# Gene expression based predictive biomarkers in the systemic therapy of solid tumors

Synopsis of PhD thesis

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# 1. Introduction

In my thesis, I investigate systemic therapy resistance and I focus on identification of predictive biomarkers for chemotherapy and targeted therapy resistance of solid tumors. Resistance is common in all tumor types, and it is the primary reason of systemic therapy failure. Predictive biomarkers can improve the effectiveness of the therapy, can help to maximize the cost-effectiveness of the treatment, and minimize the drug exposure of the patients. Furthermore, understanding the resistance mechanisms can unveil new drug targets. Predictive and prognostic biomarkers are important tools for rational therapy design, and the introduction of biomarkers to clinical practice is expected to refine the current clinical protocols.

I identified biomarkers for therapy resistance in two specific systems: predictive biomarkers of carboplatin resistance of ovarian carcinoma and predictive biomarkers for five tyrosine kinase inhibitors (sunitinib, sorafenib, lapatinib, erlotinib, gefitinib).

# 2. Aims

In the platinum resistance study, the aim was to identify biomarkers of platinum resistance and biomarkers of survival after platinum treatment through the following steps:

- Building a database containing clinical data of platinum treated ovarian carcinoma patients and corresponding microarray data.

- Identifying biomarker candidates based on the database by searching for genes whose high expression correlates with worse therapy response.

- Characterizing the effect of expression and silencing of the biomarker candidates on carboplatin resistance *in vitro.*

- Validation of biomarker candidates which were effective *in vitro* in clinical samples with qRT-PCR and immunohistochemistry.

In the targeted tyrosine kinase inhibitor resistance biomarker study, the goal was to identify predictive biomarkers of sunitinib, sorafenib, lapatinib, erlotinib and gefitinib resistance through the following steps:

- Characterizing the resistance profiles of 45 cancer cell lines against the five tyrosine kinase inhibitors *in vitro.*

- Identify genes (using the microarray data of the 45 cell lines) whose level of expression associates with resistance. Validation of the resistance biomarkers in the cell lines.

- Clinical testing of the sunitnib resistance biomarkers in renal cell carcinoma samples by immunohistochemistry.

# 3. Methods

## 3.1. Methods of the carboplatin resistance study

### 3.1.1. Database, bioinformatics

A database of ovarian cancer transcriptomic datasets including treatment and response information was set up by mining the GEO and TCGA repositories. Microarray data were renormalized, and Receiver Operating Characteristics (ROC) analysis was performed to identify genes whose high expression correlated with worse response to platinum therapy.

### 3.1.2. In vitro experiments

The most significant biomarker candidates were *in vitro* functionally evaluated in four epithelial ovarian cancer cell lines (ES-2, CAOV-3, OVCAR-3, SKOV-3).

MTT assay was used to test the carboplatin sensitivity of the cell lines. Cells were seeded onto 96 well plates and were treated with nine different concentrations of carboplatin for 48 hours in six repeats. Plates were stained with MTT, and the relative viability (compared to vehicle treated controls) was computed for each measurement point. I determined dose-response curves and calculated the IC50 doses with GraphPad Prism.

After measuring the carboplatin sensitivity of each cell line, I investigated the effect of the silencing (by siRNA-s) of the biomarker candidates in the carboplatin sensitivity of the cell lines. The silencing was performed with 30 nM siRNA concentration and Lipofectamine RNAiMax

transfection reagent. The silencing efficacy was measured by qRT-PCR. The eight biomarker candidates were silenced in each cell lines, and cells were treated with IC50 carboplatin dose for 48 hours. I used negative control siRNA as a control. Viability was measured by MTT.

I used flow cytometry to analyze the effect of the silencing of the biomarker candidates on the apoptotic activity of carboplatin treated CAOV-3 cells. After transfection and 48 hour carboplatin treatment FITC Annexin V Apoptosis Detection Kit I was used to measure the apoptotic ratio.

The silencing of MEK1 resulted in significant sensitizing effect and caused apoptotic activation after carboplatin treatment. I tested PD0325901, a MEK1 inhibitor, in the SKOV-3 and ES-2 cell lines alone and in combination with carboplatin to detect possible synergistic effects. MTT test was used to measure the cytotoxic effects.

