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Molecular background of acquired resistance of *BRAF*^{V600E} mutated human melanoma

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INTRODUCTION

Malignant melanoma of the skin is potentially the most serious type of skin cancer for both men and women, with an ever-increasing global occurrence. Although it only accounts for 1% of all skin cancers, it is responsible for the great majority of skin cancer-related deaths.

In men, melanoma mainly occurs on the back, while in women it is most common on the legs. The individual risk of emerging melanoma increases with fair skin, blond or red hair, blue eyes, a greater number of nevi, and freckles phenotypes. Additionally, emerging evidence suggests that excessive ultraviolet-B (UVB) radiation exposure during childhood, particularly sunburns, is linked to an increased risk of developing melanoma. Furthermore, the accumulation of genetic and epigenetic alteration promotes uncontrolled cell proliferation, and escape of melanoma cells from programmed cell death in response to DNA damage that leads to melanoma. In addition to this, a series of gene mutations in mitogen-activated protein kinase (MAPK) signalling molecules, results in constitutive activation of the MAPK pathway leading to uncontrolled cell proliferation.

A number of therapies for advanced stage melanoma have been approved over the last decade by the Food and Drug Administration (FDA). The treatment options were determined based on the location of the lesions, stage, and genetic profile of the tumours. The treatment options may include the following parameters: surgical resection, radiation, chemotherapy, photodynamic therapy (PDT), targeted therapy, or immunotherapy alone or in combination. However, surgical treatment is still the primary clinical option for melanoma patients with stages I-IIIB.

The most common genetic alterations in malignant melanoma include the activating mutations in the *BRAF* oncogene, 45-60% of patient's tumours carry mutations within this gene. *BRAF* mutations are one of the most effective therapeutic targets for advanced stage and metastatic melanoma. The most promising treatment strategy for metastatic and unresectable *BRAF*-mutated melanomas is the use of BRAF inhibitors (vemurafenib, dabrafenib, encorafenib etc.), either alone or in combination with MEK inhibitors (cobimetinib, trametinib, binimetinib etc.). Other effective therapeutic options include immune checkpoint blockade therapies such as anti-PD-1, anti-PD-L1 and anti-CTLA-4, either alone or in combination. With these breakthroughs therapeutic treatment options, patients with metastatic melanoma have experienced increased median overall survival from 9 months to more than 2 years, and in some instances, have achieved long-term remission or complete response.

Molecular background of malignant melanoma

Among all tumour types, malignant melanoma is characterized by the highest mutational burden. The disease is triggered by both hereditary and environmental factors (UV radiation). Melanoma development is primarily due to sporadic-, rather than inherited mutations. Approximately 10% of primary melanoma cases are familial and the *CDKN2A* tumour suppressor gene is the most commonly altered gene in familial cancers.

Using high throughput molecular technologies and bioinformatics analyses, a large amount of melanoma tissue samples has been analysed and classified into four subgroups according to activating gene mutations, including *BRAF*- (~50%); *N-Ras*, *K-Ras*, and *H-Ras*- (~25%); *NF1*-mutant melanomas (~15%), and triple-wild-type melanomas (~10%). Additionally, copy number alterations including amplifications of the *KIT*, *MITF*, *CCND1*, and *CDK4* genes, and deletions of the *PTEN* and *CDKN2A* tumour suppressor genes are characteristic for the triple-wild-type tumours. Other genetic alterations include activating mutation of the

promoter region of the *TERT* gene (30-80%). In addition, numerous tumour suppressor genes, including *NF1*, *PTEN*, and *ARID2*, are commonly mutated in melanoma.

MAPK/ERK pathway

Malignant cells emerge as a result of alterations in key signalling pathways. Melanoma cell proliferation signalling is primarily mediated by MAPK pathway (Ras/Raf/MEK/ERK), which is over-activated in roughly 80-90% of cases. Additionally, this signalling pathway has been shown to contribute to melanoma progression such as growth, angiogenesis, invasion, and resistance to therapy. The majority of *BRAF* mutations occur at the V600E position (Val600Glu), while in the *RAS* gene, the majority of mutations are associated with the replacement of glutamine at position 61 ($NRAS^{Q61}$) for an arginine, lysine, or leucine (Q61R/K/L), although it may also affect *HRAS* and *KRAS*.

A cascade of serine/threonine protein kinases, known as the MAPK/ERK pathway, functions within the context of extracellular mitogenic stimuli and transcriptional programming in order to induce downstream signalling. Several sequential events trigger the MAPK pathway, such as the *RAS* phosphorylating *RAF* kinase, which subsequently phosphorylates and activates *MEK1/2*, which phosphorylates and nuclear translocate *ERK1/2*, resulting in cell cycle progression and growth.

Current and novel targeted therapies in melanoma

Since our understanding of the molecular pathways and related genetic abnormalities implicated in malignant melanoma has grown, effective immunotherapies and targeted therapies for unresectable stage III and IV melanoma are now available to restore cellular homeostasis. However, surgery, as the primary treatment option for localized melanoma, remains a very successful disease management approach. Until 2011, dacarbazine chemotherapy was the

"gold standard" for advanced-stage melanoma. Since 2011, the FDA has approved a variety of therapy approaches that have significantly improved overall survival (OS) for patients with metastatic melanoma.

