Final Report

Novel biomarkers for diagnosis and prognosis of adrenal tumors
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Background: Between 2017-2021, as a national reference center for rare endocrine diseases, and full member of ENDO-ERN (Endocrine Reference Network) and the co-chair of the Endocrine Genetic Tumor Syndromes Main Thematic group we collected and maintained the Hungarian database of patients presenting with these symptoms. Adrenal gland and tumors arising from both adrenal cortex and medulla are part of these syndromes. Their genetic background is heterogeneous and multiple genes involved in their development and pathogenesis. This grant significantly helped in development, validation and introducing into clinical practice next generation sequencing (NGS) based comprehensive mutation screening methods. These methods allowed us to perform these genetic analysis cost efficiently and resulted in identification of mutation carriers, as well as novel genotype-phenotype associations. During the last 4 years we analyzed over 1000 patients with clinical diagnosis of one of these syndromes. Of them, approx. 20% carried germline pathogenic or likely pathogenic variants which clarified the clinical diagnosis and helped to provide them the most accurate therapy. Beside the clinical usefulness, several basic science results were obtained and novel prognostic and therapeutical targets came into our vision. Our detailed results are summarized by our original objectives.

Objective 1: Comprehensive mutation screening of genes involved in molecular genetics of adrenal adenoma (*CYP21A2, TP53, ARMC5, KCNJ5, PRKAR1A, GNAS, PDE11A, GR*)
We developed and successfully validated the ADRENALSEQ panel (covering 25 genes - *ARMC5, ATP1A1, ATP2B3, CACNA1D, CACNA1H, CYP11A1, CYP11B1, CYP11B2, CYP17A1, CYP21A2, NR0B1 (DAX1), GNAS, HSD3B2, KCNJ5, MC2R, MRAP, NR3C1, NR3C2, NR5A1, PDE11A, POR, PRKAR1A, SRY, STAR, TP53*) implicated in adrenal tumor pathogenesis. We tested 396 samples.

Of these, we identified 14 patients carrying pathogenic *TP53* mutations, one with *STAR*, one *CACNA1D*, one *PTEN* (Tömböl et al) and one *SF1* gene carrier. We identified the first case with *ARMC5* mutation (Hella et al.). Several novel genotype-phenotype associations were revealed. Of these, two unique families were further studied. One patient having bilateral periadrenal mass, further confirmed as PEComa (Perivascular epithelioid cell tumors) carried two *PTCH1* mutations (Igaz P et al). Another case with PEComa carried a novel splice *TP53* mutation. Further functional studies proved the pathogenicity of this variant. We plan two manuscripts about the *TP53* mutations in hereditary cancer cases and about the novel phenotype. The novel findings have been presented at national conferences and one manuscript containing the PTCH1 cases is under revision in Journal of Molecular Genetics). Regarding the *CYP21A2* several methodological developments have been developed in order to achieve the most accurate technique. The similarity between the real *CYP21A2* gene and its pseudogene, *CYP21A1* makes that the PCR based methods result in diagnostic inconsistency. Therefore, long range PCR with copy number analysis and haplotyping are needed for the diagnostic workflow (Doleschall et al.-2017). Our methods have been updated and presented and national meeting and one manuscript is under review, Doleschall et al. 2021; Csitary et al.).

Objective 2: Developing a targeted resequencing panel for comprehensive mutation screening of genes involved in molecular genetics of pheochromocytomas/paragangliomas-Pheo/PGL (*RET, VHL, SDHA, SDHB, SDHC, SDHD, SDHAF2, TMEM127, MAX, MDH2, MEN1, EGLN1*)
Similarly to genetic study described above, for Pheo/PGL studies we used our ENDOGENE panel. version 2. Totally 284 samples were analysed. Important novel findings (unusual genotype-phenotype associations ie. carotid paraganglioma with *VHL* mutation) were revealed. A manuscript has been published in Frontiers in Genetics about an ultra rare association of *BRCA1 and RET* mutations (Sarkadi et al. 2017). We collected and reevaluated all cases harboring *MEN1* gene mutation. The Hungarian MEN1 database revealed novel genotype-phenotype associations, the most important that neuroendocrine tumors in young individuals represent a major genetic risk for this syndrome. Our paper was published in Endocrine (Kovesdi et al.). The complex analytical validation of our method and bioinformatical workflow was published in Cancers (Sarkadi et al. 2021). This study serves as a starting point for accreditation of our method in routine molecular genetic analysis of patients with Pheo/PGL. Based our results some neighboring countries (Serbia, Romania) through ERN Network asked as to test their patients. Related to this topic we actively participated and participate in several international collaborations. Of these collaborations high impact publications were published (Larsen et al, 2020; Castinetti et al 2019; Krauss et al. 2019; Neumann et al. 2018, Bausch et al. 2017).