### 3.1.3. Clinical validation

Fresh-frozen and paraffin embedded (tissue microarray, TMA) samples from ovarian cancer patients were collected. RNA was isolated from the fresh-frozen samples with RNeasy kit. After DNase treatment, concentration and quality check (NanoDrop, Bioanalyzer), I performed reverse transcription. qPCR was used to investigate the expression level of the four biomarker candidates that were efficient *in vitro*.

TMA blocks were cut into 4µm thick sections for immunohistochemistry. After antigen retrieval and peroxidase blocking, the sections were incubated using anti-MEK1 antibody (dilution 1:50)

overnight. The average intensity of four samples per patient was computed for statistical analysis.

## 3.2. Methods of the tyrosine kinase inhibitor resistance study

### 3.2.1. *In vitro* experiments

45 cell lines from various cancer types were cultured, and their sensitivity against five clinically approved tyrosine kinase inhibitors (lapatinib, sorafenib, sunitinib, erlotinib, gefitinib) were tested. Cells were seeded onto 96 well plates and were treated with three concentrations of each drug for 72 hours. MTT assay was used to evaluate the viability. I determined resistance index for each cell line against each compound. Cell lines were ranked based on their increasing resistance indices, and the sensitive, resistant and intermediate cell lines were determined for each compound.

### 3.2.2. Bioinformatics, identification of biomarker candidates

Raw microarray data for the cell lines was gathered from the caArray database, which contains full microarray data of the cell lines in triplicate measurements. After normalization of the data, two statistical algorithms (Rank Products and Significance Analysis of Microarrays) were utilized to identify the genes whose levels of expression were able to discriminate between sensitive and resistant cell lines.

### 3.2.3 Biomarker validation in the cell lines

I isolated RNA from 40 cell lines, and after reverse transcription I measured the expression of 95 selected genes with TaqMan real-time PCR in a Micro Fluidic Card System. The 95 genes composed of the 63 most significant biomarker candidates that we identified and further 32 biomarkers from literature.

### 3.2.4. Clinical validation

Pretreatment paraffin embedded TMA samples from sunitinib treated renal cell carcinoma patients were collected to validate the sunitinib biomarker candidates. Ventana automatized staining system was used for immunohistochemistry. RAB17 (dilution: 1:200), LGALS8 (1:50), EPCAM (1:100) and CD9 (1:300) antibodies were tested. The average staining of two samples per patient was computed for statistical analysis.

# 4. Results

## 4.1. Results of the carboplatin resistance biomarkers study

### 4.1.1. Database, bioinformatics

I identified 1,452 patients in eight datasets meeting our criteria (available therapy response, survival and microarray data) in GEO and TCGA. Of these patients, 1,145 received platinum-based chemotherapy. ROC analysis was performed for all genes, and the eight genes showing the

highest AUC value and highest significance were selected for further experiments. The strongest biomarker candidates are JRK (AUC: 0.62; $p = 1.34 \times 10^{-7}$), CCT3 (AUC: 0.62; $p = 3.5 \times 10^{-7}$,RTF1 (AUC: 0.62; $p = 5.87 \times 10^{-7}$), MEK1 (AUC: 0.61; $p = 1.75 \times 10^{-6}$), FUBP1 (AUC: 0.61; $p = 2.25 \times 10^{-6}$), CNOT8 (AUC: 0.61; $p = 3.07 \times 10^{-6}$), NFATC2IP (AUC: 0.61; $p = 3.69 \times 10^{-6}$), CSDE1 (AUC: 0.6; $p = 4.18 \times 10^{-6}$). Besides the high AUC values, high expressions of JRK ($p = 3.2 \times 10^{-5}$), CNOT8 ($p = 2.2 \times 10^{-4}$), FUBP1 ($p = 0.014$) and MEK1 ($p = 0.0078$) also correlated with worse relapse-free survival.

### 4.1.2. In vitro experiments

Silencing four of the eight investigated genes resulted in significant sensitization effect against carboplatin in all four cell lines, namely RTF1, CSDE1, CNOT8 and MEK1 ($p < 0.01$).

