Role of succinate dehydrogenase in pheochromocytomas and paragangliomas

PhD thesis

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1. INTRODUCTION

1.1. Succinate dehydrogenase and function

Succinate dehydrogenase (SDH) described first by Albert Szent-Györgyi in the middle of the 1930's is part of both the citric acid cycle (CAC) and respiratory electron transfer chain (ETC)/oxidative phosphorylation. SDH catalyzes the oxidation of succinate to fumarate in the mitochondrial matrix and transfers electrons to ubiquinone without pumping protons across the mitochondrial inner membrane.

1.1.1. The function of succinate dehydrogenase

The Krebs cycle consists of chain of chemical reactions in order to generate energy for cells. The whole CAC takes place in the mitochondrial matrix. It uses carbohydrates, fats, amino acids and proteins to oxidase acetyl-coenzyme A (acetyl CoA). The main source of acetyl CoA comes from the glycolysis, but can be derived from fatty acid oxidation as well. The intermediates of the CAC serve as substrate for biosynthetic pathways.

The CAC result in a total of four molecules of ATP, ten molecules of NADH, and two molecules of $FADH_2$. Electrons from NADH and $FADH_2$ are then transferred to molecular oxygen through the oxidative phosphorylation.

Succinate dehydrogenase catalyzes the 7^{th} step of the CAC (tricarboxylic acid cycle or Krebs cycle). It catalyzes the oxidation of succinate to fumarate along with the reduction of ubiquinone (Coenzyme Q) to ubiquinole, by transferring electrons thru FAD-$FADH_2$.

Succinate dehydrogenase or complex II is also involved in the oxidative phosphorylation (OXPHOS) or electron transport chain, representing the major source of cellular energy. The OXPHOS takes place in the inner membrane of the mitochondria and consists of four complexes; complex I, II, III and IV. The fifth complex is the ATP synthase, which uses the proton gradient to synthesize 32 to 34 ATP molecules. The flow of electrons from NADH and $FADH_2$ thru the protein complexes is associated with pumping protons to the intermembrane space of the mitochondria, which results in a proton gradient and builds up

the transmembrane potential. This is necessary for driving complex V to synthetize ATP. However, complex II is not coupled with a proton pump and transfers electrons to ubiquinone without contributing to the proton gradient.

1.1.2. The structure of SDH

SDH consists of a hydrophilic head that protrudes into the matrix compartment and a hydrophobic tail that is embedded within the IM with a short segment projecting into the soluble intermembrane space. The hydrophilic head consists SDHA (flavoprotein) and SDHB (iron sulphur protein), forming the catalytic core. Here are the binding sites for FAD cofactor and succinate. Three iron-sulphur clusters can be found in the SDHB subunit and these clusters mediate electron transfer to ubiquinone.

The hydrophobic tail consists of SDHC and SDHD subunits. The enzyme complex binds to membrane through these subunits. The structure of the enzyme complex is constructed of six transmembrane helices containing one heme b group and a ubiquinone-binding site.

1.1.3. Chromosomal localization of genes encoding the SDH subunits

All four subunits of SDH or complex II are encoded by genes located in the nuclear genome. *SDHA* encoding gene is mapped to the p arm of chromosome 5 at locus 15, *SDHB* gene is localized on the p arm of chromosome 1 at locus 36. *SDHC* gene is encoded on the q arm of chromosome 1 at locus 23. *SDHD* and *SDHAF2* genes are encoded on the q arm of chromosome 11 at locus 23.1 and 13, respectively.

Genetic mutations of these genes are associated with familial paraganglioma syndrome, childhood T-cell acute leukaemia and gastric stromal tumours.

1.2. Pheochromocytoma and paraganglioma

1.2.1. Definition, anatomical distribution

The term pheochromocytoma means "dusky-colored tumour" and it was historically derived from the color change that occurs when the tumour tissue was immersed in chromate salts. Pheochromocytomas are rare catecholamine-producing tumours arising from neural crest-derived chromaffin cells in the adrenal gland. Chromaffin cells are also found in the sympathetic ganglions; sympathetic extra-adrenal paragangliomas are generally confined to the lower mediastinum, abdomen, and pelvis (e.g. the aortic chemoreceptors and the Zuckerkandl-organ), and are typically hormone secreting. In contrast, parasympathetic paragangliomas are located predominantly on the skull base, neck, and upper mediastinum (e.g. carotid artery/body).

