Cell cycle dependent gene and microRNA expression and its' clinical significance in adrenocortical cancer

Ph.D. theses

Vince Kornél Grolmusz M.D.

Semmelweis University
Clinical Medicine Doctoral School

Advisor: Attila Patócs, Ph.D., associate professor

Opponents: Erika Tóth, Ph.D., Head of Department
Csaba Sőti, Ph.D., D.Sc., associate professor

Final Examination Committee
Head: Zsuzsa Schaff, Ph.D., D.Sc., professor emerita
members: Erika Hubina, Ph.D., senior physician
Tamás Orbán, Ph.D., senior research fellow

Budapest
2016

I. Introduction

The cell cycle is the recurrent cycle of eukaryote cells, during which they grow, duplicate their DNA and divide to two daughter cells. The cell cycle is the consequence of tightly controlled mechanisms involving transcriptional, post-transcriptional, translational regulations and protein degradation. During tumorigenesis, key cell cycle regulators display aberrant expression, playing a role in uncontrolled proliferation. Generally, the elevated expression of key cell cycle cyclins and cyclin dependent kinases and the decreased expression of cyclin dependent kinase inhibitors and other tumor suppressor genes are present in neoplasms compared to healthy, untransformed tissues.

Synchronization is the most widely used method for the study of cell cycle dependent mechanisms. During the synchronization procedure the cell cycle of the tissue culture is inhibited at a certain point of the cell cycle by serum starvation or by the application of cell cycle inhibitors. After reintroducing fresh cell culture media time-course experiments are performed to assess cell cycle related mechanisms. With the use of synchronization procedures followed by high-throughput gene expression profiling using microarray experiments the cell cycle dependent transcription program has been successfully identified in budding yeast and human cells as well. However, human diploid cells lose their synchronization relatively soon after the removal of the synchronization agent and only a subset (50-70%) of the cells engage in continuous divisions. Moreover, synchronization procedures in general, and the application of DNA replication inhibitors such as thymidine in particular, result in the perturbance of cell cycle machinery, producing growth imbalance and unscheduled expression of cyclins.

MicroRNAs (miRNAs) are short, ~ 21-26 nucleotide long noncoding RNA molecules regulating gene expression on the post-transcriptional level *via* RNA interference. MiRNA-mediated post-transcriptional silencing has been shown to regulate

the expression of the majority of the human genome and exerts pathogenic importance during tumorigenesis.

Adrenocortical cancer (ACC) is a rare, highly aggressive malignancy with poor prognosis. The incidence of ACC is 0.7-2.0/million person/year, and 5-year survival is only 22-37%. The histopathological diagnosis of ACC is challenging. In addition to the histopathological markers of proliferation (e.g. Ki67 index), Weiss score, a pathomorphological scoring system incorporating nine histomorphological parameters is used for the characterization of malignancy of ACCs.

II. Aims

1. My aim was to optimize and apply a DNA content based fluorescence activated cell sorting (FACS) to segregate cells residing in different cell cycle phases (cell cycle sort) without the use of classical synchronization techniques. With the application of high-throughput expression analyses I aimed to detect, analyze and compare the cell cycle dependent transcription program in primary human untransformed fibroblasts and human transformed (adrenocortical and cervical) cancer cells. Moreover, I aimed to compare the results of the cell cycle sort based and the synchronization based experiments.

2. Thereafter, by an integrative high-throughput miRNA expression profiling using microarray, quantitative real-time PCR (qRT-PCR)-based TaqMan Low Density Array (TLDA) and small RNA sequencing, I aimed to analyze the cell cycle dependent miRNA expression alterations.

3. By comparing the malignancy signature of ACC with the cell cycle dependent transcription program of a human ACC cell line, my aim was to detect a novel biomarker of proliferation which may facilitate the histopathological diagnosis of ACC. Moreover, I aimed to analyze the expression of this novel biomarker (RRM2 – ribonucleotide reductase M2 subunit) in ACC samples with different proliferation characteristics.

