Exploring the expression patterns and pathogenic relevance of microRNAs in adrenal tumours

Ph.D. Theses

Dr. Zsófia Tömböl
Semmelweis University
Clinical Medicine Doctoral School

Programme Chairman: Prof. Károly Rácz MD PhD DSc
Tutor: Dr. Peter Igaz MD MSc PhD

Opponents: Dr. Edit Buzás MD PhD DSc
Dr. Tamás Orbán PhD

Head of Examination Committee:
Prof. Ilona Kovalszky MD PhD DSc

Members of Examination Committee:
Dr. Csaba Fekete MD PhD DSc
Dr. Artur Beke MD PhD

Budapest
2011

I. INTRODUCTION

The wide-spread use of high-throughput molecular techniques has lead to rapid development of molecular biological research in many fields. A new science has been developed to analyze the vast amount of data obtained by these techniques, i.e. bioinformatics. A major field of current research involves the molecular and “in silico” analysis of microRNAs (miRNA, miR).

miRNAs are short, 20-24 nucleotide long, non-coding, single stranded RNA molecules that are regarded as the endogenous mediators of RNA interference. The phenomenon of RNA interference was first described as RNA degradation following the administration of exogenous double stranded RNA molecules. Short interfering RNA (siRNA) molecules formed from these exogenous RNA molecules specifically bind and inhibit their complementary mRNA targets, and thereby constitute a basic mechanism of posttranscriptional gene expression regulation. siRNAs are widely used for modulating gene expression in molecular biological research. Further studies have shown that RNA interference is mediated not only by exogenous RNAs, but also by endogenous RNA molecules coded in the genome.

miRNA genes are found in genomes of most divergent species including humans and their expression is cell and tissue specific. The number of miRNA coding genes in humans is over one thousand. miRNAs bind complementary messenger RNA molecules and thereby result in the posttranscriptional inhibitions of their targets. A single miRNA may inhibit several hundreds of complementary mRNAs,

moreover a single mRNA molecule can be affected by several miRNAs. The potential targets of miRNAs are identified by bioinformatical approaches at present.

miRNAs as participants in epigenetic regulation play important roles in the fine tuning of gene expression. They are involved in the regulation of signal transduction, cell proliferation, apoptosis, cell differentiation, metabolism, embryogenesis and in the maintenance of homeostasis. Based on the various functions of miRNA targets, altered expression of miRNAs is expected in the pathogenesis of several diseases. Most literature data refer to the pathogenic roles of miRNAs in tumourigenesis. Characteristic miRNA expression patterns have been described in several solid and hematologic tumours, whose study may help tumour classification, the definition of malignancy and even for prognosis.

miRNA expression patterns have been studied in several tumours of the endocrine system including pituitary, thyroid, parathyroid, endocrine pancreas and ovarian tumours. As no data have been available on the miRNA expression alterations in adrenal tumours at the beginning of our studies, I have studied the miRNA expression patterns of adrenocortical and adrenomedullary tumours, and searched for miRNA biomarkers that can be used for the classification of these tumours and the definition of malignancy. I have examined whether formalin-fixed, paraffin-embedded (FFPE) samples are suitable for the analysis of miRNA expression patterns in pheochromocytomas. I have also tried to explore altered biological pathways affected by miRNA

regulation in the pathogenesis of these tumours by bioinformatical analysis. These pathogenic pathways may be targets of drug treatment in the future.

II. AIMS OF OUR STUDIES

i. Analysis of miRNA expression patterns in normal adrenocortical tissues and sporadic adrenocortical tumours (hormonally inactive, cortisol-producing benign, cortisol-producing malignant).
ii. Study of miRNA expression patterns in sporadic and hereditary benign and sporadic recurring pheochromocytomas.
iii. Exploring the applicability of formalin-fixed paraffin-embedded (FFPE) tissue blocks for the study of pheochromocytoma miRNA expression patterns.
iv. Attempt to identify miRNA biomarkers useful for the differentiation of malignant adrenocortical tumours and recurring pheochromocytomas from other tumour types.
v. Development of a novel method for the establishment of tissue-specific miRNA targets by the use of parallel mRNA expression profiling in adrenocortical tumours.
vi. To identify basic pathogenic pathways influenced by miRNAs in a posttranscriptional manner using bioinformatical methods.