Early diagnosis and resection of the tumour can cure most of the cases. The diagnosis is difficult because clinical features/symptoms can mimic other diseases or can be very unspecific or uncharacteristic.

1.2.2. Etiology/Genetic background and associated hereditary syndromes

Pheochromocytomas and paragangliomas are mainly sporadic tumours, formerly about 10% of all tumours were associated with hereditary syndromes, including multiple endocrine neoplasia type 2 (MEN2), von Hippel-Lindau syndrome (VHL) and neurofibromatosis type 1 (NF1). A small percent of PHEO/PGL were associated with Carney-triad, Carney-Stratakis syndrome and more rarely with MEN type 1. In the last decade several genes (*SDHx, TMEM127, MAX, KIF1B, FH, EGLN1*) were discovered as genetic causes of pheochromocytoma and paraganglioma, making the prevalence of hereditary PHEO/PGL 30%–35% of all cases.

The common feature of hereditary syndromes is that they show an autosomal dominant inheritance, meaning that the affected individual receives one mutant gene from one of his/her parents.

2. OBJECTIVES

During my PhD training I aimed to collect and to summarize the evidence of the role of *SDHx* variants in the pathogenesis of PHEO/PGL. My specific aims were:

- *What was the prevalence of germline mutations in the SDHx, SDHAF2, MAX and TMEM127 genes in Hungarian patients with apparently sporadic PHEO/PGLs?*
- *Was there any genotype-phenotype association in patients with PHEO/PGL?*
- *Were novel mutations present among Hungarian patients?*
- *What was the prevalence of SDHx polymorphisms in patients with sporadic MTC, sporadic PHEO, healthy subjects and RET mutation carriers?*
- *Did the polymorphisms of the SDHx genes influence clinical manifestations of the disease in a cohort of subjects harbouring RET mutations?*
- *Did this prevalence differ in sporadic MTC, sporadic PHEO, healthy subjects from RET mutation carriers?*
- *What were the levels of the two Krebs cycle metabolites, succinate and fumarate in*
- *tumour tissue?*
- *What was the succinate to fumarate ratio in human plasma samples?*
- *What was the succinate to fumarate ratio in mouse pheochromocytoma (MPC) and mouse tumour tissue (MTT) cells?*
- *Did this difference propose the implementation of succinate/fumarate measurements in clinical diagnosis?*

3. METHODS

3.1. Germline mutation prevalence in Hungarian patients with pheochromocytoma and/or paraganglioma

3.1.1. Patients

Our database containing the clinical and laboratory data of 129 patients diagnosed and followed up at the 2nd Department of Medicine, Faculty of Medicine, Semmelweis University with clinical diagnosis of PHEO/PGL between 1998 and 2014 was reviewed in order to select cases for comprehensive genetic testing. All patients underwent genetic counselling and written informed consent was obtained before genetic analysis.

Of these patients, the clinical diagnosis was confirmed by pathological examination of the surgically removed tumour tissues in 92 cases. Mutation screening of the *RET* and *VHL* genes identified 4 *RET* mutation carriers and 4 patients with germline *VHL* mutations. In two cases the specific phenotype features indicated neurofibromatosis type 1. These patients were excluded from this current analysis and *SDHB, SDHC, SDHD, SDHAF2, MAX* and *TMEM127* mutation analysis was performed in 82 cases.

3.1.2. Genetic testing

Genetic testing of the *RET, VHL, SDHB, SDHC, SDHD, SDHAF2, MAX* and *TMEM127* genes using bidirectional Sanger sequencing. Blood DNA was extracted using commercially available DNA extraction kits. Large deletion analysis of the *SDHB, SDHC* and *SDHD* genes were performed using multiplex ligation probe amplification.