Apoptotic activity after carboplatin treatment in RTF1, CNOT8, MEK1, CSDE1 siRNA transfected cells were measured by flow cytometry. Silencing of MEK1 in CAOV-3 cells caused significant increase in the number of apoptotic cells ($p = 0.0365$) (Annexin V positive cells) and significant decrease in the number of viable cells ($p = 0.0341$) (Annexin V and propidium iodide negative cells) after 48 hours of carboplatin treatment.

The selective MEK1 inhibitor PD0325901 was effective alone in both investigated cells. The combination treatment with carboplatin had stronger cytotoxic effect compared to monotherapies of the

compounds ($p < 0.0001$). Combination of sub-optimal dose of carboplatin with PD0325901 resulted in strong cell death ($p < 0.0001$).

#### 4.1.3. Clinical validation

Fresh-frozen pretreatment ovarian cancer samples were collected from 34 carboplatin treated patients. I measured the expression of the four best biomarker candidates. In the Kaplan-Meier analysis, I found that the lower expression of MEK1 significantly correlated with longer relapse-free survival ($HR = 5.8; p = 0.003$).

Pretreatment samples from 59 platinum treated tumors were paraffin embedded for immunohistochemistry. High staining intensity of MEK1 significantly correlated with worse overall survival after platinum treatment ($HR = 4.2; p = 0.03$).

### 4.2. Results of the tyrosine kinase inhibitor resistance study

#### 4.2.1. In vitro experiments

I investigated the sensitivity of 45 cell lines against five tyrosine kinase inhibitors. I separated the resistant, sensitive and intermediate cell lines against each compound.

#### 4.2.2. Bioinformatics, identification of biomarker candidates

SAM and Rank Products algorithms were used to identify the potential resistance biomarkers against the five tyrosine kinase inhibitors.

### 4.2.3. Biomarker validation in the cell lines

45 of the 63 genes associated with resistance based on the microarray data were able to predict the resistance/sensitivity in the PCR validation with $p < 0.05$ significance. For 23 biomarkers the p value was lower than 0.001. The highest significance was achieved by ITGB4($p = 0.005$) and TFAP2C ($p = 0{,}004$) of the erlotinib-resistance associated biomarkers, by ADA ($p = 0.003$) of the gefitinib-associated genes, by FAT4 ($p = 0.011$) of the sorafenib associated genes and by FURIN and ME1 ($p = 0.011$) of the lapatinib biomarkers. The most significant sunitinib biomarkers in the PCR evaluation were KRT18 ($p = 0.001$), LGALS8 ($p = 0.019$), RAB17 ($p = 0.002$), CD9 ($p = 0.002$) and PPL ($p = 0.001$). Meanwhile, only seven of the 32 genes previously described in the literature as associated with resistance against the targeted tyrosine kinase agents were able to predict the sensitivity/resistance of the cell lines. Only two genes (ANXA3 and RAB25) were correlated to resistance against four compounds.

### 4.2.4. Clinical validation

39 renal cell carcinoma samples were collected from sunitinib treated patients. Kaplan-Meier analysis was preformed to evaluate the correlation between immunohistochemical staining and survival.

In the cell lines, the expression levels of LGALS8, RAB17 and EPCAM were lower in the resistant cell lines compared to the resistant lines. We presumed that high staining correlates with sensitive phenotype and better survival. The increased staining intensity of LGALS8 ($p =$

0.026) and RAB17 ($p = 0.018$) and the frequency of positive cells for EPCAM ($p = 0.01$) and LGALS8 ($p = 0.01$) were correlated to better survival.

# 5. Conclusion

1. Predictive biomarker candidates of carboplatin therapy were identified using the gene expression and clinical data of more than 1000 ovarian carcinoma patients.

2. Silencing or pharmacological inhibition of MEK1 sensitizes the ovarian carcinoma cell lines against carboplatin *in vitro*. High expression (evaluated in RNA and protein level) of MEK1 correlates with wrong survival in carboplatin treated ovarian carcinoma patients.

3. Utilizing measurements of the sensitivity and gene expression profile of 45 cell lines, predictive biomarkers of five clinically approved tyrosine kinase inhibitors were identified.

4. The new biomarkers of sunitinib resistance LGALS8, RAB17 and EPCAM are able to predict survival of sunitinib treated renal cell carcinoma patients clinically.

