The complex approach to pheochromocytoma's and paraganglioma's genetics and tumour biology

PhD thesis

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INTRODUCTION

Pheochromocytomas and paragangliomas (Pheo/PGL) are rare, chromaffin cell derived tumours. More than 40% of these tumours occur due to an inherited mutation in one of the 17 documented Pheo/PGL genes. The diverse genetic background poses a great challenge in research and in the routine clinical practise as well. Moreover, there is no specific prognostic and therapeutic factor for the rarely occurring malignant phenotype of these tumours.

AIMS

1. Genetics

Our goal was to develop a next generation sequencing panel for the accelerated and cost effective assessment of inherited endocrine tumour syndromes. Moreover, we present the evaluation of the phenotype modifier effect of two different hereditary syndromes in a unique case of a patient who carried two distinct germline mutations.

2. Tumour biology

We aimed to validate novel prognostic and therapeutic factors for Pheo/PGL and to develop an *in vitro* model for *SDHB* mutation associated malignant Pheo/PGL.

METHODS

1. Genetic testing of the patients

After genetic counselling and obtaining informed consent all patients of the Endogene Panel 1.0 validation cohort underwent genetic testing for the *RET, VHL, SDHB, SDHC, SDHD, MAX* and *TMEM127* genes, whereas the patient and her family presented in our case report underwent the genetic testing of *RET*, *BRCA1* and *BRCA2* genes using conventional methods including PCR followed by Sanger sequencing. Blood DNA was extracted using commercially available DNA extraction kits. Bidirectional DNA sequencing of all these genes and large deletion analysis of the *VHL, SDHB, SDHC,* and *SDHD* and *TMEM127* genes were performed using multiplex ligation probe amplification. All patients gave informed consent and our study was authorized by ETT-TUKEB (ETT-TUKEB 4457/2012/EKU).

2. Assembly of the Endogene Panel

In the first version of Endogene Panel (Endogene Panel 1.0) the covered genes included the *EGLN1*, *EPAS1*, *FH*, *KIF1B*, *MAX*, *MEN1*, *NF1*, *RET*, *SDHA*, *SDHAF2, SDHB*, *SDHC*,

SDHD, *TMEM127* and *VHL* genes. During the development of the panel novel Pheo/PGL were introduced in the literature, therefore the second version of the Endogene Panel (Endogene Panel 2.0) the *GOT2*, *MDH2* and *SLC25A11* genes were added to the panel. For targeted library preparation we used a hybridization based Roche NimbleGene SeqCap technology was used. This technique enables a more specific library preparation compared to PCR based techniques. Probes were designed for every exon of each gene, therefore a >98% coverage was achieved. Beside the exons, the 30 nucleotide long splice regions could be assessed as well with these probes.

The micro format of the MiSeq Reagent kit was used for Endogene Panel 2.0, whereas the nano format was used for Endogene Panel 2.0. The sequencing was carried out on Illumina MiSeq sequencing device.

3.Variants assessment

The genome data generated by the sequencing was assessed with GATK (Genome Analysis Toolkit) Best Practices. The adapter sequences were removed with Cutadapt software. The raw FASTQ format data was aligned to the UCSC hg19 human reference genome with BWA algorithm. The reads below quality score 20 were removed with Samtools. PCR duplicates were removed with Picard MarkDuplicates (http://broadinstitute.github.io/picard) software. The indel realignment and the recalibration of the quality score was carried out with GATK software. The categorization of the variants was carried out with online available SNPEFFECT, SIFT, ClinVar, Varsome and PolyPhen applications. The results of the Endogene panel 2.0 was analysed again with the GATK FUNCTATOR algorithm.

The prevalence and the clinical impact of the variants was assessed with dbSNP, the American Exome Project Variants Server (National Heart, Lung, and Blood Institute Exome Sequencing Project Exome Variant Server (http://evs.gs.washington.edu/EVS/)), Hapmap, ClinVar, Varsome and 1000Genomes databases.
All the variants went under the same bioinformatics filtering during what the variants outside the exome (UTR), synonymous and low-read count (<10 read/variant) variants were removed.

