double knockdown, SLC22A18. In SNB19 cells the combined knockdown of SDHD and SLC22A18 resulted in significant apoptosis resistance compared to single knockdown of SDHD (Fig. 6F).

Gene expression changes characteristic for SDH-related PGL/PCC by the combined loss of SDHD and SLC22A18 or CDKN1C

SDHD PGLs display distinctive gene expression patterns compared to paragangliomas and pheochromocytomas linked to other genes. Unsupervised hierarchical cluster analysis of gene expression in SNB19 and SHSY5Y cells showed that while cell type is the primary determinant of clustering (Supplementary Material, Fig. S5), a large number of genes are significantly differentially expressed in both SNB19 cells (Supplementary Material, Table S1) and SHSYSY cells (Supplementary Material, Table S2) depending on single SDHD, or double knockdown together with SLC22A18 or CDKN1C. Focusing on pathways believed to play a role in PGL/PCC (12,13) and exploiting the pathway database KEGG, we selected functional gene sets for analysis, including oxidative phosphorylation, citrate cycle (TCA cycle), apoptosis, glycolysis, VEGF signalling pathway, pathways in cancer including HIF, glutathione metabolism and beta-alanine metabolism.

Analysis using the global test revealed a synchronized suppression of mitochondrial functions in SDHD knockdown SNB19 cells compared to scrambled control cells, characterized by significant differential expression of components of the oxidative response and TCA cycle (Table 2). Interestingly, double knockdown of SDHD together with either CDKN1C or SLC22A18 in SNB19 cells led to greater (significant) differential expression of additional PGL/PCC-associated pathways (Table 2). These changes were not observed in SHSYSY cells using the global test (Supplementary Material, Fig. S5). To identify further cellular functions that might be affected by the observed gene expression changes, we performed a series of comparisons using Ingenuity Pathway Analysis (IPA). This analysis...
revealed that double knockdown of SDHD and SLC22A18 or of SDHD and CDKN1C strongly decreased apoptosis and cell death-associated gene expression in both SNB19 cells (Fig. 7A) and SHSY5Y cells (Fig. 7B), compared to single SDHD knockdown. In addition, both double knockdowns induced gene expression signatures for increased cell proliferation and cell survival compared to single SDHD knockdown.

Protein expression of SLC22A18 and CDKN1C and somatic mutation analysis in SDHD mutant tumours without loss of chromosome 11

Most SDHD-linked HNPGLs show loss of the entire maternal copy of chromosome 11 (16–18), effectively preventing further genetic or functional analysis of genes and gene products found on the maternal chromosome. However, surveying 60 SDHD mutant tumours, we identified four (6.6%) tumours that were heterozygous (no LOH) for microsatellite markers on chromosome 11, indicating retention of chromosome 11 (Fig. 8A and Supplementary Material, Table S3). Reasoning that retention of maternal chromosome 11 would lead to an alternative pathway of inactivation of a bona fide SDHD modifier gene, we analysed SLC22A18 and CDKN1C protein loss in all 60 tumours by IHC. Interestingly, four tumours with retention of chromosome 11 showed similarly reduced expression levels of SLC22A18 and CDKN1C compared to tumours showing loss of chromosome 11 (Fig. 8B and C).

To investigate whether somatic mutation in CDKN1C or SLC22A18 might underlie protein loss, we analysed all exons of both genes by Sanger sequencing. While no variants were found in CDKN1C, a missense variant was found in the coding region of SLC22A18, c.65G > A (p.Arg22Gln) in tumours and in normal matched DNAs in two cases. However, as this variant was frequent in a large population database (http://exac.broadinstitute.org), this variant is unlikely to explain CDKN1C or SLC22A18 inactivation in this specific group of SDHD mutant PGLs/PCCs.