Following the discovery of *BRAF* mutations in numerous malignancies, including cutaneous melanoma, molecular targeted treatments were developed. As a result of this breakthrough, selective BRAF inhibitors were tested as single agents in patients with metastatic melanoma and demonstrated exceptional clinical efficacy. Following the success of single-agent *BRAF* inhibition, BRAFi/MEKi combination were studied based on *BRAF* inhibition's clinical efficacy and the significance of downstream MAPK pathway signalling.

BRAF and MEK inhibitors

In the last two decades, the targeting of melanoma-bearing mutations has been refined through the implementation of new molecular approaches. MAPK signalling is regulated by a serine-threonine kinase known as *BRAF*, and 50% of melanomas that do not have persistent UV exposure carry a mutation in *BRAF* gene. Mutations in the *BRAF* gene lead to increased proliferation and growth of cancer cells through activation of MAPK signalling pathway.

In 2011, vemurafenib (a selective BRAF inhibitor) was approved for the treatment of unresectable metastatic melanomas with $BRAF^{V600E}$ mutations by the FDA. In addition to vemurafenib, other BRAF inhibitor with the same mechanism of action, dabrafenib and encorafenib, were approved by the FDA for the treatment of unresectable metastatic melanomas with $BRAF^{V600E}$ mutations.

Approaches that target downstream signalling of driver oncogenes are effective in overcoming resistance to BRAF inhibitors. Since *MEK* is a downstream signalling component in the MAPK pathway through which *ERK1/2* is stimulated; hence, the FDA and the European Medicines Agency (EMA) have approved series of MEK1/2 inhibitor (trametinib, cobimetinib, and binimetinib) as monotherapy or in combination with BRAF inhibitors to treat *BRAF*-mutant

advanced melanoma. *MEK1/2* blockade results in a reduction of cell proliferation in tumour cells, as downstream signalling. Following the first two BRAF and MEK inhibitor combination (dabrafenib+trametinib, vemurafenib+cobimetinib), FDA authorized a third BRAF and MEK inhibitor combo, encorafenib+binimetinib in 2018, for patients with unresectable or metastatic melanoma with *BRAF* $^{V600E/K}$ mutations. The BRAF and MEK inhibitors combination shows higher response rates, longer progression-free survival, and longer survival over chemotherapy.

Resistance to BRAF inhibitors

Activation of the MAPK pathway is essential for many cellular processes, such as cell growth and differentiation. Furthermore, in normal cells, the physiological upstream negative feedback response inhibits sustained MAPK-pathway activation; but, in melanoma cells with *BRAF*^{V600} mutations, this mechanism is dysregulated, resulting in constitutive MAPK pathway activation. Targeting *BRAF*^{V600} mutant cells by using selective BRAF inhibitor become one of the most successful treatment strategies to treat melanoma.

Patients that acquired BRAF inhibitor resistance were shown to have reactivation of the MAPK pathway, higher levels of *pERK*, and multiple *MEK1/2* mutations. In light of this, a combination of BRAF and MEK inhibitors (BRAFi/MEKi) was selected instead of BRAFi alone since it provides more potent inhibition of the MAPK pathway with durable response and lower rates of drug-induced toxicity. Unfortunately, even when combination treatment is used, the majority of patients develop resistance and tumour relapse.

BRAFi resistance is primarily due to MAPK pathway activation, or activation of alternative survival and proliferation pathways. There are three chronologically distinct phases of resistance: (i) within a day, changes in cellular signalling result in a new homeostasis; (ii) within a month, epigenetic, immuno-, and micro-

environmental adaptation results in tolerance; and (iii) after months (to years), genetic mutations result in the outgrowth of resistant clones.

Importance of 3D cell culture

The tumour infiltrating cells and tumour types are exceedingly diverse, adding to the complexity of the disease. As a result, developing novel therapies is a persistent, critical, and difficult process. *In vitro* drug screening is a low-cost, commonly used method for identifying and selecting drugs of greater therapeutic potential. Two-dimensional (2D) flat monolayer cell culture is currently the most commonly used method for cell-based assay analysis because it is easy and convenient. However, 2D cultures do not accurately mimic tumour complexity or human physiology. In 2D cell culture, cells developed more contact space with the plastic surface than neighbouring cells, resulting in decreased cell-to-cell interaction and cell to extracellular matrix interaction. The irrelevant 2D environment can generate misleading results when it comes to cancer cell predicted responses to anticancer drugs.

The primary limitation of conventional 2D cell culture systems led to the development of 3D cell culture systems, which promise to reduce inconsistency between cell-based assays and *in vivo* drug screening. Three-dimensional (3D) culture systems are becoming more common due to their potential to replicate tissue-like structures more efficiently than monolayer cultures. This approach helps cells to expand and communicate with their surroundings. Taken together, it has the potential to minimise the gap between conventional 2D culture and animal research, and it is critical for future tumour biology studies.

OBJECTIVES

Despite intensive research efforts, which have significantly improved melanoma patient survival, therapy resistance to the targeted mono- and combined therapies remains unsolved problems. The major focus of our study was to

investigate molecular alterations associated with acquired resistance in malignant melanoma cell line models.