3.2. The G12S polymorphism of the *SDHD* gene as a phenotype modifier in patients with MEN2A syndrome

3.2.1. Patients

Written informed consent was obtained from all patients and family members who participated in the study at the 2nd Department of Medicine, Faculty of Medicine, Semmelweis University. Patients underwent a complete clinical examination, laboratory testing, including serum basal calcitonin measurement, plasma parathyroid hormone, urinary catecholamine metabolites, and imaging studies, including cervical ultrasonography, thoracal and abdominal computed tomography (CT), and whole-body metaiodobenzylguanidine scintigraphy (MIBG).

3.2.1.1. Patients with MEN2 syndrome

77 patients with germline *RET* proto-oncogene mutations who were members of 21 unrelated families with MEN2 syndrome were identified by genetic screening at our centre. 55 had MEN2A (mean age at diagnosis: 33.4±17 years; range: 7–76 years), three had MEN2B (mean age at diagnosis: 15.6±5 years; range: 10–20 years), and 19 had FMTC (mean age at diagnosis: 23.7±16.8 years; range: 2–57 years). The presence of PHEO and MTC were confirmed by histological examination of surgically removed tumours. Total thyroidectomy was performed in all patients with germline *RET* mutation in the symptomatic group and was also offered to all individuals from the asymptomatic group.

3.2.1.2. Patients with sporadic MTC

47 unrelated patients with histologically confirmed MTC were evaluated. There were 15 men (age, mean ± SD, 44.7±13.3; range: 28–82 years) and 32 women (age, mean ± SD, 47.7±12,3; range: 23–76 years).

3.2.1.3. Patients with apparently sporadic PHEO

48 unrelated patients with histologically confirmed sporadic adrenal pheochromocytomas were evaluated. There were 16 men (age, mean ± SD, 36±14; range: 13–66 years) and 32

women (age, mean ± S.D, 42±14; range: 19–64 years). The mutation analysis of *RET* exons 10–14 and the entire *VHL, SDHB*, and *SDHD* genes revealed no disease-causing mutations. Patients with confirmed *VHL* (five patients), *SDHB* (one patient), or *SDHD* (one patient) mutations were excluded from the study. Five patients were initially thought to have sporadic pheochromocytoma, but were later identified as having a disease-causing *RET* mutation and were included in the study as *RET* mutation carriers. MTC, either by elevated serum calcitonin or by postoperative histology, had been diagnosed in all of these patients. Genetic counselling and genetic screening for all first-degree relatives have been offered.

3.2.2. Genomic DNA was isolated from peripheral blood using commercially available DNA extraction kits in accordance with the manufacturers' instructions. *RET* proto-oncogene mutations were detected by direct sequencing. Mutation analysis of the *VHL, SDHB*, and *SDHD* genes in cases of apparently sporadic PHEO were performed by direct sequencing of the entire coding region of the *VHL*, *SDHB*, and *SDHD* genes, and large deletion analysis of the *VHL, SDHB, SDHC*, and *SDHD* genes performed using multiplex ligation probe amplification.

3.2.3. The nucleotide change of G to A, which corresponds to the G12S polymorphism, results in the preservation of the BanI restriction cleavage site. To identificate this site in our patients we used restriction fragment length polymorphism (RFLP). Samples from patients with positive results were examined by direct DNA sequencing.

3.3. Biochemical consequences of *SDHx* mutations, succinate to fumarate ratio in *SDHB/D* associated paragangliomas

3.3.1. Materials

PHEO/PGL tissue samples were collected at the National Institutes of Health (NIH) under clinical protocol 00-CH-0093, approved by the Institutional Review Board of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD). Tissue samples were frozen in liquid nitrogen shortly after surgical removal of a tumour. All patients underwent genetic testing for known PHEO/PGL susceptibility genes except *SDHAF2* and neurofibromatosis 1 (*NF1*); the diagnosis of the latter was based on clinical grounds.