Discussion

Our goal in this study was to identify genes that, upon knockdown together with SDHD, would enhance cellular characteristics previously associated with paraganglioma/pheochromocytoma (PGL/PCC). The Hensen model postulates that tumour formation in SDHD-linked PGL/PCC occurs upon loss of the SDHD wild type gene together with a maternally-expressed tumour modifier gene probably located in the 11p15 region (17). Our evaluation of protein expression in the chief cell component of SDHD-mutated tumours showed that KCNQ1 and PHLDA2 were expressed and thus excluded as candidates, whereas CDKN1C, SLC22A18 and ZNF215 all showed loss of protein expression consistent with the Hensen model. The protein expression of the candidate genes H19 (noncoding RNA) and KCNQ1DN (noncoding RNA) could not be explored for obvious reasons, or due to lack of reliable antibodies (OSBPL5).

Using two distinct neural-derived cell lines, we then developed stable single and double knockdowns of SDHD in combination with the candidate genes OSBP5, CDKN1C, SLC22A18 and ZNF215. Consistent with earlier reports (11,23,24), we showed...
knockdown of SDHD results in a disturbed metabolism indicated by changed levels of TCA cycle metabolites and ATP in cells, and by differential gene expression of components of the oxidative response and TCA cycle. We anticipated that SDHD gene knockdown together with the knockdown of the relevant 11p15 tumour modifier gene would enhance PGL-related cellular characteristics compared to SDHD knockdown alone. Indeed, additional knockdown produced small but significant increases in cell proliferation and apoptosis resistance. Although relatively modest enhancements, similar changes found in benign, slow-growing SDHD mutant PGLs are also small. Large changes would in fact be intrinsically suspect. Most importantly, comparative analysis of gene expression confirmed these broader functional differences by showing decreased levels of apoptosis and increased cell proliferation compared to single knockdown.

Results from the cell line-based functional assays were further supported by the finding that SDHD mutant tumours with either retention or loss of chromosome 11 showed equally low levels of SLC22A18 and CDKN1C protein expression. SDHx mutations are associated with DNA hypermethylation and histone methylation (25), suggesting a possible mechanism underlying the lowered expression of SLC22A18 and/or CDKN1C in SDHD mutant tumours with retention of chromosome 11.

A limitation of our in vitro work is that all observations were made with tumour cell lines that have already acquired genetic changes that allow them to tumourigenic growth properties. It is therefore remarkable that the knockdown of SDHD with or without a concomitant knockdown of 11p15 candidate genes was nonetheless capable of causing additional cellular phenotypes resembling those found in primary PGLs (22). While it would have been more appropriate to perform knockdowns in normal carotid body cells, these are currently unavailable as in vitro cell lines. Likewise, reintroduction of SDHD, SLC22A18, or CDKN1C into a PGL tumour cell line to revert the phenotype wasn’t possible for the same reason.

The concept that SDHD knockdown (or knockout) alone is insufficient to trigger tumourigenesis in the carotid body is supported by work carried out in genetically engineered mice. No engineered mouse germline knockout of Sdhb, Sdhc, or Sdhd has developed tumours to date (26–28), and conditional tissue-specific homozygous knockout leads only to severe apoptotic loss of neuronal and chromaffin cells and early death of newborn mice (29). Starting from what we understand of SDHD PGLs in man - the almost complete resistance to tumour development of carriers of maternally-inherited, and the loss of entire maternal chromosome 11 – we would argue that the loss of mitochondrial complex II activity in chromaffin cells can only be tolerated on a background of other genetic changes that allows them to overcome cellular lethality. Simultaneous loss of SDHD and SLC22A8 and/or CDKN1C may create a favourable genetic landscape via a single genetic event, whole chromosome loss of chromosome 11 (30).

Loss-of-function mutations in CDKN1C are associated with Beckwith-Wiedemann syndrome, an overgrowth disorder related to disruption of imprinted expression of 11p15 (31). CDKN1C, encoding the cyclin-dependent kinase inhibitor 1C, inhibits cell cycle...
progression and may therefore lead to increased cell proliferation when lost in SDHD mutant PGLs. SLC22A18 is a member of a family of polyspecific transporters and multidrug resistance genes and has been reported to be a tumour suppressor candidate and a substrate for RING105, a conserved E3 ubiquitin ligase (32). Genetic mutations in SLC22A18 are rare, with isolated reports of point mutations in a breast cancer cell line (33), a rhabdomyosarcoma cell line (34), and Wilms’ tumours and lung tumours (35,36). In glioma cells, SLC22A18 has a pro-apoptotic function and confers drug resistance (37) and more recently, downregulation of SLC22A18 in colorectal cancer cell lines has been shown to lead to slower growth by inducing G2/M arrest (38), supporting a role for SLC22A18 as a tumour suppressor in certain cell types. Our results showed that the combined loss of SDHD and SLC22A18 leads to apoptosis resistance and may, in combination with the increased cell proliferation, result in tumour formation in SDHD mutant PGLs. Future studies should address this mechanism, together with the triple knockdown of these genes to assess possible synergistic interactions.

