# Final report

Title: **Utilization of cell culture techniques and a systems biology approach to develop a model of resistance against targeted therapy agents**

OTKA: **PD83154**

PI: **Dr. Balázs Győrffy**

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## Contents

# 1. THE RESEARCH PROJECT

## 1.1. Experimental studies

The experimental research project was executed as planned in the original grant application. **According to the grant reporting guideline, there is no need for repeated description for the already published results.** For a brief summary, please see section 1.4.2.

## 1.2. Clinical studies

In contrast to the original work plan we did not had sufficient number of lung cancer samples to validate our model. However, by initiating a collaboration with the Dept. of Urology of the Semmelweis University, we acquired a cohort of Sunitinib-treated renal cell cancer samples and validated a set of genes in these. For results, please see section 1.4.2.

## 1.3. Project participants

This grant was a PD (postdoctoral), and the realization of the project included the originally planned participants. The clinical participant delivering the RCC samples were included as collaboration partners in the study.

## 1.4. Main publications of the OTKA project

### 1.4.1. Current Cancer Drug Targets (2012, last author)

In this study, we gave a comprehensive overview of current advances in the systemic treatment of metastatic renal cell carcinoma. Renal cell carcinoma (RCC) was chosen as the subject of the review publication, because we have used RCC samples to validate our strongest genes. For RCC, chemotherapy and immunotherapy failed to deliver decisive results in the systemic treatment of metastatic renal cell carcinoma. Agents representing the current standards operate on members of the RAS signal transduction pathway. Sunitinib (targeting vascular endothelial growth factor), temsirolimus (an inhibitor of the mammalian target of rapamycin - mTOR) and pazopanib (a multi-targeted receptor tyrosine kinase inhibitor) are used in the first line of recurrent disease. A combination of bevacizumab (inhibition of angiogenesis) plus interferon is also first-line therapy. Second line options include everolimus (another mTOR inhibitor) as well as tyrosine kinase inhibitors for patients who previously received cytokine. We reviewed the results of clinical investigations focusing on survival benefit for these agents. Additionally, trials focusing on new agents, including the kinase inhibitors axitinib, tivozanib, dovitinib and cediranib and monoclonal antibodies including velociximab were also discussed.

### 1.4.2. PLoS One (2013, last author)

Because of the low overall response rates of 10–47% to targeted cancer therapeutics, there is an increasing need for predictive biomarkers. We aimed to identify genes predicting response to five already approved tyrosine kinase inhibitors. We tested 45 cancer cell lines for sensitivity to sunitinib, erlotinib, lapatinib, sorafenib and gefitinib at the clinically administered doses. A resistance matrix was determined, and gene expression profiles of the subsets of resistant vs. sensitive cell lines were compared. Triplicate gene expression signatures were obtained from the caArray project. Significance analysis of microarrays and rank products were applied for feature selection. Ninety-five genes were also measured by RT-PCR. In case of four sunitinib resistance associated genes, the results were

validated in clinical samples by immunohistochemistry. A list of 63 top genes associated with resistance against the five tyrosine kinase inhibitors was identified. Quantitative RT-PCR analysis confirmed 45 of 63 genes identified by microarray analysis. Only two genes (ANXA3 and RAB25) were related to sensitivity against more than three inhibitors. The immunohistochemical analysis of sunitinib-treated metastatic renal cell carcinomas confirmed the correlation between RAB17, LGALS8, and EPCAM and overall survival. In summary, we determined predictive biomarkers for five tyrosine kinase inhibitors, and validated sunitinib resistance biomarkers by immunohistochemistry in an independent patient cohort. The primary results of the OTKA project were published in this paper.

## 2. ADDITIONAL PUBLICATIONS RELATED TO THE OTKA PROJECT

These studies were not directly included in the original research plan. However,

1. in these projects we focused on cancer therapy response and survival,
2. various resources of the OTKA support were used,
3. the OTKA support is also acknowledged in each of these papers.

Below I give a brief summary for these projects.