We included four groups of tumours: *SDHB* (10 PGLs), *SDHD* (5 PGLs), apparently sporadic (6 PHEOs, 4 PGLs), and *NF1* (2 PHEOs). *NF1*-related PHEOs were included because of the genetic background of the mouse PHEO (MPC) and mouse tumour tissue (MTT) cells used in the in vitro experiments. The MPC and MTT cell lines were used as described previously. MTT cells are known to be more aggressive than MPC cells and show aggressiveness similar to human disease.

3.3.2. Silencing of SDHB in MPC and MTT cells

Early passages of MPC and MTT cells were transduced with lentiviral particles carrying either shRNA targeted against *mSDHB* or control shRNA. To evaluate the degree of *SDHB* silencing in MPC and MTT cells, Western blot analysis was performed.

3.3.3. Metabolic measurements

Procedures for the determination of succinate and fumarate have been described elsewhere and are briefly related here. The organic acids were analyzed as their tertiary butyl dimethylsilyl ether derivatives using gas chromatography-mass spectrometry (GC-MS) in the electron impact mode and quantified using the 13C-labeled internal standards for each analyte. Samples for analysis were prepared by perchloric acid extraction as previously described. The 13C-labeled internal standards were added in two-fold excess of the

concentrations of the individual analytes in the tumour tissue to the neutralized PCA extracts. Samples were analyzed on an Agilent 5973 quadrupole GC-MS (Agilent).

4. RESULTS

4.1. Germline mutations in Hungarian patients with pheochromocytoma and paraganglioma

Eleven patients were identified to carry mutation in one of the PHEO/PGL associated genes. Together with our previous data demonstrating mutations in *RET* (n=4) and *VHL* (n=4) genes, the prevalence of germline disease-causing mutations in Hungarian patients with apparently sporadic, non-syndromic PHEO/PGL was 21.1% (19/90; 11 of 82 cases, 4 *RET* and 4 *VHL* mutation carriers). For mutation detection bilateral involvement and multiple tumours had the most positive predictive value. The prevalence of bilateral tumours was significantly higher in mutation carriers than in genetically negative cases (8 of 11, 72.8% vs. 3 of 71, 2.1%; $p<0.001$).

The mutation spectrum observed in our patients was heterogeneous, the most frequent mutations were detected in the *SDHB* gene (7 different of which 4 were novel mutations), Three patients had *TMEM127* mutations (two novel) and one had mutation in the *SDHD* gene. All novel *SDHB* mutation have been submitted to TCA Mutation Database and the new *TMEM127* mutations to dbSNP database. No mutations in *SDHC, SDHAF2,* and *MAX* were identified in our patients.

Genotype-phenotype associations

Comparison of the main demographic and clinical data of the genetically positive and negative cases indicated that genetically positive patients were younger, their PHEO/PGL was more frequently malignant, and 72% of cases had bilateral or multiple tumours. As expected the malignancy was the highest (3 out of 7 cases) in patients with *SDHB* mutations. Two patients with mutations *SDHB*:c758G>A -Cys253Tyr- and the novel *SDHB*: c.586T>G -Cys196Gly- were lost because of metastatic disease by the age of 35 years. In these patients multiple metastases in bone and liver were observed. In the third case with malignant PGL the novel *SDHB*: c728G>A Cys243Tyr mutation was identified. In this patient an intraabdominal PGL with multiple bone metastases was diagnosed.

Another important finding was that the *SDHB* associated tumours were mainly intraabdominal PGLs (6 out of the 7 cases). In one case with the novel *SDHB* c607G>T Gly203Stop mutation pheochromocytoma and renal cell carcinoma with oncocytic feature was detected at age of 19 years**.** The solid architecture, cytoplasmic inclusions of flocculent material and intratumoural mast cells as the main characteristics for *SDHB* associated renal cell carcinomas could be identified.

Head-neck PGLs were detected in a patient harbouring the *SDHB:* c286+1G/A mutation, and in a patient with *SDHD* c.147-148 insA frameshift mutation. In the latter case an intraabdominal PGL was also removed. After 4-8 years follow-up no malignancy was observed in these cases.