III. METHODS

Patients and tissue samples

Tissue samples from adrenocortical tumours were collected at the 1st Department of Surgery of Semmelweis University, whereas intact adrenal tissues were obtained from the Department of Urology. Altogether 36 frozen samples were examined: 10 normal adrenals, 10 hormonally inactive adenomas, 9 cortisol-producing adenomas and 7 cortisol-producing adrenocortical cancer tissues.

In the second part of my work, 33 tumour samples from pheochromocytomas were studied: 8 MEN2-associated (multiple endocrine neoplasia 2), 6 VHL-related (von Hippel-Lindau syndrome), 5 NF1-associated (neurofibromatosis 1), 9 sporadic benign and 5 sporadic recurring pheochromocytomas have been examined. Mutation analysis of candidate genes involved in hereditary pheochromocytoma pathogenesis (*RET*, *VHL*, *SDHB*, *SDHC* and *SDHD*) have been studied by direct DNA-sequencing in all patients. Diagnosis of NF1 was based on the clinical picture. Diagnosis of recurring pheochromocytoma was established in cases, when tumour recurrence occurred on the same side from where the primary pheochromocytoma was removed. We have studied primary tumours. As we have not disposed of sufficient number frozen tissue samples, FFPE tissue blocks were used in our analysis. In three cases frozen tissues were also available.

RNS isolation

TriReagent (Molecular Research Center Inc.) was used for the isolation of total RNA for miRNA expression profiling in adrenocortical tumours, whereas RNeasy Lipid Tissue Mini Kit (Qiagen GmbH) was applied for further mRNA profiling. Ambion RecoverAll Total Nucleic Acid Isolation Kit (Applied Biosystems) and Qiagen miRNeasy Mini Kit (Qiagen GmbH) was used for RNA isolation from FFPE and frozen pheochromocytoma samples, respectively.

miRNA expression profiling by high-throughput techniques

16 frozen adrenocortical samples (4 inactive adenomas, 4 cortisol-producing adenomas, 4 carcinomas and 4 normal adrenal tissues) were studied by parallel miRNA and total mRNA expression profiling. miRNA expression profiling was performed by TaqMan Low Density Array (TLDA) Human MicroRNA Panel v1.0 (Applied Biosystems). 21 FFPE pheochromocytoma samples (6 sporadic benign, 5 sporadic recurring, 5 MEN2, 5 VHL) and 3 frozen samples were analyzed on 8x15k Agilent Human miRNA Microarray Rel12.0 platform.

Validation of miRNA expression data by qRT-PCR

Results of miRNA expression studies were validated and sample sizes were extended by quantitative real-time RT-PCR (qRT-PCR). NF1-associated pheochromocytomas were included at this stage.

14 and 5 significantly differentially expressed miRNAs in adrenocortical tumours ($p < 0.05$), and pheochromocytomas ($p < 0.01$),

respectively, were selected for validation and sample size extension. *RNU48* was used as the housekeeping miRNA in adrenocortical samples, whereas *hsa-miR-324-3p* and *hsa-miR-320b* showing the least standard deviation in signal intensities were chosen as housekeepers in pheochromocytomas.

Whole genome expression microarray in adrenocortical tumours

16 adrenocortical tissues were subjected to whole genome mRNA expression studies by Agilent 4x44 k Whole Human Genome Oligo Microarrays (Agilent Tech. Inc.) using double fluorescent marking.