4. During an *in vitro* approach, I aimed to analyze the effect of certain antineoplastic drugs on the expression of RRM2, a novel biomarker of proliferation in human ACC cell line.

III. Materials and Methods

III.1. Cell culture and procedures

Cell culture procedures were performed on human primary (human dermal fibroblast from adult – HDFa) and cancer (adrenocortical – NCI-H295R and cervical – HeLa) cells, according to the manufacturer's instructions.

III.1.1. Flow cytometry

Cell suspensions were stained by a viable DNA stain (Vybrant DyeCycle Orange, Molecular Probes, Life Technologies) and were sorted according to the DNA content of the cells in G1, S and G2 phases (cell cycle sort) by fluorescence activated cell sorting (FACS) on a FACSAria III cell sorter (Becton-Dickinson). The purity of sorted populations was verified by FACS reanalysis. Data was analyzed by BD FACSDiva v6.1.3 software.

The effect of certain antineoplastic drugs on the apoptosis and cell cycle distribution of NCI-H295R cells were analyzed by flow cytometry. After treatments, cells were stained with propidium-iodide and analyzed on a FACSCalibur flow cytometer (BD Biosciences). Data was analyzed by Cell Quest Pro and Winlist softwares.

III.1.2. Treatments with certain antineoplastic drugs

The effects of gemcitabine (G6423, Sigma-Aldrich Chemical Co., concentration: 5×10^{-6} mol/dm^3), mitotane (N12706, Sigma-Aldrich Chemical Co., concentration: 5×10^{-6} mol/dm^3), 9-cis-retinoic acid (sc-205589A, Santa Cruz Biotechnology, concentration: 5×10^{-5} mol/dm^3) alone or in combination on NCI-H295R human ACC cell line were investigated. Eight treatment protocols were performed (control, gemcitabine, mitotane, 9-cis-retinoic acid, gemcitabine+mitotane, gemcitabine+9-cis-retinoic acid, mitotane+9-cis-

retinoic acid, gemcitabine+mitotane+9-cis-retinoic acid) for 24, 48 and 72 hours. The effects of different treatments on proliferation, apoptosis, cell cycle distribution, gene and protein expression were investigated.

III.1.3. Proliferation assay

The effect of certain treatment regimens on proliferation was analyzed using alamarBlue reagent (DAL1025, Thermo Fischer Scientific) in 96-well plates.

III.1.4. Cortisol measurements from the media of NCI-H295R cells

Following treatments, the effect of gemcitabine and mitotane on cortisol production of NCI-H295R cells were analyzed by liquid chromatography followed by mass spectrometry (LC-MS). LC-MS measurements were performed on Perkin-Elmer Flexar FX10 UHPLC coupled to a Sciex 5500 QTRAP mass spectrometer.

III.2. RNA isolation, gene and miRNA expression analyses

Total RNA was isolated with miRNeasy Mini Kit (Qiagen), according to the manufacturer's instructions.

III.2.1. High-throughput gene and miRNA expression analyses

Gene expression microarray analysis on total RNA obtained from G1- S- and G2-sorted HDFa, NCI-H295R and HeLa cells on Agilent whole human genome 4x44K microarray slides (Agilent Technologies) according to the manufacturer's instructions were performed. Data was analyzed with GeneSpring 12.6 (Agilent Technologies) software; the

affected biological pathways were identified by Ingenuity Pathway Analysis (IPA, Ingenuity Systems) software.