B*ecause m*ajority of currently available data on drug resistance have been obtained from 2D *in vitro* cell cultures, we aimed to*:*

- develop reproducible three-dimensional melanoma spheroid models from *BRAF*^{V600E} mutant melanoma cell lines that are sensitive and resistant to a BRAF inhibitor (BRAFi)
- compare the gene expression signature of the sensitive and resistant melanoma cell lines grown under 2D and 3D cell culture conditions,
- define genes that are differently expressed between differently cultures cells.

In parallel, our aim was to explore the molecular background associated with acquired resistance of melanoma cells during combinatory treatment using BRAF and MEK inhibitors:

- in order to reach our goal, we established human melanoma cell lines resistant to encorafenib (BRAF inhibitor) plus binimetinib (MEK inhibitor),
- evaluated the invasive properties of drug-sensitive and drug-resistant cell lines,
- studied the effect of "drug holiday" on cell proliferation and protein expression in the drug sensitive and resistant cell lines,
- investigated and compared the gene expression pattern of BRAFi/MEKi resistance using RNAseq analyses,
- defined the biological functions of the differently expressed genes linked to the development of resistance.

MATERIALS AND METHODS

Cell lines and culture conditions

A total of 9 human melanoma cell lines were included into our study. These cells were purchased from the Coriell Institute for Medical Research (Camden, New Jersey, USA). All cell lines were cultured at 37°C in a humidified incubator with 5% CO_2 and 95% air using RPMI 1640 (Lonza Group Ltd, Basel, Switzerland) supplemented with 10% of foetal bovine serum (FBS) (Gibco, Carlsbad, California, USA), 2 mmol/l glutamine, and 50 mg/ml gentamycin sulphate. Once the cells had reached 70% confluence, they were passaged with trypsin (IITD PAN, Wroclaw, Poland) in standard 25 cm^2 flasks. The WM1366 cell line was wild-type for the $BRAF^{V600E}$, but exhibited the *NRAS* mutation, while the WM3211 cell line was wild-type for both gene.

Development of BRAFi (PLX4720) resistant cell lines under 2D and 3D cell culture conditions

BRAFi (PLX4720) resistant cell lines were established using two cell lines (WM983A and WM983B), by continuously increasing the concentration of a vemurafenib analogue PLX4720. The resistant cell lines were designated as $WM983A^{RES}$ and $WM983B^{RES}$.

To develop spheroids, BRAFi sensitive and resistant cells were seeded using $1.8x10^4$ cells/well into Corning® Costar® Ultra-Low Attachment (6 well) plates containing RPMI-1640 supplemented with 2 mmol/l glutamine and 50 mg/ml penicillin and streptomycin. After 72 hours the medium was supplemented with 10%, FBS. The cells were grown for one week, then the visible spheroids were transferred into a cell culture flask (T/25) and leaved to attach (~ 6 hours). After attachment, the spheroids were washed, cell debris were removed using 1xPBS. Spheroids were assigned as $WM983A^{SPH}$, $WM983B^{SPH}$, $WM983A^{RES\text{-}SPH}$ and $WM983B^{RES\text{-}SPH}$.

Development of BRAFi (encorafenib) + MEKi (binimetinib) resistant cell lines

Nine melanoma cell lines (WM983A, WM983B, WM278, WM1617, WM902B, WM793B, WM35, WM1366 and WM3211) were treated in combination with encorafenib (BRAFi: ENCO) + binimetinib (MEKi: BINI). Resistant cell lines were generated through long-term high-dose treatment from six cell lines using increasing concentrations of ENCO+BINI. Unless otherwise indicated, the resistant cells were maintained in complete medium supplemented with 200 nmol/l inhibitor mixture to prevent resistance loss. Comparisons of the different parameters (cell viability, invasive potential, protein expression, and transcriptome profile) between the sensitive and resistant cells were performed at the same passage number.

Cell proliferation assay

To assess the viability of cells, WST-1 assay were used in accordance with the manufacturer's instructions. In brief, 5 x 10^3 cells were seeded in triplicate on 96-well plates and grown in 100 µL growth medium for 24 hours. On the following day, the medium was replaced with fresh growth medium enriched with the combination of 1 µmol/l of ENCO+BINI for 72 hours. DMSO was used during the control experiment. Then, 10 µL WST-1 reagent was directly added to each well, and cells were incubated at 37°C for three hours. Absorbance at 440 nm was measured using an Epoch™ Microplate Spectrophotometer (BioTek Instruments, Winooski, Vermont, USA), reference absorbance was set to 650 nm. The viability of the cells was defined as follows: dividing the absorbance of the ENCO+BINI treated cells by that of the DMSO-treated control cells (the absorbance of control cells was defined as 100%).

Drug holiday experiment

In the drug holiday experiments, resistant cells (5 × 10^3 cells/well/100 µL medium) were seeded into 96-well plates in triplicate containing combination of

200 nmol/l ENCO+BINI in each well for 24 hours. The next day, cells were divided into two groups: the first half of each cell line was switched to growth medium enriched with drug for 72 hours, while the other half was retained in growth medium containing the DMSO (0.5 %) for 72 hours. Then, 10 µL of WST-1 reagent was directly added to each well, and the cells were incubated at 37°C for the following three hours. Absorbance was measured as described above. Percentage of viable cells was calculated from relative absorbance.