4. Cell lines

All cell lines were obtained from American Type Culture Collection (ATCC). Cell cultures were incubated at 37°C in a humidified 5% CO_2/95% air atmosphere.

PC12 cells (rat pheochromocytoma cell line) were grown in 75-cm^2 flasks in F-12 Kaigh's modification, containing 15% horse serum, 5% foetal bovine serum and 1% penicillin-streptomycin. Culture media was replaced three times a week. Cells were removed from flasks for subculture and for plating into assay dishes using Trypsin-EDTA solution.

HeLa cells (human cervix carcinoma cell line) were grown in 75-cm^2 flasks in Dulbecco's modified Eagle medium/HamF12 containing 10% FBS and 1% penicillin-streptomycin. Culturing was performed as it is described above in case of PC12 cells.

H295R cells (human adrenocortical carcinoma) were grown in 75-cm^2 flasks in Dulbecco's modified Eagle medium/HamF12 containing HEPES buffer, L-glutamine and pyridoxine HCl. Additional supplements were added to the medium, including 0.00625 mg/ml insulin, 0,00625 mg/ml human transferrin and 6.25 ng/ml selenous acid 1.25 mg/ml bovine serum albumin, 2.5% nu-serum and 1% penicillin-streptomycin. Culturing was performed as it is described above in case of PC12 cells.

5. *Sdhb* gene silencing

PC12 cells were seeded in 6-well plates for 24 hours before transfection with two Silencer Select small interfering RNAs (siRNA A: Sequence (5'→3': GAUUAAGAAUGAAAUCHAUtt, siRNA ID: #s151576; siRNA B: Sequence (5'→3': GCAAAGUCUCGAAAAUAUAtt, siRNA ID: #s220846) targeting SDHB using RNAiMAX Reagent according to the manufacturer's protocol. For negative control, cells cultured under identical conditions were transfected with non-targeting Silencer Select siRNA. Specific effect of siRNA against *Sdhb* was verified by western blot analysis.

6. Protein extraction and Western Blot

Total protein was extracted with M-Per reagent, according to the manufacturer's instructions. Protein concentrations were determined by BCA Assay. Total protein was separated by 10-15% SDS polyacrylamide gel electrophoresis, transferred to a PVDF membrane and incubated overnight with primary antibody against SDHB (5ug/mL). Spectra Multicolor Broad Range Protein Ladder was used as a protein ladder. For loading control membranes were stripped and re-probed using mouse anti-β-actin (1:25000). Anti-mouse HRP-conjugated

IgG was used as secondary antibody (1:2000). Band intensities were quantified using Image J software.

7. Biochemical inhibition of SDH enzyme

500 mM stock solutions of itaconate were prepared with nuclease free water; pH 7.2 was adjusted with NaOH. Cells were seeded onto 6-well plates. After 24 hours incubation, the used medium was replaced by fresh medium, after washing with PBS. 25 mM itaconic acid was added in the wells. Nuclease free water was used as control.

2mM stock solution of atpenin A5 was prepared with absolute ethanol. Cells were seeded onto 6-well plates. After 24 hour incubation, the used medium was replaced by fresh medium, after washing with PBS. 1 μM itaconic acid was added in the wells. Absolute ethanol in the same treatment volume was used as control.

8. Metabolite concentrations

Cells were grown in 6-well plates. All experiments (treatment with itaconic acid and siRNA transfection) were made in 3 replicates except for 24h itaconic acid treatment of PC12 cells, where 9 biological replicates were carried out.