Three high-throughput screening method (microarray, qRT-PCR-based TLDA and small RNA sequencing) were used for cell cycle dependent miRNA expression profiling on total RNA obtained from G1- S- and G2-sorted HDFa, NCI-H295R and HeLa cells. Microarray measurements were performed on Agilent 8×15K Human miRNA Microarray Release 12.0 slides (Agilent Technologies) on cell cycle sorted HDFa and NCI-H295R samples. TLDA measurements were performed on TaqMan Human MicroRNA Array A and B cards (Applied Biosystems, Life Technologies) from cell cycle sorted NCI-H295R samples. Small RNA sequencing was performed on Illumina HiSeq2000 platform from cell cycle sorted NCI-H295R and HeLa samples. Data was analyzed by GeneSpring 12.6 (Agilent Technologies), Real-Time StatMinerTM (Integromics, Granada, Spain) and edgeR (version 3.8.6) softwares.

Results of high-throughput gene and miRNA expression analyses were validated by qRT-PCR using predesigned TaqMan probes.

III.2.2. Analysis of gene expression levels and cell cycle transcriptional dynamics in primary and cancer cells

Analysis of gene expression levels and cell cycle transcriptional dynamics in different cell types were performed by investigating the changes of 124 genes found to be cell cycle dependently expressed in HDFa and HeLa cells. Upon combined normalization of all cell cycle sort-based gene expression microarrays, normalized intensity values in each cell type in each cell cycle phase were compared. For the analysis of the gene expression dynamism during cell cycle progression the absolute values of fold changes between cell cycle phases were calculated and were subjected to comparison between HDFa, NCI-

H295R and HeLa cell types. Results of qRT-PCR experiments in 10 out of these 124 genes were also subjected to these analyses.

III.3. Protein isolation and Western blot

Samples were thawed on ice, sonicated with ultrasound and incubated on ice for 30 minutes. Thereafter, samples were centrifuged. Protein concentration was determined by the method developed by Bradford. Protein samples were subjected to electrophoretic separation on a 10% poliacrilamide gel and were blotted on polyvinylidene fluoride (PVDF) membrane. After blocking, membranes were incubated overnight with rabbit anti-pospho-CDC-2 (Tyr15) antibody (Cell Signaling Technology, catalog No.: 9111, dilution: 1:500) or goat anti-RRM2 antibody (Santa Cruz Biotechnology, catalog No.: sc-10846, dilution: 1:200). Thereafter, membranes were incubated with the corresponding secondary antibodies. After exposure to SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific), signals were visualized by Kodak Image Station 4000MM Digital Imaging System. Protein loading was monitored by incubating with anti-β-actin antibody (Cell Signaling Technology, catalog No.: 4967, dilution: 1:2000), followed by incubating with secondary antibody and subsequent visualization.

III.4. *In silico* reanalysis of former microarray experiments

The results of the cell cycle dependent transcription program obtained by cell cycle sorting were compared with previous studies identifying the cell cycle dependent transcription program using synchronization procedures. Moreover, in the case of cell cycle dependent transcription program of HeLa cells, the affected biological pathways were analyzed of the following gene lists: genes unique to HeLa cell cycle sort experiment, genes unique to HeLa synchronization experiment and the overlap between HeLa cell cycle sort and synchronization experiments. Additionally, the malignancy signature of ACC (gene expressional differences of malignant ACCs and benign adrenocortical adenomas)

was identified and compared with the cell cycle dependent transcription program of NCI-H295R human ACC cell line.

III.5. Immunohistochemistry on ACC samples

Formalin-fixed paraffin-embedded tissue sections of twelve ACCs, formerly diagnosed histopathologically were chosen for immunohistochemical analysis. 4μm-thick slides were deparaffinized, rehydrated, and the endogenous peroxidases were inhibited. After antigen retrival non-specific binding was blocked. Rabbit monoclonal anti-Ki67 (clone SP6, RM-9106, ThermoScientific, dilution: 1:100) or goat polyclonal anti-RRM2 (sc-10846, Santa Cruz Biotechnology, dilution: 1:100) was used overnight at 4°C. Visualization was performed using DAB Chromogen Kit (Biocare Medical). Ki67 index was determined as the portion of positive cells upon examining 1000 cells. For the characterization of RRM2 immunoreactivity a previously used score system was used.