### 2.1. Cell Reports (2013, second author)

Prognosis for patients with estrogen-receptor (ER)-negative basal breast cancer is poor, and chemo-therapy is currently the best therapeutic option. In this study, we have generated a compound-mutant mouse model combining the activation of b-catenin and HGF (Wnt-Met signaling), which produced rapidly growing basal mammary gland tumors. We identified the chemokine system CXCL12/CXCR4 as a crucial driver of Wnt-Met tumors, given that compound-mutant mice also deficient in the CXCR4 gene were tumor resistant. Wnt-Met activation rapidly expanded a population of cancer-propagating cells, in which the two signaling systems control different functions, self renewal and differentiation. Molecular therapy targeting Wnt, Met, and CXCR4 in mice significantly delayed tumor development. The expression of a Wnt-Met 322 gene signature was found to be predictive of poor survival of human patients with ER-negative breast cancers. We concluded that targeting CXCR4 and its upstream activators, Wnt and Met, might provide an efficient strategy for breast cancer treatment.

### 2.2. International Journal of Cancer (2012, last author)

Transcriptomic analysis of global gene expression in ovarian carcinoma can identify dysregulated genes capable to serve as molecular markers for histology subtypes and survival. Our aim in this study was to validate previous candidate signatures in an independent setting and to identify single genes capable to serve as biomarkers for ovarian cancer progression. As several datasets are available in the GEO today, we were able to perform a true meta-analysis. First, 829 samples (11 datasets) were downloaded, and the predictive power of 16 previously published gene sets was assessed. Of these, eight were capable to discriminate histology subtypes, and none was capable to predict survival. To overcome the differences in previous studies, we used the 829 samples to identify new predictors. Then, we collected 64 ovarian cancer samples (median relapse-free survival 24.5 months) and performed TaqMan Real Time Polimerase Chain Reaction (RT-PCR) analysis for the best 40 genes associated with histology subtypes and survival. Over 90% of subtype-associated genes were confirmed. Overall survival was effectively predicted by hormone receptors (PGR and ESR2) and by

TSPAN8. Relapse-free survival was predicted by MAPT and SNCG. Thus, in this project we successfully validated several gene sets in a meta-analysis in large datasets of ovarian samples.

### 2.3. PLoS One (2012, last author)

Developing chemotherapy resistant cell lines can help to identify markers of resistance. Instead of using a panel of highly heterogeneous cell lines, we assumed that truly robust and convergent pattern of resistance can be identified in multiple parallel engineered derivatives of only a few parental cell lines. Parallel cell populations were initiated for two breast cancer cell lines (MDA-MB-231 and MCF-7) and these were treated independently for 18 months with doxorubicin or paclitaxel. IC50 values against 4 chemotherapy agents were determined to measure cross-resistance. Chromosomal instability and karyotypic changes were determined by cytogenetics. TaqMan RT-PCR measurements were performed for resistance-candidate genes. Pgp activity was measured by FACS. All together 16 doxorubicin- and 13 paclitaxel-treated cell lines were developed showing 2–46 fold and 3–28 fold increase in resistance, respectively. The RT-PCR and FACS analyses confirmed changes in tubulin isoform composition, TOP2A and MVP expression and activity of transport pumps (ABCB1, ABCG2). Cytogenetics showed less chromosomes but more structural aberrations in the resistant cells. We surpassed previous studies by parallel developing a massive number of cell lines to investigate chemoresistance. While the heterogeneity caused evolution of multiple resistant clones with different resistance characteristics, the activation of only a few mechanisms were sufficient in one cell line to achieve resistance.