All samples were measured in triplicates. Concentrations of metabolites were normalized to DNA concentration isolated from cells plated, incubated and treated in the same manner as cells used for metabolite analysis. The cells were trypsinized and DNA was extracted using semiautomatic DNA isolation protocol with QIAcube instrument. The concentration of the extracted DNA samples was measured with NanoDrop 1000 Spectrophotometer. The normalized concentrations were multiplied by 1000, therefore the final concentration was μmol/μg. Intracellular metabolites (lactate, pyruvate, citrate, α-ketoglutarate, succinate, fumarate, malate, glutamate, aspartate) were measured. In brief, the cells were quenched in liquid nitrogen and extracted by mixture of MeOH–chloroform–H_2O (9:1:1) and vortexed at 4 °C. After centrifugation (15,000xg, 10 min, 4 °C) the clear supernatants were kept at −80 °C until liquid chromatography-mass spectrometry (LC-MS) measurements. The concentrations of lactate, citrate, succinate, fumarate, malate, glutamate and aspartate were assessed by using calibration curves obtained with the dilution of analytical grade standards in the range of 0.5-50 μM. LC-MS assays were used by Perkin-Elmer Flexar FX10 ultra-performance liquid chromatograph coupled with a Sciex 5500 QTRAP mass spectrometer. Chromatographic separation was carried out on a Phenomenex Luna Omega C18 column (100×2.1 mm, 1.6

μm). The mobile phase consisted of water and methanol containing 0.1% (v/v) formic acid. The MS was operating in negative electrospray ionization mode. For the measurements the following settings were adjusted – source temperature: 300°C ionisation voltage: -4000 V, entrance potential: -10 V, curtain gas: 35 psi, gas1: 35 psi, gas2: 35 psi, CAD gas: medium. Multiple reaction monitoring (MRM) mode was applied to perform quantitative analyses. All samples were measured in triplicates.

9. AlamarBlue – Viability assay

AlamarBlue test was used to determine the viability effects of itaconic acid and atpenin treatment after 24, 48 and 72 hours in PC12, HeLa and H295R cells and in PC12 cells after transfection of siRNA against *Sdhb* or mock siRNA and after co-treatment with BPTES. The assay was performed in 96-well plates. All treatments at each time point and siRNA transfections were performed in 6 replicates, outliers were excluded before the statistical analysis. For studying the viability changes with AlamarBlue assay, PC12 cells were plated in 100 μL cell culture media at a density of 5000 cells/well for 24h treatment; 2500 cells/well for 48 h treatment and 1700 cells/well for 72-hour treatment. HeLa cells were plated in 100 μL cell culture media at a density of 3000 cells/well for 24-hour treatment; 1500 cells/well for 48 hour treatment; 1000 cells/well for 72 hour treatment. H295R cells were plated onto 96-well culture plates in 100uL cell culture media at a density of 10000 cells/well for 24-hour treatment; 5000 cells/well for 48-hour treatment; 3500 cells/well for 72 hour treatment. After 24 hours, cell media was replaced by fresh media, and itaconate, atpenin or siRNA against *Sdhb* treatment was performed. After the given incubation time, 10 μL AlamarBlue, was added to each well. After 1 hour and 15 minutes incubation at 37°C, fluorescence was measured in the 560-590 nm range using Varioskan Flash plate reader.

10. Cell proliferation assay

Sulforhodamine B (SRB) assay was used for evaluation of proliferation of PC12 cells. The cells were seeded onto 96-well plates at a density of 2500 cells/well. Each measurement was performed six replicates. After incubation with the indicated drug concentrations for 24/48/72 h, cells were fixed by cold 10% trichloroacetic acid for 60 min in 4°C, washed with water and dried. After drying, cells were incubated with 0.4% sulforhodamine B for 15 min in room teperature. After washing with 1% acetic acid, the protein-bound dye was dissolved in 10 mM

Tris. The absorbance at 570 nm was measured in LabSystems Multiskan RC/MS/EX Microplate Reader.