Identification of potential mRNA targets of significantly differentially expressed miRNAs by bioinformatical methods

TargetScan, PicTar and miRBase Targets algorithms were used for the establishment of potential miRNA mRNA targets. For the analysis of different prediction algorithms, a novel software application by Microsoft Visual FoxPro 9.0 was developed. This database management software is suitable for corresponding the different target identifiers applied by these algorithms and to identify all potential predicted targets and overlapping targets.

Since whole genome mRNA expression data were available for our adrenocortical samples, we attempted to perform a tissue-specific target prediction approach by bioinformatical methods.

Pathway analysis

The biological functions of miRNA targets and mRNA expression alterations were studied by Ingenuity Pathway Analysis (IPA) 7.1 software (Ingenuity Systems).

Statistical analysis

A complex statistical methodology was applied for the analysis of our results including ANOVA and different post-hoc tests (Scheffé, Tukey, Fisher), and Benjamini-Hochberg correction for mRNA microarray studies. GeneSpring (Agilent), STATISTICA 8.0 (StatSoft Inc.) and SPSS15 software were used for the analysis. Receiver Operating Characteristics (ROC) analysis was applied for the establishment of biomarkers.

IV. RESULTS

miRNA expression profiling in adrenocortical tumours

22 miRNAs were identified as significantly differentially expressed in the miRNA expression profiles of different adrenocortical tumours following normalization and statistical analysis (ANOVA, $p < 0.05$). 14 miRNAs were selected for validation and sample size extension, and 6 among these could be validated to be significantly differentially expressed among different study groups (*hsa-miR-184, hsa-miR-210, hsa-miR-214, hsa-miR-375, hsa-miR-503, hsa-miR-511*).

Expression of *hsa-miR-184* and *hsa-miR-503* was significantly overexpressed in malignant adrenocortical cancer compared to healthy adrenal tissues and benign adenomas (hormonally inactive and cortisol-producing). *hsa-miR-210* expression was significantly elevated in malignant tumours compared with cortisol-producing benign adenomas. In contrast, *hsa-miR-511* was significantly underexpressed in adrenocortical cancer compared to all other groups, whereas *hsa-miR-214* was underexpressed in cancer tissues in relation to normal tissues and inactive adenomas. *hsa-miR-375* was significantly overexpressed in normal adrenals compared to cortisol-producing benign and malignant tumours.

In our search for biomarkers applicable for the differentiation of benign and malignant adrenocortical tumours, $dCT_{hsa\text{-}miR\text{-}511}$-$dCT_{hsa\text{-}miR\text{-}503}$ turned out to be the best for the establishment of malignancy with 100% sensitivity and 97% specificity.

Whole genome mRNA expression microarray in adrenocortical tumours

16 adrenocortical samples (4 per group: normal adrenals, inactive adenomas, cortisol-producing adenomas, adrenocortical cancer) were subjected to parallel transcriptomics analysis.

Altogether, expression of 614 genes was found to be significantly differentially expressed among the groups studied. Several of these genes have already been described in other microarray studies on adrenocortical tumours. We have validated the malignancy-associated overexpression of *topoisomerase 2A (TOP2A)* by qRT-PCR.

Identification of tissue-specific miRNA targets in adrenocortical tumours

Predicted targets of significantly differentially expressed miRNAs (*hsa-miR-184*, *hsa-miR-210*, *hsa-miR-214*, *hsa-miR-375*, *hsa-miR-503*, *hsa-miR-511*) in adrenocortical tumours were examined by our database managing software. Based on the results of the three algorithms, altogether 17868 predicted miRNA-mRNA interactions were found for the six significant miRNAs.

We have developed a novel tissue-specific target prediction approach using the results of parallelly performed whole genome mRNA expression studies on the same samples for reducing the lists of potential targets, and false positive predictions and for the identification of biologically relevant targets. As mRNAs not co-expressed with their potential miRNA regulators cannot be under miRNA regulation, these

mRNAs were filtered out from the list of potential targets by our database managing software.