# 6. List of publications

## 6.1. Publications related to the thesis

Pénzváltó Z, Lánczky A, Lénárt J, Meggyesházi N, Krenács T, Szoboszlai N, Denkert C, Pete I, Győrffy B. (2014) MEK1 is associated with carboplatin resistance and is a prognostic biomarker in epithelial ovarian cancer. BMC Cancer, 14:837. IF: 3,319

Pénzváltó Z, Surowiak P, Győrffy B. (2014) Biomarkers for systemic therapy in ovarian cancer. Current Cancer Drug Targets, 14(3): p. 259-73. IF: 3,582

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Tegze B, Szállási Z, Haltrich I, Pénzváltó Z, Tóth Z, Likó I, Győrffy B. (2012) Parallel evolution under chemotherapy pressure in 29 breast cancer cell lines results in dissimilar mechanisms of resistance. PloS One, 7(2): p. e30804. IF: 3,730

Pénzváltó Z, Mihály Z, Győrffy B. (2009) Génexpresszió mérésén alapuló multigénes prognosztikai és prediktív előrejelzés emlőtumorokban, Magyar Onkológia, 53(4): p. 351-9.

## 6.2. Presentations, posters

Pénzváltó Z, Lánczky A, Lénárt J, Meggyesházi M, Krenács T, Szoboszlai N, Denkert C, Pete I, Győrffy B: *Carboplatin rezisztenciát előrejelző biomarkerek azonosítása petefészek tumorokban,* Magyar Klinikai Onkológusok Társaságának Kongresszusa, Budapest, 2012

Győrffy B, Lánczky A, Pete I, Denkert C, Krenacs T, Meggyeshazi N, Pénzváltó Z.: *Inhibition of MEK1 increases carboplatin sensitivity in ovarian cancer,* American Society of Clinical Oncology, Chicago, 2014 **(J Clin Oncol 32:5s, 2014 (suppl; abstr 5557))**

Pénzváltó Z, Lánczky A, Győrffy B: *Identifying predictive biomarkers of carboplatin resistance in ovarian cancer,* European Cancer Congress, Amsterdam, 2013, **(European Journal of Cancer 49, S737-S738)**

Pénzváltó Z, Lánczky A, Győrffy B: *Biomarkers of platinum resistance in ovarian cancer,* 17th International AEK Congress, Heidelberg, 2013

Pénzváltó Z, Lánczky A, Győrffy B: *A carboplatin rezisztencia prediktív biomarkerei petefészek tumorokban,* Magyar Onkológusok Gyógyszerterápiás Tudományos Társasága 7. Kongresszusa, Budapest, 2013

Pénzváltó Z, Lánczky A, Győrffy B: *Predictive biomarkers of carboplatin resistance in ovarian cancer,* Semmelweis Egyetem PhD Tudományos Napok, Budapest, 2013

Pénzváltó Z. *A RAS/ErbB útvonalon ható célzott terápiás szerekkel szembeni rezisztencia biomarkerek fejlesztése,* Magyar Klinikai Onkológusok Társaságának Kongresszusa, Budapest, 2012

Mihály Z, Pénzváltó Z, Győrffy B: *Utilizing microarray for investigation of trastuzumab resistance biomarkers,* Magyar Klinikai Onkológusok Társaságának Kongresszusa, Budapest, 2012

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Pénzváltó Z, Tegze B, Szász AM, Sztupinszki Z, Likó I, Szendrői A, Győrffy B: *Öt tirozin kináz inhibitorral szembeni rezisztencia faktorok azonosítása 45 sejtvonalon,* Magyar Onkológusok Társaságának Kongresszusa, Budapest, 2011

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Tegze B, Munkácsy G, Pénzváltó Z, B Győrffy: *Rezisztens sejtvonalak létrehozása MDA-MB-231 és MCF7 emlőrák sejtvonalakból a*

*párhuzamosan kialakuló kemorezisztencia kialakulásának modellezésére*, Semmelweis Egyetem PhD Tudományos Napok, Budapest, 2010

Pénzváltó Z, Mihály Z.: *Doxorubicin intracelluláris lokalizációja és a kemorezisztencia közötti összefüggés sejtvonalakban,* Semmelweis Egyetem Tudományos Diákköri Konferencia, Budapest, 2010

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