11. Cellular respiration

Seahorse XF96 Analyser (Agilent Technologies, USA) was used to assess real-time oxygen consumption rate (OCR), reflecting mitochondrial oxidation and extracellular acidification rate (ECAR), based on previous descriptions. PC12 cells were plated in 100 µL complete medium at 30.000 cells/well density onto 96-well Seahorse plates (Agilent Technologies, USA) 24h prior to the assays. Itaconate (25 mM) or atpenin (1 uM) treatment was carried out 24h or 48h before the assays, whereas transfection with siRNA against *Sdhb* or mock vector 48h before the assays. BPTES treatment was carried out 24h prior to the assay. On the day of the assay complete medium was removed and was replaced by a medium containing (in mM): 120 NaCl, 3.5 KCl, 1.3 $CaCl_2$, 1.0 $MgCl_2$, 20 HEPES, 10 glucose at pH 7.4. The basal OCR and ECAR values were calculated after 1.5h incubation at this condition.

During the measurements freshly prepared glutamine (4 mM) and/or metabolic inhibitors/modulators (oligomycin 2µM, 2,4-dinitrophenol- DNP 200 µM and antimycin A + rotetone 1-1µM) were injected into each well to reach the desired final working concentration.

12.Statistical analysis

All data are expressed as mean ±SD except where it is indicated otherwise. Statistical analysis was performed using GraphPad Prism 6 software (GraphPad Software Inc., La Jolla California USA). Gaussian distribution of data was evaluated with Shapiro-Wilks test. In case of normally distributed data the differences were analysed by Student's t-test, otherwise by rank sum test. Correlation in case of normally distributed data was calculated with Pearson-test, otherwise Spearmen-test was used. *P* values of <0.05 were considered to be statistically significant.

RESULTS

1. The co-occurrence of germline *BRCA1* and *RET* mutations

A 16-year-old female patient was investigated for hypothyroidism due to hair loss. During the routine physical examination, two thyroid nodules were identified, therefore the patient was referred to the Endocrine Unit of the 2nd Department of Medicine, Semmelweis University for

further investigation in 2013. The thyroid and neck ultrasound, fine needle aspiration biopsy, CT scan, calcitonin and CEA serum levels pointed to the diagnosis of medullary thyroid carcinoma (MTC). Although the serum calcitonin and CEA levels significantly decreased after the surgery, complete biochemical remission has not been achieved since and the serum calcitonin levels has shown uncertain progression. Recurring tumour and other metastases were not confirmed during the postoperative follow-up.

After genetic counselling the patient underwent genetic testing of *RET* gene, and a pathogenic heterozygous mutation (p.Cys634Trp) was identified. The p.Cys634Trp is a well-known mutation associated with MEN2A syndrome therefore the patient underwent detailed clinical, laboratory and imaging studies for manifestations of MEN2A. Until the present day, the proband is still free of any MEN2A associated lesions.

Due to the fact that MEN2A syndrome is inherited in an autosomal dominant way, genetic counselling and genetic testing of first degree relatives is indicated. The detailed family history revealed that the patient's mother and her aunt had breast cancer and both died due to brain metastases. This unexpected clinical presentation was suspicious to hereditary breast cancer syndrome. However, no samples for mutation analysis of *BRCA1*/2 genes were available from these two affected relatives. Hereditary breast cancer also is an autosomal dominant disorder mutation, and thus, analysis is recommended for the first degree relatives. Accordingly, the mutation analysis of *BRCA1*/2 genes identified a pathogenic, heterozygous frameshift *BRCA1* mutation (*BRCA1* Ile90Serfs, NC_000017.10:g.41256905_41256917 delTGAAAAGCACAAA) in the proband. Clinical examination and breast magnetic resonance imaging (MRI) revealed no sign of malignancy of breast or ovarian cancer. After these striking results, the patient's family members underwent genetic counselling again and, after obtaining informed consent, underwent mutation testing for the *RET* exon 11 and *BRCA1* genes. The proband’s brother (age: 36 years) and his two sons (age: 4 and 6 years) were also heterozygotes for the *RET* p.C634W mutation but the *BRCA1* mutation was absent in them. In her brother two thyroid masses and elevated serum calcitonin level were observed. Total thyroidectomy was performed and his serum calcitonin returned to normal range. Two sons of the proband’s brother underwent prophylactic thyroidectomy and the histopathological examination of the removed tissues showed no signs of MTC. The proband’s father did not carry the *RET* mutation and the *BRCA1* mutation.