In-vitro invasion assay

The invasive potential of the inhibitor (ENCO+BINI)-sensitive and -resistant melanoma cells was evaluated using BioCoat Matrigel Invasion. Shortly, for BRAFi/MEKi-sensitive cells, the upper part of the invasion chamber was filled with a serum-free cell suspension (500 µL), and growth medium with 10% FBS was used (as a chemoattractant) in the lower chamber. For BRAFi/MEKi-resistant cells, we filled the upper chamber with 500 µL of the melanoma cells (cells were kept in serum-free medium containing a 200 nmol/l combination of the inhibitors). The culture medium in the lower chamber was supplemented with 10% FBS (as a chemoattractant) and a 200 nmol/l combination of ENCO+BINI. The invaded cells were fixed with ice-cold methanol after 24-h incubation and stained with haematoxylin-eosin. Invasive cells were counted under microscope (200x magnification) in seven different areas.

Protein expression analysis

The Proteome Profiler™ Human XL Oncology Array Kit was obtained from R&D Systems and the experiments were performed according to manufacturer's protocol. The protein expressions (labelled spots on the membrane) were exposed using the Azure c300 Chemiluminescent Imaging System and were analysed using AzureSpot (Version: 2.2.167) software. The intensity of the positive control (reference spot) was considered 100%.

RNA Isolation and Microarray Hybridization

RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) was used for total RNA isolation. Concentration of RNA was measured using NanoDrop ND-1000 UV-Vis spectrophotometer. Only samples with ratios >1.8 (measured at 260/280 nm) were included in further analysis. An Agilent 2100 Bioanalyzer was used to evaluate sample quality before RNA sequencing (Agilent Technologies Inc., Santa Clara, CA, USA).

RNA sequencing and RNA-seq data analyses

To obtain global transcriptome data, high throughput mRNA sequencing analysis was performed on an Illumina sequencing platform. An Agilent BioAnalyzer with Eukaryotic Total RNA Nano Kit was used for checking RNA integrity (RIN). RNA samples with integrity number >7 were accepted for the library preparation process. Library preparations, sequencing, and primary data analysis were performed at Genomic Medicine and Bioinformatics Core Facility of the University of Debrecen, Hungary.

Gene Expression Analysis of Melanoma Cells Cultured Under 2D and 3D Cell Culture Conditions

Following the filtering steps of the data, 9,653 genes were used in gene expression analyses. To reveal the differentially expressed genes between cells growing in 2D and 3D, paired t-tests with a random variance model were applied, considering a P-value 0.01 or less to be statistically significant. The microarray data were deposited in the Gene Expression Omnibus repository (http://www.ncbi.nlm.nih.gov/gds) under accession number GSE114443 and GSE148638.

Gene Expression Analysis of ENCO+BINI sensitive and resistant cell lines

The Fragments Per Kilobase Million (FPKM) number was used to quantify gene expression. To determine significantly expressed genes in each sample, the

following criteria were applied: fold change ≥2 and p-value ≤0.05. The RNA-Seq data were deposited into the Gene Expression Omnibus (GEO) repository (http://www.ncbi.nlm.nih.gov/gds) under accession number GSE186108.

Pathway Analysis

Significant pathways associated with specific gene expression signatures were identified using the EnrichR web-based application (http://amp.pharm.mssm.edu/Enrichr/#). Only significantly altered signalling pathways (p-value ≤0.05) were included in the analyses. Benjamini-Hochberg adjustment (FDR <0.05) was applied as a cut-off, and pathways with ≥5 significantly differentially expressed genes were considered and used to define molecular pathways associated with the differentially expressed genes.

Quantitative Real-Time PCR

Quantitative real-time PCR (qRT-PCR) was used to define the relative mRNA expression of selected genes using a Light Cycler 480 Real-Time PCR System. cDNA synthesis was performed applying High-Capacity cDNA Reverse Transcription Kit with random primers as described in the supplier's protocol; 600 ng of total RNA for each reaction was used. SYBR Premix Ex Taq (Takara Holding Inc., Kyoto, Japan) was applied to carry out the qRT-PCR reaction. GAPDH (glyceralde-hyde-3-phosphate dehydrogenase: Hs9999 9905_m1) was used as a reference gene, and the Livak method (2^-ddCT equation) was applied for the qRT-PCR data analyses.

Statistical Analysis

Statistical analysis was performed using SPSS (IBM SPSS 19.0, SPSS Inc., Chicago, IL, USA) and Graph Pad Prism 9 (Graph Pad Software Inc., San Diego, CA, USA) software. Pearson's correlation coefficient was calculated to correlate the RNA-seq and qRT-PCR data. The cellular parameters were statistically

analysed using the Mann–Whitney–Wilcoxon test. Only a p-value <0.05 was considered statistically significant.