In conclusion, this study has identified two credible candidate 11p15 tumour modifier genes that may be involved in SDHD-linked PGL/PCC, and provides further insight into the consequences of SDHD knockdown in cells with a neuronal background.

Materials and Methods
Selection of cell lines
As no human SDH-related PGL tumour cell line is currently available, we selected developmentally similar neural crest-derived cell lines. Cell lines carrying somatic mutations in PGL/PCC-linked susceptibility genes, including VHL and MAX mutations, were excluded based on information from the Catalogue of Somatic Mutations in Cancer (COSMIC) database. SNB19 (glioblastoma) and SHSY5Y (neuroblastoma) cell lines were selected based on a karyotype that demonstrated two copies of chromosome 11 (Supplementary Material, Fig. S6). SHSY5Y cells were heterozygous for chromosome 11 as detected by microsatellite markers, while SNB19 cells were homozygous for chromosome 11 (Supplementary Material, Fig. S7A). To establish the parental origin of chromosome 11 in the cells, we determined the methylation status of the two imprinted domains at 11p15.5 [H19-differentially methylated region (DMR) and KvDMR]. When both parental copies of chromosome 11 are present, the H19-DMR/KvDMR methylation rate ratio should be around one (39). SHSY5Y cells showed an average methylation rate of 0.75 ± 0.08 for H19-DMR and 0.65 ± 0.1 for KvDMR, resulting in a ratio of 1.1

Figure 5. Increased cell proliferation in cells with knockdown of SDHD and CDKN1C or SLC22A18. (A) SHSY5Y cells with stable knockdown of SDHD demonstrate a reduced cell proliferation compared to scrambled control cells, measured in real-time using xCelligence. (B) Using propidium iodide and FACS analysis, DNA content histograms showed a decreased S phase fraction and increased G0/G1 fraction in SDHD knockdown cells compared to scrambled cells. The acquired FACS data were analysed by ModFit LT software (Verity Software House, Inc.). (C) Using xCelligence, SHSY5Y cells with stable knockdown of SDHD and CDKN1C or (D) SLC22A18 show increased proliferation compared to single knockdown of SDHD. (E) No differences in cell proliferation were observed when comparing single knockdown of SDHD to combined knockdown of SDHD and OSBPL5 or (F) SDHD and ZNF215.
The average methylation rate for H19-DMR in SNB19 cells was 0.16 ± 0.10, while the average methylation rate for KvDMR was 0.005 ± 0.03, suggesting loss of imprinting. Nonetheless, the clear RNA expression of H19 (expressed from the maternal allele) and absence of expression of IGF2 (expressed from the paternal allele) indicated that chromosome 11 in SNB19 cells shows a maternal expression pattern. Both cell lines were therefore considered suitable.

Patients and samples

A tissue microarray (25) comprising 100 PGL and 17 PCC paraffin-embedded specimens yielded 5 micrometer sections for immunohistochemistry (IHC). Related patient clinical and (Supplementary Material, Fig. S7B). The average methylation rate for H19-DMR in SNB19 cells was 0.1 ± 0.1, while the average methylation rate for KvDMR 0.005 ± 0.03, suggesting loss of imprinting. Nonetheless, the clear RNA expression of H19 (expressed from the maternal allele) and absence of expression of IGF2 (expressed from the paternal allele) indicated that chromosome 11 in SNB19 cells shows a maternal expression pattern. Both cell lines were therefore considered suitable.