TMEM127 mutations were detected in three patients. Two of them had PHEO (one bilateral) while in the third patient with the novel mutation (*TMEM127*: c467T>A, -Leu155Stop) PHEO and PGL of the head-neck region was also observed. These tumours showed no malignancy. The youngest patient harbouring *TMEM127* associated tumour was 22 years old.

4.2. The G12S polymorphism of the *SDHD* gene as a phenotype modifier in patients with MEN2A syndrome

As mutations of the *SDHx* cause hereditary PHEO/PGL and PHEO is part of MEN2 we hypothesized that variants of *SDHx* genes might be genetic modifiers in MEN2. Therefore all patients with MEN2 from our biobank was tested for *SDHx* mutation. Eight of the 55 patients with MEN2A (15.5%) had the G12S variant, whereas it was absent in the MEN2B and FMTC groups. No patient with sporadic MTC and/or sporadic PHEO carried this variant. Among the 100 population-based, healthy control individuals, only one individual carried this variant (prevalence, 1%). No association between the G12S polymorphism of the S*DHD* gene and the incidence of PHEO or hyperparathyroidism in *RET* mutation carriers was observed, and the age of disease manifestation was similar in G12S carriers

and in non-carriers (43±9 versus 40±3 years in probands and 29.6±19.3 versus 32.5±20.5 years in non-carriers). Among probands with *RET* mutations, carriers of the G12S had higher serum calcitonin levels compared with those who did not carry the *SDHD* G12S variant (6,864±11,111 versus 1,250±932 pg/ml), but the difference was not significant. Among family members with *RET* mutations, serum calcitonin levels were similar in G12S carriers and non-carriers 436±876 versus 393±556 pg/ml).

\4.3. Biochemical consequences of *SDHx* mutations, succinate to fumarate ratio in *SDHB/D* associated paragangliomas

The present study included 27 tumour samples and *SDHB* silenced and control MPC and MTT cells. The samples included 10 *SDHB* PGLs, 5 *SDHD* PGLs, 2 *NF1* PHEOs, and 10 apparently sporadic PHEOs/PGLs.

2.3.1. Succinate concentration in tumour tissues

In *SDHB*-related PGLs, the results showed a mean succinate concentration of 2.692 ± 1.979 mmol/L with the COV of 0.71, in comparison with the apparently sporadic PHEO/PGL group (0.219 ± 0.066 mmol/L; COV of 0.3; $P = .0009$). One *SDHB*-related PGL showed a much higher succinate level than the other *SDHB* tumours (7.916 mmol/L). This sample is a significant outlier ($P < .05$ with a Z value of 2.29). In the *SDHD* group, the mean succinate concentration was 2.078 ± 0.491 mmol/L (COV of 0.24); this value was higher ($P < .05$) than in the apparently sporadic PHEO/PGL group. The *NF1* PHEOs showed a similar mean succinate concentration (0.364 ± 0.165 mmol/L; COV of 0.454) to apparently sporadic PHEOs/PGLs.

2.3.2. Fumarate concentration

In *SDHB*-related PGLs, the results showed a mean fumarate concentration of 0.015 ± 0.007 mmol/L with the COV of 0.46, in *SDHD*-related PGLs 0.046 ± 0.029 (COV of 0.62), and in apparently sporadic PHEOs/PGLs 0.038 ± 0.016 mmol/L (COV of 0.43). Fumarate

concentrations were significantly lower in the *SDHB*-related compared with *SDHD*-related and apparently sporadic PHEOs/PGLs (P = .005, P = .0008, respectively). The *NF1* PHEO group showed a mean fumarate concentration of 0.057 ± 0.018 mmol/L (COV of 0.324).

2.3.3. Succinate-to-fumarate ratio in tumour tissues

The mean succinate-to-fumarate ratio was high in *SDHB*- and *SDHD*-related PGLs, 238.6 ± 327.2(COV of 1.37) and 60.24 ± 36.58 (COV of 0.60), in contrast to apparently sporadic PHEOs/PGLs 6.3 ± 2.0 (COV of 0.31) (P = .0376, P = .0003, respectively). The *NF1* PHEOs had a mean succinate-to-fumarate ratio of 6.204 ± 0.87 with the COV of 0.14.