2. Endogene Panel

2.1. The development of Endogene Panel version 1.0

The hybridization based Endogene Panel 1.0 was capable of the simultaneous sequencing of *EGLN1*, *EPAS1*, *FH*, *KIF1B*, *MAX, MEN1*, *NF1*, *RET*, *SDHA*, *SDHB*, *SDHC*, *SDHD*, *SDHAF2*, *TMEM127* and *VHL* genes.

For the analytical validation, 17 patients with 12 verified pathogenic mutations (2 RET, 5 SDHB, 2 TMEM127, 2 MEN1 and 1 VHL) were used. All the previously detected pathogen variants were identified. After optimizing the bioinformatics pipeline and filtering, 68 false positive variants, 2 VUS (variant of unknown significance) and 25 benign polymorphisms were identified.

The Endogene Panel 1.0 was tested in the routine clinical setting on 24 samples which had no previous genetic diagnosis. Out of the 24 patients, 10 carried a total number of 11 pathogenic variants (1 *VHL*, 3 *SDHB*, 7 *NF1*). It is worth noting that in a patient the panel sequencing identified two different *SDHB* mutations which were verified with Sanger sequencing: beside a p.R90 frameshift mutation, an *SDHB* p.T88I variant was found as well. In another, young female patient a *VHL* mutation was associated with a single neck PGL.

2.2. Development of the Endogen Panel version 2.0.

With the experiences gained during the development of Endogene Panel we altered the library preparation by applying a different sequencing kit and 3 additional Pheo/PGL genes (*GOT2*, *MDH2* and *SLC25A11)* were added to the list of sequenced genes. The bioinformatics pipeline was improved as well in order to identify true positive variants in the regions with a relatively low coverage. The effectiveness of the developed Endogene Panel 2.0 was tested on 38 patients with no previous genetic diagnosis. Pathogen variants were identified in 10 patients (26%) in the *SDHB* (3 patients), *FH* (2 patients), *NF1* (4 patients) and *VHL* (1 patient) genes. A total number of 8 VUS calls were made as well. In 3 cases these were found alongside pathogenic mutations, but in 3 patients the pathogenic role of these variants has been anticipated as they are not represented in any of the used genetic databases. In the remaining two patients, variants were labelled as VUS due to their expected phenotype modifier effects.

3. Tumourbiology

3.1. SDH Impairment Had an Overall Positive Effect on Cell Viability Without Significant Changes in the Proliferation in PC12 Cells

Successful SDH inhibition with *Sdhb* gene silencing and itaconate treatment significantly increased the PC12 cell line's viability. Atpenin treatment yielded an increase in cell viability, but not at a significant level in the PC12 cell line Cell proliferation of PC12 cells measured with SRB assay was not affected by SDH impairment either with *Sdhb* knockdown or itaconate/atpenin treatment.

HeLa cells showed significant decrease in cell viability upon itaconate treatment, whereas atpenin treatment increased HeLa cells' viability. The H295R cell line showed an overall negative response to all SDH impairment methods.

3.2. Metabolite profiles

In all cell lines, after all Sdhb inhibition the succinate level were increased parallel with decreasing the fumarate concentration.

In case of PC12 cells, *Sdhb* silencing and itaconate treatment significantly decreased intracellular glutamate levels without lactate accumulation. On the other hand, the reduced intracellular glutamate concentration in case of atpenin treatment was accompanied with lactate accumulation.