Cell culture

SNB19 cells and HEK293 cells were obtained from DSMZ (ACC 305 and ACC 325, Braunschweig, Germany) and cultured in Dulbecco’s Modified Eagle Medium (DMEM, Life Technologies, Paisley, UK) supplemented with 10% fetal bovine serum and penicillin/streptomycin (Life Technologies). SHSY5Y cells were obtained from European Collection of Cell Cultures via Sigma Aldrich (St. Louis, USA, Catalogue no. 86012802). SHSY5Y cells were cultured in DMEM-F12 (Life Technologies, Paisley, UK), supplemented with 15% fetal bovine serum and penicillin/streptomycin, and maintained at 37°C in a humidified atmosphere of 5% CO2 in air.

Patients and samples

A tissue microarray (25) comprising 100 PGL and 17 PCC paraffin-embedded specimens yielded 5 micrometer sections for immunohistochemistry (IHC). Related patient clinical and
genetic data for tumours included in the study is provided in the Supplementary Material, Table S4. All samples were handled according to the Dutch Code for Proper Secondary Use of Human Materials approved by the Dutch Society of Pathology (www.federa.org). SDHD mutant FFPE samples were used for DNA extraction. In addition, we included eight fresh frozen SDHD tumour samples and paired blood samples for DNA extraction. The samples were handled in a coded (pseudonymised) fashion according to procedures agreed with the LUMC ethical board (P12.082).

LOH analysis by microsatellite genotyping

DNA from SNB19 and SHSY5Y cells was isolated using the Wizard Genomic DNA purification kit (Promega, Fitchburg, USA) according to the manufacturer’s instructions. Representative tumour areas from FFPE samples were selected to punch 3 cores of 0.6 mm in diameter for DNA isolation. FFPE and fresh frozen tumour sections were incubated overnight with proteinase K at 60°C and DNA was isolated using the Qiagen FFPE DNA kit or QIAamp DNA Mini Kit (Qiagen Benelux B.V., Venlo, The Netherlands), respectively, following manufacturer’s instructions. All DNA samples were genotyped for microsatellite markers located on chromosome 11 (primer sequences available upon request). For each marker, 30 nanograms of DNA was amplified over 40 cycles using FastStar Taq DNA Polymerase (Roche). Forward primers were labelled with a 6-FAM, HEX or NED fluorophore (Sigma-Aldrich, St. Louis, MO, USA). Amplicons of microsatellite markers were run on an ABI 3730 genetic analyzer and data were analysed using Gene Marker software (Soft Genetics, State College, PA 16803, USA), with ABI GeneScan Rox 400 as the internal size standards. LOH of markers in tumour samples was calculated using the allelic imbalance ratio: \[ \text{AIR} = \frac{\text{Tumour1/Tumour2}}{\text{Normal1/Normal2}}. \]

Tumours were regarded as positive for LOH when the mean allele ratio between tumour and blood was <0.7 for all informative markers, as described earlier (19). In cases where no matching blood DNA sample was available, allele peak ratios were compared to DNA samples with the same or very similar allele combinations. Some markers were either not informative in the patient or did not perform well enough with tumour DNA to give a reliable result and were therefore excluded.

Karyotyping

Conventional cytogenetic analysis on GTG-banded chromosomes from cultured SNB19, HEK293 and SHSY5Y cells was performed according to standard techniques. Briefly, 17 h before
harvesting the cells, 200 μl FdU (5 μM) was added to the cells. Then, the cells were incubated with 200 μl BrdU (14 mg/ml) for another 5–6 h. Finally, colcemid was added 15 min before harvesting and metaphase spreads were prepared according to standard protocols.

Bisulphite-modified PCR and sequencing

DNA (300 ng) from SNB19 and SHSY5Y cells was bisulphite-treated using the EZ DNA methylation kit (Zymo research, Irvine, USA). Bisulphite-treated DNA was then amplified by PCR using primers specific for modified DNA designed with Meth primer (40). Primer sequences for H19 were 5'-GGTTT TAGTGTGAAATTTTTT-3' (forward) and 5'-CCATAAAATATCCT ATTCACAAATAAC-3' (reverse), and 5'-TTAGGGAGTTTTTT GGAGGGT-3' (forward) 5'-ACCC AACCAATACCTCATAC-3' (reverse) for KvDMR1. The PCR program consisted of an initial denaturation step at 94 °C for 15 min followed by 44 cycles of 20 s at 94 °C, 30 s at 55 °C for KvDMR1 and 52.5 °C for H19, followed by 5 min at 72 °C. Sanger sequencing of PCR products was performed using standard protocols, and methylation rates were evaluated using ESME software (41).