III.6. Statistical analysis

Upon the microarray data, differently expressed genes and miRNAs between G1, S and G2 phases were detected by one-way ANOVA followed by Tukey's Honestly Significant Difference post hoc test and Benjamini-Hochberg correction for multiple measurements. On the TLDA dataset one-way ANOVA was used to detect significantly altered expression. For the small RNA sequencing data, the classical exact T-Test followed by Benjamini-Hochberg correction for false discovery rate were applied. Student's two-sided paired samples T-test was used to detect difference in normalized expression of genes expressed in a cell cycle dependent manner between various cell types. Student's two-sided independent samples T-test was used to detect difference in absolute values of fold change of cell cycle dependently expressed genes of various cell types. Results of qRT-PCR

measurements were subjected to Student's two-sided independent samples T-test. For correlation analyses Pearson's and Spearman's methods were used.

For detecting differences between drug-treated compared to untreated control groups at each time point in relative proliferation rate, cortisol concentration, apoptosis and cell cycle distribution, Students' two sided independent samples T-test was used.

In all comparisons p-value < 0.05 was considered statistically significant.

IV. Results

IV.1. The cell cycle dependent gene transcription program

Our optimal cell cycle sorting was able to differentiate cells residing in various cell cycle phases in all of the three cell types used (HDFa, NCI-H295R and HeLa cells). The FACS reanalysis and the Western blot of p-cdc-2 on cell cycle sorted samples validated the successful sorting.

Gene expression profiling, followed by rigorous statistical analysis detected 55 mRNA transcripts in NCI-H295R cells and 252 mRNA transcripts in HeLa cells to be expressed in a cell cycle dependent manner. Statistical analysis of HDFa microarray data failed to detect genes with significantly altered expression. In the HDFa experiment gene expression changes of greater than a twofold change (FC2) between cell cycle phases were subjected to further analysis. Gene expression changes observed by qRT-PCR experiments of six genes chosen upon microarray analysis confirmed the microarray results in all of the three cells. As a further confirmation of our method, cell cycle associated molecular and cellular functions were the most concerned biological pathways determined by the functional bioinformatics analysis in all three cell types.

Pearson's method showed significant correlation between gene expression changes observed in synchronization based and cell cycle sort based experiments in the case of primary fibroblasts and HeLa cells, confirming previous synchronization experiments by a synchronization-free method in unperturbed cells.

Additional analysis was performed on the HeLa cell cycle dependent transcriptional program to analyze the possible difference in biological processes affected by cell cycle sort and synchronization procedures. All biological processes detected in the unique HeLa

cell cycle sort list were detected in the overlap list, however, interestingly, five out of eight processes detected in the unique HeLa synchronization list were unique to this list of genes.

Upon the analysis of the magnitude of gene expression alterations during cell cycle progression in untransformed and cancer cells, significantly lower expression values were found in primary untransformed compared to cancer cells in G1, S and G2 phases as well. Moreover, a robustly higher difference in mean fold change of gene expression was observed in G1/S transition in primary fibroblasts compared to cancer cells based on both microarray and qRT-PCR results.

IV.2. Analysis of cell cycle dependent miRNA expression

Three high-throughput platforms (microarray, TLDA and small RNA Sequencing) of miRNA expression were used to detect cell cycle dependent miRNA expression. Among them, microarray displayed the lowest dynamic range and was unable to detect miRNAs of altered expression between cell cycle phases in HDFa and NCI-H295R cells. TLDA performed on RNA isolated from sorted NCI-H295R cells detected 8 miRNAs of altered expression between cell cycle phases. Among the three platforms used in our study, small RNA sequencing was found to have the largest dynamic range in detection of miRNA expression alterations, although only 11 miRNAs were found to have statistically significant expression changes between cell cycle phases in HeLa cells. However, gold standard qRT-PCR measurements failed to validate any significant changes observed by the high-throughput analyses.