RESULTS

Development and characterization of BRAF inhibitor resistant melanoma cell lines growing under traditional and 3D cell culture conditions

Gene expression profiles of BRAFi (PLX4720) sensitive melanoma cell lines cultured under 2D and in 3D conditions

Gene expression profiles of BRAFi sensitive (2D cell culture designated as WM983A and WM983B; 3D cell culture designated as $WM98A^{SPH}$ and $WM983B^{SPH}$) melanoma cell lines were compared (data obtained by using the Affymetrix Human Gene 1.0 microarray). This analysis resulted in 1049 differently expressed genes between the two types of cell culture conditions. Among the 1049 genes 562 were overexpressed) and 487 downregulated. In order, to determine the functional and biological significance of the differentially expressed 1,049 genes in the BRAFi-sensitive cells, we performed pathway enrichment analysis. This analysis revealed that the upregulated genes are involved in a variety of biological pathways mainly in the regulation of cell cycle, G2/M checkpoints, p53 signalling pathway, DNA replication, Rho GTP-ase signalling, DNA repair, cellular senesce and other cancer-related signalling pathways.

The functional role of downregulated genes were clustered into the following pathways: cellular responses to external stimuli and stress, regulations of lipid metabolism by peroxisome proliferator-activated receptor alpha signalling by TGF-beta family members, interleukin-4 and interleukin-13 signalling, TP53 regulated transcription of cell death, and other cancer related pathways. The functional involvement of differently expressed genes between the sensitive adherent and spheroid cells was validated by using the web-accessible program: Database for Annotation, Visualization and Integrated Discovery (DAVID) https://david.ncifcrf.gov/.

Gene expression profiles of PLX4720 resistant melanoma cell lines cultured under 2D and in 3D conditions

BRAFi resistant melanoma cell lines were developed from the PLX4720 sensitive cell lines. The morphology of the resistant cell lines were different from the sensitive cells under 2D cell culturing conditions, showing elongated, fibroblast-like shape. The resistant cell lines grow similarly such as the sensitive cells during the spheroid formation.

Gene expression of the differently cultured BRAFi resistant cells was analysed using using the Affymetrix Human Gene 1.0 microarray. Gene expression analysis of the resistant adherent melanoma cell lines ($WM983A^{RES}$ and $WM983B^{RES}$) and their corresponding spheroids ($WM983A^{RES\text{-}SPH}$ and $WM983B^{RES\text{-}SPH}$) revealed 297 significantly differentially expressed genes. Based on the analysis 72 genes were upregulated and 225 genes were downregulated in the resistant spheroids compared to the resistant adherent cells. Pathway analysis of the differentially downregulated genes revealed that these genes are mainly involved in cellular and mitochondrial translation, axon guidance pathway, ROBO receptor regulation and signalling, G2/M checkpoints, and other cancer related pathways. Upregulated genes in the resistant spheroids were not significantly enriched in any pathway according to our criteria.

Comparison of the gene expression signature of the BRAFi sensitive and resistant cell lines during spheroid formation

In order to describe similarities and differences of gene expression between the sensitive and resistant cell lines associated with spheroid formation, we compared the expression patterns of overexpressed and downregulated genes of the WM983A and WM983B cell lines. Based on these data, we found 447 genes which were downregulated only in the sensitive- and 185 genes only in the resistant spheroids, respectively. The number of upregulated genes in the BRAFi sensitive spheroids was 556, while 66 genes were detected in the resistant spheroids. During the analysis, we were able to identify 46 genes that were

commonly altered in both types of spheroids. Forty shared genes were downregulated including *MMP16, IGF1R, FLOT1* and *CEP19* and the 6 commonly upregulated genes included the followings: *HIST1H2BM, DDAH1, UCP2, MBD3L5, DEFB124* and *MLF2*.

Furthermore, we discovered an inverse gene expression trend for several genes when compared the gene expression differences between sensitive and resistant spheroids. Ten of the 712 genes that were upregulated in resistant spheroids were downregulated in sensitive spheroids but upregulated in resistant ones. In contrast, the expression of three genes (*SCN8A*, *RING1*, and *ABHD4*) was downregulated in sensitive spheroids while upregulated in resistant ones. Surprisingly, inversely expressed genes seem to be involved in the cell cycle (*CENPF*, *LOXL2*, and *BNIP3*) and epigenetic gene regulation (*HIST1H2BB*).

Comparing the fold change gene expression levels that were generated using the Affymetrix microarray analysis with the qRT-PCR results, we found that five out of the eight genes tested (*DCUN1D1, CMSS1, ZNF639, ABHD4, and HIST1H2BB*) showed the same direction of gene expression changes in the sensitive- and in the resistant spheroids. In addition, we observed a strong correlation between the Affymetrix array and qRT-PCR expression data in case of the *ABHD4* and *SCN8A* genes ($R > 0.7$; P-value < 0.05).

Molecular alterations associated with acquired resistance during combined treatment of BRAF and MEK inhibitors on BRAFV600E mutated melanoma cell lines

The effect of BRAF and MEK inhibitor treatment on viability of melanoma cell lines

To investigate the effect of ENCO+BINI treatment on melanoma cell viability, we treated 9 melanoma cell lines with the combination of 1 μmol/l drug mixture for 72 hours. Our results revealed that only melanoma cells with BRAF mutations showed significant ($p < 0.05$) decrease in cell viability during the treatment. The decrease of cell viability was not uniform within the *BRAF* mutated cell lines. In

contrast, we did not detect significant changes in cell viability in the WM1366 *(NRAS*^{Q61L} mutant) and WM3211 (*BRAF/NRAS* wild-type) melanoma cell lines.