Objective 3. Role of sequence variants of the mitochondrial genome in adrenal tumorigenesis
The sequence variants of mitochondrial genome have been tested with a commercially available MitoSeq NGS panel. We used for method development pituitary tumor tissues. Our results were included in one manuscript which was published in Journal of Endocrinological Investigations (Nemeth et al. 2019.).

Objective 4a: Functional consequences of genetic alterations on gene expression levels in different types of adrenal tumors using transcriptome sequencing of RNA isolated from tumor tissues.

For this specific aim we started to select cases for our MEN1 database. Of these cases several had adrenal adenoma along with pancreatic neuroendocrine (PANNET) tumors. The main cause od mortality in this clinical entity is linked to PANNET we decided to analyze first these cases. MicroRNAs are small noncoding RNA molecules whom expression is tissue specific. Therefore, we hypothesized that PANNET may contain microRNAs which are related to MEN1. We systematically evaluated the MEN1 targeting microRNAs and their expression were studied in PANNET tumors. We successfully identified MEN1-related microRNAs, and, in addition we demonstrated their prognostic role in neuroendocrine pancreatic neoplasm. Our paper was published in European Journal of Endocrinology (6).

Based on these results we aimed to further pursue this topic in other cases with various neuroendocrine tumors. The serum Chromogranin A is the only widely used serum biomarker for diagnosis and for prognosis of these cases. However, its diagnostic accuracy is dependent on histology and its sensitivity and specificity are around 60-85%. We hypothesized that circulating microRNA together with CgA would serve as a novel biomarker in metastatic

pancreatic neuroendocrine tumors. We identified a set of microRNAs which along with chromogranin Am increased the diagnostic sensitivity of CgA (7). This study was a pilot study demonstrating that for other clinical conditions tissue-specific microRNAs may be useful markers both is differential diagnosis and for prognosis.

There are some still ongoing studies. In H295R adrenocortical cells we developed a three dimensional modelling condition (see later). We compared the biological properties of 2D, 3D and xenographs of H295R cells in basal condition and after mitotane treatment. Total RNA was isolated and transcriptome sequencing was performed. Our results demonstrated that the 3D model is more relevant for assessment of the biological processes than than the 2D models. Based on the transcriptomics data a novel potentially therapeutical target came into our sight. To demonstrate that indeed this molecule would be a novel drug for adrenocortical cancer several novel experiments have been started. As I expect later this year or early next year we will able to submit a new manuscript containing these data.

Objective 4b: In vitro functional studies in PC12 rat pheochromocytoma cell line for evaluation the oncometabolite role of succinate

Subunits of succinate dehydrogenase genes (SDHx) are genetic factors for malignant pheo/PGL. As we shown in Objective 2, several patients carry mutations in one of SDH genes. Of these, the malignant potential is associated with SDHB mutations. Our aim was to decipher the consequences of inhibition of succinate dehydrogenase enzyme in various cell lines (PC12, HeLa, H295R) in order to identify the molecular mechanism leading to malignant phenotype associated with *SDHB* mutation. Itaconic acid, atpenin and siRNA against SDHB inhibitions were performed, intracellular metabolites were measured by HPLC tandem mass spectrometry. We confirmed that itaconic acid inhibits SDH enzyme and resulted in a more viable PC12 cells compared to HeLa or H295R cells. Glutamine was identified as the main source for metabolism input in this cell line. Based on these in vitro results we performed an immunhistochemistry analysis of SDHB and glutaminolysis type 1 in pheochromocytoma/paraganglioma tissues. Malignant behavior of these tumours associated with GLS1 immunopositivity. Manuscript, containing these data has been published in Cancers (Basel) (Sarkadi et al. 2020).

In addition, based on our expertise in mitochondrial energetics a strong collaboration with Eotvos Lorand University has been established. Together with Vellai-Takacs Krisztina we evaluated the biological and biochemical features of C elegans model containing deletion od Sdhb genes. Important findings have been identified and even in this worm the pseudohypoxia mechanism was demonstrated associating with Sdhb malfunction. Importantly, other mechanisms including fertility and developmental issues were also revealed. One important manuscript (Saskoi et al) has been published in Dis Mech Models).