IV.3. Identification of a novel proliferation marker in ACC

Upon the reanalysis of the malignancy signature of ACC and the cell cycle dependent transcription program of NCI-H295R human ACC cell line, the majority of the genes of the cell cycle dependent transcription program were present in the malignancy

signature as well. Among them, *RRM2* (ribonucleotide reductase subunit M2) had the largest upregulation in S compared to G1 phase and was therefore chosen for further analysis. RRM2 is a subunit of the ribonucleotide reductase complex (RR), which catalyzes the formation of deoxyribonucleotides from ribonucleotides, which is a rate limiting process during DNA synthesis.

Successful validation of cell cycle dependent expression of *RRM2* in ACC cell line NCI-H295R was performed on cell cycle sorted samples on both mRNA and protein level. RRM2 expression rises in S phase, acquiring an important role in the creation of the ribonucleotide reductase complex.

Upon immunohistochemical analysis of RRM2 and Ki67 in 12 selected ACC samples of various proliferative activities, RRM2 expression was found to be tightly correlated with the widely used proliferation marker Ki67 index.

IV.4. Effects of different antineoplastic drug treatments on NCI-H295R human ACC cell line

The effects of gemcitabine, mitotane and 9-cis-retinoic acid given alone or in combination on NCI-H295R human ACC cell line were analyzed.

Although all treatment regimens decreased proliferation of ACC cells after 72 h, cells treated with gemcitabine alone or in combination showed the largest decrease in proliferation compared to other treatments. Additionally, gemcitabine-mediated decrease in proliferation was detectable at shorter treatment times as well. These differences may partly be the results of the cytotoxic effects of gemcitabine, revealed by higher levels of apoptosis in gemcitabine-treated cells.

Although mitotane treatment inhibited cortisol production as expected, gemcitabine treatment had no effect on cortisol production.

Effects on cell cycle distribution showed a larger portion of G1 phase cells upon gemcitabine treatment. However, 24 h mitotane and 9-cis-retinoic acid treatments and especially their combination resulted in a larger portion of G2 phase cells.

Independently from treatment duration, *RRM2* was found to be upregulated in response to gemcitabine treatment. Combination with other drugs did not alter this effect. Conversely, mitotane and 9-cis-restinoic acid had no effect on *RRM2* expression. Western blot analysis confirmed the gemcitabine-mediated alterations on protein level.

V. Conclusions

1. With the application of cell cycle sorting, I was able to analyze the cell cycle dependent mechanisms without the well known consequences of inhibitive synchronization procedures. My data confirmed numerous members of the cell cycle dependent transcription program in various cell types. It has been already acknowledged that members of the cell cycle dependent transcription program are overexpressed in the malignancy signature of various cancers. My results hypothesize phase-dependent and phase-independent expressional alterations in the background of this observation. Moreover, I hypothesize that lower dynamic expression changes of genes involved in the cell cycle dependent transcription program observed in cancer cells compared to primary, untransformed cells reflect the consequences of malignant transformation.

2. The application of various high-throughput platforms (microarray, TLDA, Small RNA Sequencing) for miRNA profiling on three cell types showed that miRNA expression dynamics are unaltered during the active cell cycle at the G1/S and S/G2 transitions.

3. By comparing the malignancy signature of ACC with the cell cycle dependent transcription program of NCI-H295R human ACC cell line, I identified ribonucleotide reductase subunit M2 (RRM2) as a novel biomarker of proliferation in ACC. Immunohistochemical analysis of human ACC samples confirmed RRM2's possible application in the histopathological diagnosis of these tumors.

4. I confirmed the cytotoxic effects of gemcitabine in NCI-H295R human ACC cell line. The elevated expression of the gemcitabine-target RRM2 in response to gemcitabine treatment is presumably the result of an emerging chemoresistance against gemcitabine, which should be overcome for successful clinical applications.