Contrary to PC12 cells, HeLa and H295R cells both displayed glutamate and lactate accumulation upon itaconate treatment.

Atpenin treatment yielded an universal response in all cell lines as the decreased glutamate concentrations were accompanied by lactate accumulation.

3.3. GLS-1 expression correlates with SDH inhibition

Based on the metabolomics and cell viability measurements we sought to assess the importance of glutamine/glutamate metabolism, especially the mitochondrial uptake of glutamine by glutaminase-1 (GLS-1). A significant increase in GLS-1 expression after *Sdhb* knockout and itaconate treatment was observed in PC12 cells. On the contrary, GLS-1 expression decreased after atpenin treatment in PC12 cells.

HeLa cells expressed a significant increase in GLS-1 expression upon itaconate treatment. On the other hand, GLS-1 expression significantly decreased after 24 h atpenin treatment in HeLa cells, but significantly increased after 48 h.

H295R cells exhibited a significant increase in GLS-1 expression upon both itaconate and atpenin treatments.

3.4. Immunohistochemistry of SDHB and GLS-1 in Pheo/PGL Tissues Points to the Importance of GLS-1 Enzyme

Based on our in vitro findings we sought to evaluate the expression level of GLS-1 in Pheo/PGLs tumour tissues in order to assess whether GLS-1 expression might serve as a marker for malignancy in Pheo/PGLs.

Increased GLS-1 expression was detected in *SDHB*-mutant tumour tissues compared to *RET*-mutant and sporadic tumours, however the difference was not significant. A total of 54% (7 of 13) of the low SDHB expressing tumours showed high GLS-1 staining while only 22% (five of 22) of high SDHB expressing tumours showed high GLS-1 staining.

GLS-1 was overexpressed in three *RET*-mutant samples. Of these three samples, in two cases malignancy was proved as they were reoccurring, invasive, and metastatic Pheos of Patient No. 4 and Patient No. 5. The third GLS-1 overexpressing *RET*-mutant Pheo sample was obtained from a patient with MEN2A syndrome with bilateral Pheo (Patient No. 6, the GLS-1 positive sample was the Pheo removed from right side).

In the three malignant sporadic Pheo/PGL samples, two showed high SDHB staining scores. All malignant sporadic samples showed low or average GLS-1 immunostaining. In case of the benign sporadic samples, out of the four samples with low SDHB scores two were accompanied by high GLS-1 immunostaining scores.

3.5. GLS-1 Inhibition in PC12 Cells both in monolayer and 3D cell culture Decreased Proliferation after SDH Inhibition Measured by SRB Assay

To test whether SDH impaired PC12 cells' proliferation is dependent on GLS-1 activity, we assessed the proliferation of the cells after selective GLS-1 inhibition with BPTES (bis-2-(5-fenilacetamido-1,3,4-tiadiazol-2il)-etil sulfide) treatment. Proliferation of PC12 cells significantly decreased when SDH inhibition was accompanied with BPTES treatment regardless of inhibitory methods.

In order to assess GLS-1 inhibition in a more relevant in vitro model, we applied 3D culturing of PC12 cells by spheroid induction using spheroid inducing media. Itaconate treatment alone did not exert a significant effect on the ratio of living. When itaconate was

accompanied by BPTES treatment in the 3D cultured PC12 cells significant decreases were observed in the living cell ratios compared to vehicle treatment.

3.6. Oxygene consumption

After biochemical characterization of SDH inhibition we assessed the mitochondrial respiration upon SDH inhibition using SeaHorse measurements in PC12 cells. BPTES treated control cells yielded the lowest basal oxygen consumption ratio (OCR), whereas itaconate the highest. Compared to control PC12 cells, itaconate yielded a significantly higher basal respiration whereas *Sdhb* knockdown resulted in significantly lower basal respiration rate compared to mock transfected cells. BPTES treatment of cells transfected with siRNA against *Sdhb* or mock transfection did not result in a significant difference in OCR values.