### 2.4. Endocrine-Related Cancer (2012, first author)

The validation of prognostic biomarkers in large independent patient cohorts is a major bottleneck in cancer research. We implemented an online tool to assess the prognostic value of the expression levels of all microarray-quantified genes in ovarian cancer patients. First, a database was set up using gene expression data and survival information of 1287 ovarian cancer patients downloaded from Gene Expression Omnibus and The Cancer Genome Atlas (Affymetrix HG-U133A, HG-U133A 2.0, and HG-U133 Plus 2.0 microarrays). After quality control and normalization, only probes present on all three Affymetrix platforms were retained (n Z 22 277). To analyze the prognostic value of the selected gene, we divided the patients into two groups according to various quantile expressions of the gene. These groups were then compared using progression-free survival (n Z 1090) or overall survival (n Z 1287). A Kaplan–Meier survival plot was generated and significance was computed. The tool can be accessed online at www.kmplot.com/ovar. We used this integrative data analysis tool to validate the prognostic power of 37 biomarkers identified in the literature. Of these, CA125, CDKN1B, KLK6, IFNG, P16, and BIRC5 were associated with survival. The combination of several probe sets can further increase prediction efficiency. In brief, we developed a global online biomarker validation platform that mines all available microarray data to assess the prognostic power of 22 277 genes in 1287 ovarian cancer patients.

### 2.5. PLoS One (2012, co-author)

Gene or protein expression data are usually represented by metric or at least ordinal variables. In order to translate a continuous variable into a clinical decision, it is necessary to determine a cutoff point and to stratify patients into two groups each requiring a different kind of treatment. Currently, there is no standard method or standard software for biomarker cutoff determination. Therefore, we

developed Cutoff Finder, a bundle of optimization and visualization methods for cutoff determination that is accessible online. While one of the methods for cutoff optimization is based solely on the distribution of the marker under investigation, other methods optimize the correlation of the dichotomization with respect to an outcome or survival variable. We illustrated the functionality of Cutoff Finder by the analysis of the gene expression of estrogen receptor (ER) and progesterone receptor (PgR) in breast cancer tissues.

### 2.6. Breast Cancer Research and Treatment (2013, last author)

To date, three molecular markers (ER, PR and CYP2D6) have been used in clinical setting to predict the benefit of the anti-estrogen tamoxifen therapy. In this study our aim was to validate new biomarker candidates predicting response to tamoxifen treatment in breast cancer by evaluating these in a meta-analysis of available transcriptomic datasets with known treatment and follow-up. Biomarker candidates were identified in Pubmed and in the 2007-2012 ASCO and 2011-2012 SABCS abstracts. Breast cancer microarray datasets of endocrine-therapy treated patients were downloaded from GEO and EGA and RNAseq datasets from TCGA. Of the biomarker candidates, only those identified or already validated in a clinical cohort were included. Relapse-free survival (RFS) up to 5 years was used as endpoint in a ROC analysis in the GEO and RNAseq datasets. In the EGA dataset, Kaplan-Meier analysis was performed for overall survival (OS). Statistical significance was set at $p<0.01$. The transcriptomic datasets included 665 GEO-based and 1,208 EGA-based patient samples. All together 68 biomarker candidates were identified. Of these, the best performing genes were PGR (AUC=0.64, p=2.3E-07), MAPT (AUC=0.62, p=7.8E-05), and SLC7A5 (AUC=0.62, p=9.2E-05). Further genes significantly correlated to relapse-free survival include FOS, TP53, BTG2, HOXB7, DRG1, CXCL10 and TPM4. In the RNAseq dataset, only ERBB2, EDF1 and MAPK1 reached statistical significance.

### 2.7. Protein and Peptide Letters (2013, co-author)

Text mining methods can facilitate the generation of biomedical hypotheses by suggesting novel associations between diseases and genes. Since many current medical hypotheses are formulated in terms of molecular entities and molecular mechanisms, here we extend the previously established RaJoLink methodology to proteins and genes, using a standardized vocabulary as well as a gene/protein network model. The proposed enhanced RaJoLink rare-term model combines text mining and gene prioritization approaches. We illustrated it utility by finding known as well as potential gene-disease associations in ovarian cancer using MEDLINE abstracts and the STRING database.