2.3.4. Succinate-to-fumarate ratio in plasma samples

The plasma mean succinate-to-fumarate ratio showed a slight increase in the *SDHB* and *SDHD* groups (3.15 ± 1.63 and 2.75±1.65) compared with the apparently sporadic group (1.61 ± 0.61), but this difference was not significant.

2.3.5. Succinate-to-fumarate ratio in MPC and MTT cells

The succinate-to-fumarate ratio was significantly higher in *SDHB*-silenced MTT cells compared with control MTTcells; 7.53 vs 2.45 (P = .0115), whereas the small elevation in the *SDHB*-silenced vs control MPC cells was not significant (1.62 vs 1.16, P = .164).

Culture supernatants were analyzed in the *SDHB*-silenced and control MPC and MTT cells, but no apparent differences in the succinate-to-fumarate ratio were determined.

The degree of *SDHB* silencing was 62% in MPC and 63% in MTT cells.

5. CONCLUSION

I summarized clinical, demographic and genetic data of Hungarian patients with apparently sporadic PHEO/PGL. Using a comprehensive mutational screening of a large series of patients with PHEO/PGL, I determined the prevalence of disease-causing mutations in this patient group. The most frequent mutations were detected in the *SDHB, TMEM127, RET and VHL* gene. This heterogeneous genetic background with six novel mutations observed in Hungarian patients was similar to other populations where no founder mutations are present. The genetic screening offered for PHEO/PGL patients in this population should cover all of the genes identified to date but the first gene for testing should be the *SDHB* for patients with intraabdominal PGL especially with malignant phenotype. The novel genotype-phenotype associations revealed may contribute to improvement of diagnostic approaches and may help to achieve a better clinical follow up of patients with PHEO/PGL. Both laboratory workload and cost of testing of all genes are still significant, but phenotype oriented guidelines allow us to set up an order of genes tested, after a negative result the remaining genes should be also examined. For most effective work the optimum would be to exclude some of the syndrome-associated genes based on the obvious phenotype features (i.e. because of typical manifestation the NF1 gene is rarely tested) and all remaining genes would be tested at the same time. Testing *KIF1B, EGLN1, FH, IDH2* and *MDH2* genes by next generation sequencing based methods would also be desired. The clinical follow-up of patients identified with pathogenic, germline mutations and their first-degree relatives is challenging. First of all, in the affected families for all first degree relatives genetic counselling followed by genetic testing should be offered.

Beside the disease-causing *SDHx* mutation I found a significantly higher prevalence of the G12S variant of the *SDHD* gene among germline *RET* mutation carriers presenting with MEN2A compared to the control group. The high prevalence of the G12S variant in these patients supports its genetic modifier role, however, we were unable to detect significant differences in the clinical presentation between G12S carriers and non-carriers. This proposal remains to be established.

For the first time I was able to demonstrate that the succinate-to-fumarate ratio could be used as a new metabolic marker for the presence of *SDHB/D*-related PGLs. Accumulation of succinate result in the inhibition of prolyl hydroxylases and consequently in the impaired degradation of hypoxia-inducible factor α (HIF1-, 2- α). HIF1-, 2-α stabilization has an impact on genes promoting tumorigenesis and cancer development with accelerated aerobic glycolysis. Based on the literature and my results, through a large prospective clinical study including other *SDH* PHEOs/PGLs, it would be possible to determine the diagnostic accuracy of succinate-to-fumarate ratio in the diagnosis of PHEO/PGL. Furthermore, following the confirmation of our initial results, we may hypothesize that intratumoural and perhaps plasma changes in the succinate-to-fumarate ratio will serve as an important indicator of potential therapies directed toward mutated SDH proteins.

6. BIBLIOGRAPHY OF THE CANDIDATE'S PUBLICATIONS

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