CDKN1C and SLC22A18 mutation analysis

All exons of the CDKN1C and SLC22A18 genes were amplified by PCR (primer sequences available on request). Twenty nanograms of genomic DNA and matched normal DNA from 6 SDHD-linked patients were amplified and primer annealing was performed at 58 °C. PCR fragments were purified using the Nucleospin gel and PCR clean-up kit (Macherey-Nagel). Sequencing was performed using standard protocols and data were analysed using Mutation Surveyor software (Softgenetics).

Tissue samples and immunohistochemistry

FFPE tissue samples used for IHC were as described in ‘patients and samples’. As control tissue, whole sections of four normal carotid bodies were included, obtained from anonymous patients at autopsy within 24 h after death. We reviewed the
histological appearance of all samples (JVMGB, JP8, ASH) and confirmed diagnoses by routine IHC staining for S-100 (detecting sustentacular cells) and chromogranin A (detecting chief cells). Mutation detection was confirmed by routine SDHA and SDHB immunohistochemical staining, as described previously (30).

Primary antibodies for IHC analysis were used as follows: Rabbit polyclonal antibody for detection of SLC2A18 (1:200, Proteintech), KCNQ1 (1:100, Sigma Aldrich), PHLD2A (1:200, Abcam), CDKN1C (1:1600, Sigma Aldrich), ZNF215 (1:200, Sigma Aldrich). Placenta and liver were used as a positive control. After antigen retrieval by exposure to microwave heating in citrate buffer, pH 6.0 at 100 °C for 10 min, sections were blocked for 30 min with 10% goat serum and incubated overnight at 4 °C with primary antibodies. Signal detection was performed with Envision+ (DAKO, Agilent Technologies, Belgium) and the chromogen 3,3′-diaminobenzidine according to manufacturer’s instructions. The results of the immunohistochemical labeling were scored semi-quantitatively: the intensity of labeling was assessed on a scale of 0 to 3 (0 = none; 1 = weak; 2 = moderate; 3 = strong), and the percentage of positive cells was assessed on a scale of 0 to 4 (0 = 0% positive; 1 = 1–24% positive; 2 = 25–49% positive; 3 = 50–74%; 4 = 75–100% positive cells). The two scores were then added to reach a total sum score ranging from 0 to 7. The scoring was performed independently by two observers blinded for clinicopathological data (ASH and JVMGB) and discrepancies were discussed. Photos of IHC sections were obtained using a Leica DFC550 camera and the Leica Application Suite, software version 4.5 (Heerbrugg, Switzerland).

Gene knockdown
To create stable cell lines with a single or double knockdown of genes, four validated MISSION® shRNA constructs and one non-validated MISSION® shRNA construct (TRCN0000231553 -237878, -147951, -344525 and -13054) targeting human SDHD (NM_003002.1), CDKN1C (NM_000076), OSTEPL5 (NM_020896), SLC2A18 (NM_002555) and ZNF215 (NM_013250), respectively (Sigma Aldrich, St. Louis, USA) or a scrambled shRNA encoding plasmid (SHC002 Sigma Aldrich) were used to produce infectious virus particles. To evaluate the transduction efficiency, the MISSION TurboGFP control plasmid (SHC003 Sigma Aldrich) was used. HEK293T cells were transfected with the shRNA constructs together with helper plasmids encoding HIV-1 gag-pol, HIV-1 rev, and the VSV-G envelope as described (42). Viral supernatants were added to SNB19, HEK293 and SHSY5Y cells in fresh medium supplemented with 8 µg/ml Polybrene (Sigma Aldrich) and the cells were incubated overnight. The next day, the medium was replaced with fresh medium. The selection was carried out using 2 µg/ml puromycin. Transduction efficiency was analysed 3 to 6 days post transduction. Experiments were performed 2–3 and 4–5 weeks after transduction of cells with shRNAs.