### 2.8. Microarrays (2013, last author)

In this review publication, we summarize the current state regarding clinical applications of microarrays in breast cancer molecular pathology. Breast cancer research has paved the way of personalized oncology with the introduction of hormonal therapy and the measurement of estrogen receptor as the first widely accepted clinical biomarker. The expression of another receptor—HER2/ERBB2/neu—was initially a sign of worse prognosis, but targeted therapy has granted improved outcome for these patients so that today HER2

positive patients have better prognosis than HER2 negative patients. Later, the introduction of multigene assays provided the pathologists with an unbiased assessment of the tumors' molecular fingerprint. The recent FDA approval of complete microarray pipelines has opened new possibilities for the objective classification of breast cancer samples. Here we review the applications of microarrays for determining ER and HER2 status, molecular subtypes as well as predicting prognosis and grade for breast cancer patients. An open question remains the role of single genes within such signatures. Openly available microarray datasets enable the execution of an independent cross-validation of new marker and signature candidates.

## 3. INDEPENDENT PUBLICATIONS

During the research project, I also participated in a set of publications not related to the OTKA project. In these, the OTKA support was not acknowledged. However, I thank OTKA for financing my primary project which also enabled to participate in these independent studies as well. These studies resulted in following publications:


1. Magnani L, Stoeck A, Zhang X, Lánczky A, Mirabella AC, Wang TL, Győrffy B, Lupien M. Genome-wide reprogramming of the chromatin landscape underlies endocrine therapy resistance in breast cancer. **Proc Natl Acad Sci U S A.** 2013 Apr 16;110(16):E1490-9. doi: 10.1073/pnas.1219992110.
2. Győrffy B, Benke Z, Lánczky A, Balázs B, Szállási Z, Timár J, Schäfer R. RecurrenceOnline: an online analysis tool to determine breast cancer recurrence and hormone receptor status using microarray data, **Breast Cancer Res Treat**, 2012;132:1025–1034.
3. Malek A, Győrffy B, Catapano CV, Schäfer R. Selection of optimal combinations of target genes for therapeutic multi-gene silencing based on miRNA co-regulation. **Cancer Gene Therapy,** 2013 May;20(5):326-9. doi: 10.1038/cgt.2013.20
4. Bockmayr M, Klauschen F, Győrffy B, Denkert C, Budczies J. New network topology approaches reveal differential correlation patterns in breast cancer. **BMC Syst Biol.** 2013 Aug 15;7:78. doi: 10.1186/1752-0509-7-78
5. Szász AM, Eklund AC, Li Q, Sztupinszki Z, Tőkés AM, Rowan A, Székely B, Kiss A, Szendrői M, Győrffy B, Szállási Z, Swanton C, Kulka J. The CIN4 chromosomal instability qPCR classifier defines tumour aneuploidy and stratifies outcome in grade 2 breast cancer. **PLoS One**, 2013;8(2):e56707. doi: 10.1371/journal.pone.0056707.
6. Porter DC, Farmaki E, Altilia S, Schools GP, West DK, Chen M, Chang DB, Puzyrev AT, Lim C, Rokow-Kittell R, Friedhoff RT, Papavassiliou AG, Kalurupalle S, Hurteau G, Shi J, Baran PS, Győrffy B, Wentland MP, Broude EV, Kiaris H, Roninson IB. CDK8 mediates chemotherapy-induced tumor-promoting paracrine activities. **Proc Natl Acad Sci U S A.** 2012 Aug 21;109(34):13799-804.
7. Budczies J, Denkert C, Müller BM, Brockmöller SF, Klauschen F, Györffy B, Dietel M, Richter-Ehrenstein C, Marten U, Salek RM, Griffin JL, Hilvo M, Orešič M, Wohlgemuth G, Fiehn O. Remodeling of central metabolism in invasive breast cancer compared to normal breast tissue - a GC-TOFMS based metabolomics study. **BMC Genomics**. 2012 Jul 23;13:334.
8. Halon A, Nowak-Markwitz E, Donizy P, Matkowski R, Maciejczyk A, Gansukh T, Győrffy B, Spaczynski M, Zabel M, Lage H, Surowiak P. Enhanced Immunoreactivity of TIMP-2 in the Stromal Compartment of Tumor as a Marker of Favorable Prognosis in Ovarian Cancer Patients. **J Histochem Cytochem**. 2012 Jul;60(7):491-501.