Establishment of ENCO+BINI resistant melanoma cell lines

To establish resistant cell line variants during combination treatment with BRAFi/MEKi, cells were treated continuously with increasing concentrations of ENCO+BINI mixture for 3-5 months starting at 1 nmol/l and the drug concentration was increased during every passage up to 200 nmol/l. Furthermore, we observed that the morphology of the drug-sensitive cells differed from that of the ENCO/BINI-resistant cells.

Because morphological changes frequently associated with the changes of cell invasiveness, and the resistant cells often show epithelial-mesenchymal transition (EMT), we determined the invasive characteristics of cell lines using a Matrigel invasion assay. Based on this experiment we observed that the WM983A^{E+BRes}, WM278^{E+BRes}, and WM902B^{E+BRes} cell lines had significantly enhanced invasive properties compared to the corresponding sensitive cell lines.

The invasive properties of two cell lines: WM983B^{E+BRes} and WM1617^{E+BRes} did not change significantly, both were originated from melanoma patient's metastatic lesion. We also observed, that the WM793B^{E+BRes} cells behaved differentially, these cells showed significantly decreased invasive potential compared to the sensitive cells.

Alterations of cancer related proteins associated with the development of acquired resistance during BRAFi/MEKi treatment

Protein expression changes during the development of the combined drug resistance were determined using Proteome Profiler Human XL Oncology Array. This array detects the expression of 84 cancer related proteins that have been spotted in duplicate on nitrocellulose membranes. These analyses showed a number of differentially expressed proteins in the resistant cell lines when compared to the drug sensitive pairs. Analysing the protein expressions, we did

not observed similar trend among the drug resistant cell lines (WM983A, WM983B, WM278, WM1617, and WM902B). Proteins with detectable differences (>10%) in at least one cell line revealed 17 differentially expressed proteins in the resistant cell lines.

The expression of the seventeen proteins were not uniform within the cell lines. Relatively high expression was seen for three proteins: Galectin, Enolase and CapG in the WM983A and WM983B drug-sensitive cell lines, the expression of these proteins were still high in the corresponding BRAFi/MEKi resistant cells. The largest number (twelve proteins) of differentially expressed proteins were recognized in the WM983A^{E+BRes} cells, compared to the sensitive ones. These proteins included Survivin, Osteopontin, Amphiregulin, EGFR, FGF and HO-1. Interestingly the expression of Galectin increased in four cell lines (between 34-100%) being the highest in the WM1617^{E+BRes} cells.

Effect of drug withdrawal on the viability and protein expression on ENCO/BINI resistant melanoma cell lines

Because drug-dependency is frequently observed during the development of acquired resistance, we cultured the resistant cell lines without the drug mixture, replacing the ENCO/BINI with DMSO (solvent of the drugs) and let the cells to grow for 72 hours. Surprisingly, the cell proliferation did not decrease significantly, and we did not observe significant cell death after 72 hours of the "drug holiday" in five cell lines (WM983A, WM983B, WM278, WM902B, and WM793B) compared to the control cell lines that were treated continuously with 200 nmol/l ENCO/BINI. These experiments clearly show that these *BRAF* mutated cell lines are not addictive to BRAFi/MEKi treatment. In contrast, opposite effect was detected in the WM1617^{E+BRes} cell line, cells behaved differently and showed significantly reduced cell proliferation**.**

We selected three resistant cell lines (WM983A^{E+BRes}, WM983B^{E+BRes}, and WM1617^{E+BRes}) and cultured the cells in the absence of the drug combination for

10 days, then measured the cell viability and protein expression changes and compared to the control resistant cells (cells were continuously treated with the inhibitor mixture).

The drug holiday results demonstrates that 3 days of drug withdrawal significantly increased the cell viability in the resistant WM983B^{E+BRes} cells, and a slight increase was observed in the WM983A^{E+BRes} cells. The viability of cells did not decrease in these two cell lines even after 10 days of drug withdrawal, the viability of cells was above the continuously (control) drug treated cells. In contrast, the WM1617^{E+BRes} behaved differentially during this experiment, the viability of the cells decreased below 85% after 3 days drug removal and the decrease was significant after 10 days of days drug withdrawal compared to the control cells.

Protein expression changes of the resistant cell lines was investigated after the drug holiday using the same Proteome Profiler (Proteome Profiler Human XL Oncology Array) as we used before. Drug withdrawal resulted in changes of seven proteins (CapG, Enolase 2, Galectin-3, HO-1/HMOX1, OPN, Survivin, and Vimentin) in all the resistant cell lines.

Ten days of "drug holiday" resulted in extensive changes in protein expression. The most uniform alterations were detected the increase expression of three proteins: CapG, Enolase 2 and OPN in all cell lines. It should be noted that in addition to the co-expressed proteins, several other proteins were also highly expressed, e.g., cathepsin S, EGFR, endoglin CXCL8, and CCL20 in the WM983B^{E+BRes} cells, and the HIF-1α, endoglin, P53, snail in the WM1617^{E+BRes} cells.