RT-PCR analysis
Total RNA from cells was isolated using the Nucleospin RNA II kit (Macherey-Nagel, Düren, Germany) according to manufacturer’s instructions. cDNA was synthesized from 1 µg RNA using the Omniscript RT kit (Qiagen, Venlo, Netherlands). Gene expression was determined using quantitative PCR and was measured in triplicate on the CFX96 Real-Time System (Bio-Rad, USA) using the iQ SYBR Green Supermix (Biorad, California, USA). The relative quantification of target mRNA was performed by the 2-ΔΔCT method (43). Results from the housekeeping genes HNRMP, TBP and HPRT were used as references. Target genes were SDHD, CDKN1C, SLC2A18, OSTEPL5, ZNF215, GLUT1, EGLN3, BNIP3, ENO1 and VEGF (Supplementary Material, Table S5).

Western blotting
Total protein was isolated using RIPA buffer (Sigma Aldrich) supplemented with “complete” protease inhibitor cocktail (Roche, Germany). The concentration of protein was determined by bicinchoninic acid protein assay (Thermo Scientific Pierce, Rockford, USA). Equal amounts of protein (35 µg) were separated by SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore). After blocking with 5% (w/v) non-fat milk powder, membranes were incubated overnight at 4 °C with the following antibodies: SDHB (Sigma Aldrich) and HIF-1α (Novus Biologicals, Littleton, USA) in a dilution of 1:500 in blocking buffer (Rockland, Gilbertsville, USA). α-tubulin was used as a loading control (1:2000, Sigma Aldrich). Visualization and quantification was carried out using the LI-COR Odyssey® scanner (Bad Homburg, Germany) and software (LI-COR Biosciences).

Microarray expression analysis
Quality control, RNA labelling, hybridization and data extraction were performed at ServiceXS B.V. (Leiden, The Netherlands). RNA concentrations were measured using the Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). The RNA quality and integrity was determined using Lab-on-a-Chip analysis on the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). Biotinylated cRNA was prepared using the Illumina TotalPrep RNA Amplification Kit (Ambion, Inc., Austin, TX, USA) according to the manufacturer’s specifications with an input of 200 ng total RNA. Per sample, 750 ng of the obtained biotinylated cRNA samples was hybridized to the Illumina HumanHT-12 v4 BeadChip (Illumina, Inc., San Diego, CA, USA). Hybridization and washing were performed according to the Illumina Manual “Direct Hybridization Assay Guide”. Scanning was performed on the Illumina iScan (Illumina, Inc., San Diego, CA, USA). Image analysis and extraction of raw expression data were performed with Illumina GenomeStudio v2011.1 Gene Expression software.

Bioinformatic analysis
Normalization and quality control were performed using the Bioconductor ‘lumi’ package of R (lumi) (44). Samples were clustered using an unsupervised hierarchical clustering method to delineate groups with biological distinction. The R package ‘Linear Models for Microarray Data’ (LIMMA) was used for the assessment of differential expression of individual genes between the different subgroups (45). Overall gene expression differences between scrambled control and SDHD knockdown, scrambled control and SDHD + CDKN1C knockdown or scrambled control and SDHD + SLC2A18 knockdown subgroups in SNB19 and SHSY5Y cells were evaluated with the ‘global test’ designed by J.J. Goeman using the R package ‘global test’ available on Bioconductor (46). We applied the global test to evaluate subtle differences between the different subgroups, as this test has greater power to detect gene sets with small effect sizes (46,47). We performed a pathway-based analysis using the global test on pathways described in the publicly available pathway database Kyoto Encyclopedia of Genes and Genomes (KEGG) annotations (48). KEGG pathway analysis of scrambled
control HEK293 cells versus SDHD knockdown was not consistent with tumour gene expression profiles of PGL with SDH gene mutations (12,49) and was therefore excluded as a relevant model. All tests, both for genes and pathways, were corrected for multiple testing based on the false discovery rate (FDR) criterion, using the Benjamini and Hochberg method (50). Comparative analysis and functional categorization of the different subgroups was performed with Ingenuity Pathway Analysis (IPA; www.ingenity.com). All data are available at the GEO database (GSE80968).