Basal respiration was then evaluated after administration of 2mM glutamine. Only minor changes were observed in itaconate, atpenin treated and *Sdhb* silenced cells. BPTES treatment only had a significant effect on *Sdhb* silenced cells when their basal oxygen consumption was compared to the OCR after glutamine admission. Similarly, a significant difference was observed when the OCR of *Sdhb* silenced cells were compared to mock transfected cells after glutamine admission. BPTES treated *Sdhb* silenced and mock transfected cells' OCRs after glutamine admission did not differ significantly.

Itaconate treatment and si*Sdhb* knockdown significantly increased maximal respiration. BPTES treatment significantly reduced the maximal OCR of both control (p = 0.0079) and si*Sdhb* silenced PC12 cells.

PC12 cells transfected with siRNA against *Sdhb* had the highest non-mitochondrial respiration which did not decrease significantly after BPTES treatment. Both itaconate and si*Sdhb* treatments significantly increased the non-mitochondrial respiration of PC12 cells.

CONCLUSIONS

1. The novel NGS methods alone are not sufficient for the correct clinical diagnosis

The next-generation sequencing made a significant landmark on the clinical diagnostics of genetic diseases. However, these methods are not replacements of the genetic consultation. In a case report, a young female patient with both MEN2 syndrome and *BRCA1* mutation was presented. Sequencing of *BRCA1* was not indicated until the genetic consultation and the attainment of the family history. After the thorough consultation the direct assessment of the

presumably affected genes became possible. Sequencing the whole genome, or even just the exome is time-consuming and expensive therefore it cannot be applied to the clinical routine. The NGS methods have to potential to become the most feasible diagnostic tool for genetic diseases when they are supplemented with a strong clinical geneticist background.

2. The Endogene Panel as an efficient diagnostic tool for molecular genetic diagnosis of the inherited endocrine tumour syndromes

The Endogene Panel is an effective tool for the rapid, accurate and cost-effective diagnosis of the inherited diseases. Respecting the limitations of the sequencing method, the Panel can be upgraded with additional genes, therefore the spectrum can be spread to other genetic diseases. In case of parallel sequencing the most important attribute is the 100% sensitivity. As the false positive calls are efficiently validated by the conventional Sanger sequencing, there is no appropriate method for the screening of false negative results.

The parallel sequencing of multiple genes highlighted the fact that unexpected genotype-phenotype correlations occur even in small number of samples. The phenotype modifier effect of multiple variants and their clinical interpretation will yield an enormous challenge in the near future's clinical genetics and oncology.

3. GLS-1 as a predictor of malignant Pheo/PGL

Inhibition of SDH via *Sdhb* silencing or itaconate had positive effect on cell viability only in PC12 cells. This can be traced back to the differences in glutamine/glutamate metabolism. One of our main goal was to establish an *in vitro* model which can aid in the identification of novel prognostic and therapeutic agents for this rare disease. The 3D model described in this study is a simple and cost-effective way to mimic the consequences of SDH impairment.

The immunohistochemical evaluation of Pheo/PGL tumours pointed to the fact that even the SDHB-associated tumours are heterogeneous. This is further supported by the fact that the remaining SDH activity differs in these tumours as differences were observed in the succinate/fumarate ratios previously.

Gene silencing with siRNA provided an adequate model for the remaining residual SDH activity. As itaconate was capable of mimicking the consequences of *SDHB* silencing, therefore it can be further used for *in vitro* modelling. The importance of glutamine/glutamate was highlighted by our study as the increase of GLS-1 was observed upon SDH inhibition. Our observations were reassured as increased GLS-1 expression was recorded in malignant

Pheo/PGL. We assume that GLS-1 could yield a valuable prognostic factor and therapeutic target for malignant Pheo/PGL, where our current options are gravely limited.

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