Identification of differentially expressed genes in resistant melanoma cell lines using RNA-Seq analysis

We performed RNA-Seq analyses to determine gene expression patterns in five ENCO/BINI-sensitive and the corresponding resistant melanoma cell lines. The number of differentially expressed genes were 1591 (fold change ≥ 2, p-value ≤

0.05). Majority of the genes were upregulated (n=1024), the number of downregulated transcripts was 567 in the resistant cell lines. The fold changes of the 10 most highly expressed genes were above 20-fold, being the highest for *CXCL12*, *COL5A1* and *ALPK2* genes. While the most downregulated genes included *DMRT2, MRGPRX4, TRIM51* and *CTD-2207A17.1* genes. Interestingly most of the genes that were downregulated in the inhibitor-sensitive cell lines became upregulated during acquiring the resistant phenotype, and the upregulated genes turned downregulated.

We validated the RNA-seq data by qRT-PCR in case of ten genes (upregulated genes: *CXCL12*, *COL5A1*, *ALPK2*, *ABCC3*, *CHST15* and downregulated genes *DMRT2*, *MRGPRX4*, *TRIM51*, *VEPH1* and *GJB1*). Seven of the ten genes examined (*CXCL12*, *COL5A1*, *ABCC3*, *CHST15*, *DMRT2*, *MRGPRX4*, and *VEPH1*) exhibited the same direction of gene expression in the parental and resistant cell lines.

Pathway analysis of differentially expressed genes

We performed pathway analysis of the differentially expressed gene in order to further understand the biological functions of genes during the development of drug-resistance. Pathway analyses were performed using the Molecular Signatures Database (MSigDB version 7.5.1.; *http://www.gsea-msigdb.org/gsea/msigdb/*). The results of this analysis highlighted that the differentially expressed genes were associated with different molecular pathways. The pathway analyses underlined that the upregulated genes were significantly associated with epithelial-mesenchymal transition, which promotes metastasis and is often associated with drug resistance and downregulated genes were associated with KRAS signalling, TNF-alpha signalling via NF-kB, inflammatory response, IL-2/STAT5 signalling, coagulation, and early oestrogen response.

DISCUSSION

Despite several advancement and options in the treatment of malignant melanoma, the disease is still the leading cause of skin cancer associated death. Approximately 40-60 % of melanomas harbour activating mutations (*BRAF*^{V600E} and *BRAF*^{V600K}) in the *BRAF* oncogene. *BRAF*^{V600E} mutation can achieve ~500-fold enhancement in the kinase activity to downstream mitogen-activated protein kinase signalling, which controls key cellular events, including proliferation, differentiation, migration, survival, and angiogenesis. Targeted inhibition of the mutant protein is the most promising therapy option for melanoma patients with advanced disease. Pre-clinical and clinical studies show that targeting *BRAF* using RAF-selective inhibitors results in remarkable tumour shrinkage in *BRAF*^{V600E} mutant melanomas. A panel of BRAF inhibitors (vemurafenib, dabrafenib, encorafenib, etc.) and a series of MEK inhibitors (cobimetinib, trametinib, binimetinib, etc.) has revolutionized the treatment of melanoma patients, and recently combination of a BRAF and MEK inhibitor (Encorafenib: Braftovi™ + Binimetinib: Mektovi®) was approved by the FDA for melanoma patients with *BRAF*$^{V600E/K}$ mutation. The combination of the small molecule inhibitors showed not only a high response rate, but a favourable toxicity profile and impressive progression-free survival (~16.9 months compared to the ~ 9 month BRAFi monotherapy) in patients with *BRAF*^{V600E} mutation. Unfortunately, even the high success of the different targeted therapies, acquired resistance develops in a large number of melanoma patients, and the mechanism of resistance remains unclear. One aims of our study was to generate reproducible three-dimensional melanoma spheroid models from BRAF inhibitor sensitive and resistant melanoma cell lines. Simultaneously, we aimed to compare the gene expression signature of the adherent and the spheroid cell lines in both drug-sensitive and drug-resistant model systems. In parallel, we aimed to explore molecular alterations associated with acquired resistance during combinatorial treatment of *BRAF*^{V600E} mutant melanoma cell lines using BRAF and MEK inhibitors.

Based on our results, the expression profiles of the two BRAFi sensitive cell line models were highly different. The differently expressed genes in the sensitive spheroids included 562 upregulated and 487 downregulated genes. Among the top 10 overexpressed genes, we identified 4 genes (*SPC25*, *CCL2*, *CCNE2* and *PLK1*) that were previously described to have functional roles in cell migration and metastasis formation, in addition, these genes are all involved in cell cycle regulation through different pathways. On the other hand, the top 10 downregulated genes are involved in various signalling pathways, such as the *CHN2* gene in the regulation of RAC1 activity, the *FOS* gene in EGFR signalling, *ITGA7* in integrin pathways and Akt signalling, and in tumour initiation and progression.