Since novel data have shown that the action of miRNAs significantly modulating protein expression is mostly realized via the degradation of target mRNAs, tissue specific targets showing opposite changes in expression as significantly differentially expressed miRNAs were searched for by the Gene Set Enrichment Analysis (GSEA) software. In the normal vs. adrenocortical cancer comparison, 1306 tissue specific targets showing opposite changes in expression as significantly expressed miRNAs were identified. 647 and 570 oppositely changing mRNA targets were retrieved in the inactive adenoma vs. cancer and cortisol-producing adenoma vs. cancer comparisons, respectively.

Pathogenic pathways in adrenocortical tumourigenesis (Ingenuity Pathway Analysis)

Studies at both transcriptional (mRNA) and posttranscriptional (miRNA target) levels revealed the damage of cell cycle G2/M checkpoint as the major pathogenic event characteristic for adrenocortical cancer.

Exploring the miRNA expression pattern and their pathogenic roles in pheochromocytomas

Three pairs of frozen and FFPE samples were studied on microarray platforms for analyzing the suitability of FFPE block-derived RNA samples in miRNA expression microarrays. Based on non-parametric

correlation studies, the Spearman coefficient was between 0.7-0.93, whereas Kendall tau correlation coefficient varied between 0.58-0.85 showing the applicability of FFPE samples for miRNA profiling.

21 pheochromocytoma FFPE samples were subjected to miRNA expression profiling by high-throughput microarray platform. Altogether 6 sporadic benign, 5 sporadic recurring, 5 MEN2 and 5 VHL pheochromocytoma samples were examined.

Following statistical analysis (one-way ANOVA, $p < 0.01$; Tukey HSD post hoc test) by the GeneSpring and STATISTICA 8.0 software with the same parameters, 14 and 16 significantly differentially expressed miRNAs were revealed among different groups, respectively.

Five miRNAs from the significant results (*hsa-miR-139-3p*, *hsa-miR-541*, *hsa-miR-765*, *hsa-miR-885-5p*, *hsa-miR-1225-3p*) were selected for further validation. Since *hsa-miR-324-3p* and *hsa-miR-320b* showed the least standard deviation among samples, the average CT (cycle threshold) values of these two miRNAs were used as housekeeping for subsequent validation. Sample size extension was performed along with qRT-PCR validation (9 sporadic benign, 5 sporadic recurring, 8 MEN2, 6 VHL), and a further study group involving NF1 samples (n=5) was also included.

Significant overexpression of *hsa-miR-139-3p*, *hsa-miR-541* and *hsa-miR-765* was observed in VHL pheochromocytomas compared with sporadic benign tumours. Moreover, significant expression alteration of *hsa-miR-139-3p* was noted between VHL and NF1

pheochromocytomas. *hsa-miR-885-5p* was overexpressed in MEN2 pheochromocytomas compared to all other groups. Significant overexpression of *hsa-miR-1225-3p* was found in sporadic recurring pheochromocytomas in comparison with sporadic benign and non-recurring pheochromocytomas. The *RNU48* gene applied in many tumour studies as a housekeeping miRNA showed significant differences in expression between MEN2 and NF1 pheochromocytomas, therefore it cannot be used as an internal control in these tumours.

Pathogenic pathways in pheochromocytomas (Ingenuity Pathway Analysis)

Fort the establishment of pathways posttranscriptionally modified by miRNAs in pheochromocytomas, predicted targets of significantly differentially expressed miRNAs were subjected to pathway analysis in all pairwaise comparisons associated with significant differences in miRNA expression.

Based on previous studies on pheochromocytoma samples: "*WNT-β-katenin* signal in sporadic benign-VHL comparison, "*MYC*-mediated apoptosis signal" in MEN2-associated tumours should be highlighted. "*Notch* signal" and "G-protein coupled receptor signaling" pathways were found to be altered in sporadic recurring pheochromocytomas relative to sporadic benign samples.