Cellular DNA content and flow cytometry

Using the Vindelov technique (51), DNA staining was performed as follows: Cells were centrifuged (500g, 5 min) and washed in PBS, then 300 µl of solution A containing trypsin (0.3 g/l, Sigma) at pH 7.6 was added and incubated for 2 h at 37 °C. Next, 225 µl of solution B containing RNase (0.5g/L, Sigma) and a trypsin inhibitor (0.1 g/l Sigma) was added, followed by a 10 min incubation at room temperature (RT). Finally, a third incubation at RT was carried out for at least 15 min after the addition of 225 µl of propidium iodide (PI) (0.42g/l, Sigma) (solution C). Samples were measured using an LSRII (BD Biosciences, Erembodegem, Belgium) flow cytometer. Detector D (BP610/20 nm) was used to collect PI fluorescence. The WinList 8.0 and ModFit 4.0.1 software packages (Verity Software House, Inc., Topsham, ME) were used for data analysis.

xCelligence

The RTCA xCelligence system (Roche Applied Sciences, Almere, the Netherlands), based on cell electrode substrate impedance detection technology, was used for proliferation assays. Cell lines were plated at a density of 10.000 cells per well in a 16-well E-Plate. The plates were loaded into the RTCA station in the cell culture incubator immediately after plating and cell index was acquired every 30 min. Experiments were performed in triplicate.

Assessment of apoptosis

SNB19 and SHSY5Y cells were stimulated with 2, 4 or 8 µM staurosporine (Sigma) or with 10, 20 or 40 µM cisplatin (Sigma). The ApoLive-Glo multiplex Assay (Promega, Madison, USA) was used to measure cell viability and apoptosis in the same sample following the manufacturer’s protocol. Briefly, the viability is measured by the activity of a protease marker of cell viability. Apoptosis is measured by the addition of a luminogenic caspase-3/7 substrate (Caspace-Glo 3/7) which is cleaved in apoptotic cells to produce a luminescent signal. Fluorescence at 400 Ex/505 Em (viability) and luminescence (apoptosis) were measured with a Victor 3 machine (PerkinElmer, Massachusetts, USA).

Nuclear fragmentation was determined in SNB19 and SHSY5Y cells stimulated with 4 µM staurosporine. Cells were fixed with 4% paraformaldehyde for 15 min. Then, the cells were washed three times in PBS and stained with 50 µg/ml 4,6-diamidino-2-phenylindole-2-HCl (DAPI; Sigma) in Vectashield mounting medium under a cover slip. Images of fixed cells were acquired on a Zeiss Axio Imager M2 fluorescence microscope equipped with an HXP 120 metal-halide lamp used for excitation. Fluorescent probes were detected using the following filters: DAPI (excitation filter: 350/50 nm) and GFP (excitation filter: 470/40 nm). Images were recorded using ZEN 2012 software.

TCA cycle metabolite quantification by LC-MS/MS

Sample preparation for biochemical analysis of SNB19 and SHSY5Y cells was performed according to (52), using ice cold 90% MeOH: CHCl₃ as extraction solvent containing ¹³C-labelled isotopes of nucleotides as internal standards. Dried samples were reconstituted in 100 µl H₂O for compatibility with the liquid chromatography-tandem mass spectrometry (LC-MS/MS) method (53). The concentrations of citric acid, α-ketoglutarate, succinate, fumarate and malate were determined by anion-exchange LC-MS/MS. The concentrations of AMP, ADP and ATP were determined by ion-pair reverse-phase LC-MS/MS (54).

Statistical analysis

IBM SPSS Statistics 20.0 for Windows software package (SPSS, Armonk, NY: IBM Corp) was used to analyse the results. The statistical significance of differences between the two groups was assessed by the Mann-Whitney U test, and the 1-way analysis of variance test was used for comparisons of more than two groups. P < 0.05 was considered statistically significant.

Supplementary Material

Supplementary Material is available at HMG online.

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