VI. List of publications related to the Ph.D. theses

1. Grolmusz VK, Toth EA, Baghy K, Liko I, Darvasi O, Kovalszky I, Matko J, Racz K, Patocs A. (2016) Fluorescence activated cell sorting followed by small RNA sequencing reveals stable microRNA expression during cell cycle progression. BMC Genomics, 17(1): 412.

2. Grolmusz VK, Karászi K, Micsik T, Tóth EA, Mészáros K, Karvaly G, Barna G, Szabó PM, Baghy K, Matkó J, Kovalszky I, Tóth M, Rácz K, Igaz P, Patócs A. (2016) Cell cycle dependent RRM2 may serve as proliferation marker and pharmaceutical target in adrenocortical cancer. Am J Cancer Res, 6(9): 2041-2053.

VII. List of publications not related to the Ph.D. theses

1. Takács E, Grolmusz VK, Vértessy BG. (2004) A tradeoff between protein stability and conformational mobility in homotrimeric dUTPases. FEBS Letters, 566(1-3): 48-54.

2. Kender Z, Torzsa P, Grolmusz K V, Patócs A, Lichthammer A, Veresné Bálint M, Rácz K, Reismann P. (2012) A metilglioxál metabolizmus szerepe 2-es típusú cukorbetegségben és szövődményeiben. Orvosi Hetilap, 153(15): 574-85.

3. Feldman K, Szappanos A, Butz H, Grolmusz V, Majnik J, Likó I, Kriszt B, Lakatos P, Tóth M, Rácz K, Patócs A. (2012) The rs4844880 polymorphism in the promoter region of the HSD11B1 gene associates with bone mineral density in healthy and postmenopausal osteoporotic women. Steroids, 77(13): 1345-51.

4. Feldman K, Likó I, Nagy Z, Szappanos A, Grolmusz VK, Tóth M, Rácz K, Patócs A. (2013) A 11-ß-hidroxiszteroid-dehidrogenáz enzim jelentősége klinikai kórképekben. Orvosi Hetilap, 154(8): 283-93.

5. Grolmusz VK, Stenczer B, Fekete T, Szendei G, Patócs A, Rácz K, Reismann P. (2013) Lack of association between C385A functional polymorphism of the fatty acid amide hydrolase gene and polycystic ovary syndrome. Experimental and Clinical Endocrinology & Diabetes, 121(6): 338-42.

6. Kender Z, Fleming T, Kopf S, Torzsa P, Grolmusz V, Herzig S, Schleicher E, Rácz K, Reismann P, Nawroth PP. (2014) Effect of metformin on methylglyoxal metabolism in patients with type 2 diabetes. Experimental and Clinical Endocrinology & Diabetes, 122(5): 316-9.

7. Grolmusz VK, Acs OD, Feldman-Kovács K, Szappanos Á, Stenczer B, Fekete T, Szendei G, Reismann P, Rácz K, Patócs A. (2014) Genetic variants of the HSD11B1 gene promoter may be protective against polycystic ovary syndrome. Molecular Biology Reports, 41(9): 5961-9.

8. Patócs A, Igaz P, Tőke J, Lendvai N, Sarkadi B, Grolmusz V, Butz H, Tóth G, Németh K, Gláz E, Kiss R, Pusztai P, Sármán B, Reismann P, Szücs N, Tóth M, Rácz K. (2016) Örökletes phaechromocytomák és paragangliomák molekuláris genetikai vizsgálatával szerzett hazai tapasztalatok. Magyar Belorvosi Archivum, 69: 83-92.

9. Patócs A, Lendvai NK, Butz H, Liko I, Sapi Z, Szucs N, Toth G, Grolmusz VK, Igaz P, Toth M, Rácz K. (2016) Novel SDHB and TMEM127 Mutations in Patients with Pheochromocytoma/Paraganglioma Syndrome. Pathology and Oncology Research, 22(4): 673-9.