V. DISCUSSION AND CONCLUSIONS

We have been among the first research groups to study and describe the miRNA expression patterns of different adrenocortical and adrenomedullary tumours. We have identified miRNA markers that can be helpful in the diagnosis of adrenocortical cancer and recurring pheochromocytomas, whose histological diagnosis is difficult. For the elucidation of the biological effects of miRNAs, a novel, tissue-specific target prediction approach has been developed. By the bioinformatical analysis of miRNA targets, we have revealed pathogenic pathways, whose altered posttranscriptional regulation may be involved in the pathogenesis of these tumours and might even represent therapeutic targets in the future.

Our results can be summarized, as followed:

i. By the high-throughput analysis of adrenocortical tumours using TaqMan approach, altogether 22 miRNAs showed significant differences in expression among experimental groups (normal, cortisol-producing, hormonally inactive benign and cancer). Cluster analysis revealed that adrenocortical cancer differed most strikingly from the other groups.

ii. 6 miRNAs could be validated by qRT-PCR. Based on their differences in expression *hsa-miR-503*, *hsa-miR-184* and *hsa-miR-210* might be oncogenic in the adrenal cortex, whereas *hsa-miR-214*, *hsa-miR-511* and *hsa-miR-375* miRNAs might be tumour suppressors.

iii. $dCT_{hsa\text{-}miR\text{-}511}$-$dCT_{hsa\text{-}miR\text{-}503}$ turned out to be the best molecular marker for malignant adrenocortical cancer. By its use, malignant tumours can be identified by 100% sensitivity and 97% specificity and it might even be used in the diagnostics of adrenocortical cancer.

iv. We have developed an integrative bioinformatical approach for the tissue-specific identification of adrenocortical miRNA targets using the results of parallel miRNA-mRNA profiling and different target prediction approaches.

v. Damage of G2/M checkpoint has been established as the major pathogenic event in adrenocortical cancer both by the analysis of transcriptional and posttranscriptional levels.

vi. Based on the good correlation of frozen and formalin-fixed paraffin-embedded (FFPE) pheochromocytoma samples, we have concluded that RNA samples isolated from FFPE blocks may be used for the study of miRNA expression profiling in pheochromocytomas.

vii. Sporadic recurring pheochromocytomas differed mostly from other groups (sporadic benign, MEN2 and VHL) based on their miRNA expression pattern, thus miRNAs might be used as biomarkers in the diagnostics of recurring tumours.

viii. Significantly different expression of five miRNAs was validated by qRT-PCR along with sample size extension. NF1-associated pheochromocytomas were also included. Overexpression of *hsa-miR-139-3p*, *hsa-miR-541* and *hsa-miR-765* was found in VHL pheochromocytomas compared to sporadic benign tumours.

Overexpression of *hsa-miR-885-5p* is a characteristic feature of MEN2 pheochromocytomas.

ix. Analysis of *hsa-miR-1225-3p* expression might be used for the identification of recurrence prone pheochromocytomas.

x. Pathway analysis of significantly differentially expressed miRNA targets revealed the posttranscriptional regulatory defect of "*Notch*-signaling" mediated by *hsa-miR-1225-3p* as a major pathogenic pathway in recurring pheochromocytomas. Changes at the transcriptomic level of this pathway have already been described in malignant pheochromocytomas.

It must be underlined, however, that these pathogenic pathways have been identified by bioinformatical predictions and therefore their experimental validation is required in the future.