Objective 5: Developing a fast, cheap high pressure liquid chromatography (HPLC) tandem mass spectrometry assay for analysis of whole genome DNA methylation status
Several attempts were carried out for parallel determination of the 5-hydroxymethylcytosine (5hmC) 5-methylcytosine (5mC), and cytosine from DNA samples isolated either from tumor tissues or blood using our HPLC-MSMS system. We used pituitary tumor DNA and it turned out that the decreased demethylation was found in samples with higher proliferation index.

Behind this results we systematically evaluated the expression of DNMT and TET enzymes and treatment of pituitary tumor cells with decitabine (demethylase inhibitor) was carried out. Our results showed that stabilizing methylation would be beneficial in growth of pituitary adenoma cells. Our manuscript was published in the Journal of Clinical Endocrinology and Metabolism (Szabo B, JCEM).

As I mentioned under Objective 4a, we performed transcriptome sequencing of 2D, 3D and xenograft models developed from H295R cells. In addition, we evaluated and finally developed cell biological methods for 3D growing pituitary and adrenal cells (Krokker et al. POR and abstract). We use these models for further studies, including one in pituitary neuroendocrine tumors and one in PC12 cells.

Summary

This proposal was completed according to the original research plan. Based on results obtained during this 4-year period, several other potential new hypothesizes and projects were further evaluated leading to important findings and prestigious publications. To date, 26 publications (IF: 110,07) were published related to the topics of this grant. Of these, 9 directly linked to this particular grant and the Acknowledgement sections of these publications contain the reference of this grant. The cumulative impact factor of these publication is: 44,09. Three manuscripts (one about *CYP21A2* analysis, one about the PTCH1 mutations in PEcoma and one about the Wnt signaling in hypercortisolism are already under revision). Of course the main results of this grant helped us in initiation of other studies and helped us in maintaining national and international collaborations. The most important collaboration is part of the Endocrine Rare Disease Network (ENDO ERN). In this network myself is the co-chair of the Endocrine tumor syndromes Main Thematic Group. Another important national collaborations has been established with Krisztian Takacs-Vellai group in developing C elegans Sdh deficient animal model. Our findings have direct clinical relevance, already some of our NGS-based sequencing methods have been introduced into every day clinical practice, allowing us to test more patients at lower cost. The diagnostic yield in this patient group is above 20% further highlighting the importance of molecular genetic testing. We would like again to thank the Office Ministry for Innovation and Technology for this support.

Publications

Objective 1:

1. Hella Z és mtsai: ACTH independens hypercortisolismust okozó macronodularis mellékvese hyperplasia ARMC5 génmutáció következtében Magyar Endokrinológiai és Anyagcsere Kongresszus 2021.08.26.-28, Eger)
2. Tömböl Zs: Cowden-szindróma esete vizsgált Magyar Endokrinológiai és Anyagcsere Kongresszus 2021.08.26.-28, Eger)
3. Igaz P és mtsai: Szomatikus és csírasejtes PTCH1 mutációk a Gorlin-Goltz szindróma egy új fenotípusában Magyar Humángenetikai és genomikai társaság XIII. kongresszusa, Szeged 2021.09.02-04)
4. Igaz P et all: Surprising genetic and pathological findings in a patient with giant bilateral periadrenal tumors: PEComas and mutations of PTCH1 in Gorlin-Goltz syndrome (**under review**, **Journal of Medical Genetics**- jmedgenet-2021-108082.
5. Doleschall M és mtsai. Fejlett genetikai diagnosztika alkalmazása veleszületett mellékvese hiperpláziában (Magyar Endokrinológiai és Anyagcsere Kongresszus 2021.08.26.-28, Eger)-előadás

6. Csitári G és mtsai: 21-hioxiláz hiányos mellékvese hyperplásiás betegek fertilitással összefüggő klinikai paramétereinek vizsgálata. (Magyar Endokrinológiai és Anyagcsere Kongresszus 2021.08.26.-28, Eger)- poszter
7. Doleschall M et al. Quantitative PCR from human genomic DNA: the determination of gene copy numbers for CAH and RCCX CNV (**under submission 2021**)

Objective 2

8. Sarkadi B, Baghy K, Sápi Z, Nyirő G, Likó I, **Patócs A**. Germline BRCA1 Mutation Detected in a Multiple Endocrine Neoplasia Type 2 Case With RET Codon 634 Mutation. **Front Genet**. 2019 Jun 11;10:544. doi: 10.3389/fgene.2019.00544
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Objective 3:

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Objective 4a

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