ARTICLES RELATED TO THE PHD THESES

1. **Tömböl Z,** Szabó P, Rácz K, Tulassay Z, Igaz P. A mikroRNS-ek jelentősége daganatos betegségekben. Orvosi Hetilap, 2007, 148: 1135-1141.
2. **Tömböl Z,** Szabó PM., Molnár V, Wiener Z, Tölgyesi G, Horányi J, Riesz P, Reismann P, Patócs A, Likó I, Gaillard RC, Falus A, Rácz K, Igaz P. Integrative molecular bioinformatics study of human adrenocortical tumors: microRNA, tissue-specific target prediction, and pathway analysis. Endocrine-Related Cancer, 2009, 16: 895-906. **IF: 4.282**
3. **Tömböl Z,** Éder K, Kovács A, Szabó PM, Kulka J, Likó I, Zalatnai A, Rácz G, Tóth M, Patócs A, Falus A, Rácz K, Igaz P. MicroRNA expression profiling in benign (sporadic and hereditary) and recurring adrenal pheochromocytomas. Modern Pathology, 2010, 23: 1583-1595. **IF (2009): 4.406**
4. Szabó PM, **Tömböl Z,** Molnár V, Falus A, Rácz K, Igaz P. MicroRNA Target Prediction: Problems and possible solutions. Current Bioinformatics, 2010, 5: 81-88. **IF (2009): 1.688.** ***Co-first author article.***
5. **Tömböl Z,** Szabó PM, Patócs A, Rácz K, Igaz P. Differences in MicroRNA expression profiles of adrenocortical tumors (Letter). Clinical Cancer Research, 2010, 16: 2915. (IF (2009): 6.747)

ARTICLES NOT DIRECTLY LINKED TO THESE PHD THESES

1. Halász Z, Tőke J, Patócs A, Bertalan R, **Tömböl Z,** Sallai Á, Hosszú É, Muzsnai Á, Kovács L, Sólyom J, Fekete G, Rácz K. High prevalence of PROP1 gene mutations in Hungarian patients with childhood onset combined anterior pituitary hormone deficiency. Endocrine, 2006, 30: 255-260. **IF: 1.805**
2. Halász Z, Bertalan R, Tőke J, **Tömböl Z,** Losonczi L, Patócs A, Sólyom J, Fekete G, Rácz K. A többszörös hypophysishormon-hiány genetikai okai. A hypophysis transzkripciós faktorok szerepe. Magyar Belorvosi Archívum, 2007, 60: 419-424.
3. Igaz P, Müllner K, Hargitai B, Igaz I, **Tömböl Z,** Rácz K, Tulassay Z. Marked chromogranin A elevation in a patient with bilateral adrenal incidentalomas, and its rapid normalization after discontinuation of proton pump inhibitor therapy - letter. Clinical Endocrinology, 2007, 67: 805-806. (IF: 3.370)
4. Igaz P, **Tömböl Z,** Szabó PM, Likó I, Rácz K. Steroid biosynthesis inhibitors in the therapy of hypercortisolism: theory and practice (Review). Current Medicinal Chemistry, 2008, 15: 2734-2747. **IF: 4.823**
5. Szabó PM, Wiener Z, **Tömböl Z,** Kovács A, Pócza P, Horányi J, Kulka J, Riesz P, Tóth M, Patócs A, Gaillard RC, Falus A, Rácz K, Igaz P. Differences in the expression of histamine-related genes and proteins in normal human adrenal cortex and adrenocortical tumors. Virchows Archiv, 2009, 455: 133-142. **IF: 2.305**
6. Szabó PM, Tamási V, Molnár V, Andrásfalvy M, **Tömböl Z,** Farkas R, Kövesdi K, Patócs A, Tóth M, Szalai C, Falus A, Rácz K, Igaz P. Meta-analysis of adrenocortical tumour genomics data: novel pathogenic pathways revealed. Oncogene, 2010, 29: 3163-3172. **IF (2009): 7.135**
7. Szabó D, Zsippai A, Bendes M, **Tömböl Z,** Szabó PM, Rácz K, Igaz P. A mellékvesekéreg carcinoma molekuláris patogenezise. Orvosi Hetilap, 2010, 51: 1163-1170.