Our BRAFi resistant spheroid cells exhibited 297 differentially expressed genes compared to the adherent resistant cells. Downregulated genes (n=225) are mainly involved in different translation pathways including axon guidance pathway, ROBO receptor signalling, G2/M checkpoints and other cancer related pathways. It was described that genes involved in axon guidance are inhibited by oncogenic B-Raf/MEK/ERK signalling in melanoma. Genes of the axon guidance signalling pathway, including plexin B1 and semaphorin 3D genes, as well as R-RAS were down-regulated in resistant spheroids. Several studies have shown that plexin B1 is associated with tumour progression and emerging as clinical biomarker. In contrast, a significant loss of the ROBO receptors are best known for mediating axon guidance through attraction or repulsion of growth cones, and their expression is known to correlate with tumour angiogenesis.

When we compared the gene expression between the sensitive and resistant spheroids, we identified a set of genes that were differentially expressed in the resistant and sensitive spheroids of both cell lines. A certain group of genes were down- or upregulated only in the sensitive and resistant spheroids, respectively. We could identify 46 genes that were altered in both type of spheroids. Most of

the common genes (n = 40) including *MMP16, IGF1R, FLOT1* and *CEP19* were downregulated and functionally involved in several types of cancers including melanoma. Upregulated genes involved only six genes: *HIST1H2BM, DDAH1, UCP2, MBD3L5, DEFB124* and *MLF2*, these genes have roles in interleukin-2 signalling pathway and negative regulation of cell proliferation. Since these genes were found to be commonly altered in both sensitive and resistant spheroids, our findings might point out that these genes are crucial for the development of melanoma spheroids.

During our analysis we discovered an inverse gene expression signature between the sensitive and resistant spheroids. The expression of three genes (*SCN8A, RING1 and ABHD4*) was downregulated in sensitive spheroids but elevated in the resistant cells. In contrast upregulation of 10 genes (*HIST1H2BB*, *CENPF*, *LOXL2*, *BNIP3*, *DCUN1D1*, *CMSS1*, *SMC3*, *ZNF639*, *IKBIP*, and *IFT57*) was detected in the sensitive spheroids, while downregulation of the same genes was detected in the resistant spheroids.

The ten genes that were upregulated in the sensitive and downregulated in the resistant spheroids are involved in tumour initiation and progression, but no detailed study was published yet in case of melanoma. These differently expressed genes require more attention during the future experiments. Generally, these results underline the gene expression signature of spheroid formation and highlight important molecular pathways that are different between 2D and 3D cell culture.

Over the past few years, melanoma research has put an emphasis on the characterization of acquired resistance at the molecular level. These findings indicate significant gene expression changes in melanoma cells that may help to explain the emergence of acquired resistance to BRAFi.

Treatment of $BRAF^{V600}$ mutant advanced melanoma using the combination of BRAF (encorafenib) and MEK (binimetinib) inhibitors was introduced into the

clinic in 2018. The combination of the dual-targeted inhibitors provides inspiring treatment options as a targeted therapy for patients with *BRAF*^{V600}-mutated melanoma with improved overall response. However, despite impressive clinical successes, many patients are diagnosed with tumour recurrence and experience a more aggressive melanoma tumour. Recently, a large number of studies has focused on identifying the underlying molecular mechanisms leading to the development of drug resistance, however, the molecular alterations in this process remain unclear.

The novelty of our study is that we were able to develop encorafenib plus binimetinib resistant melanoma cell lines after continuous BRAFi/MEKi treatment and compare the gene and protein expression differences between the drug-sensitive and drug-resistant melanoma cells. Our results highlight a number of molecular changes that arise during the evolution of acquired resistance. In contrast to the monotherapy associated BRAFi resistance development, we also observed that intermittent dosing of the drug combination (ENCO+BINI) might not be beneficial for melanoma patients with a *BRAF*^{V600E} mutation, this observation probably has clinical relevance.

From a clinical point of view, discontinuation of targeted therapy is one of the most successful approaches to prevent or delay the acquisition of drug resistance. Several preclinical and clinical studies have suggested that periodic drug dosage may be clinically beneficial. However, recently published experimental and clinical investigations have failed to establish the clinical benefits of interrupted drug dosing, and “drug holiday” has become highly controversial in terms of therapeutic improvement. In a good agreement with these findings, our data show that ENCO+BINI drug-resistant cells are not drug-addicted and do not show considerably enhanced lethality following a "drug holiday”. In summary, our data point out that intermittent treatment unlikely to increase the effectiveness of the

combined BRAFi/MEKi treatment, further research is needed to better understand intermittent drug dosing.

A number of molecular changes related to drug resistance have already been identified, including overexpression of *EGFR*, *PDGFR*, *HGF*, *IGF*, *CRAF*, *COT*, and *MITF* and downregulation of *STAG2* or *STAG3* genes. We have previously published that BRAFi (PLX4720) resistance is associated with certain cancer-related proteins, as detected using the Proteome Profiler Human XL Oncology Array. During the combination treatment, we extended our proteome profiler study to identify and compare the protein expression patterns associated with acquired resistance during BRAFi/MEKi treatment. Our protein expression analysis revealed several differentially expressed proteins in the double targeted resistant cell lines compared to their sensitive counterparts. These proteins clustered in a unique pattern; no similar expression signature was observed in the resistant cell lines.

Galectin expression was detected in all BRAFi/MEKi sensitive cell lines, and increased expression was seen in four of the five resistant cell lines. The protein galectin-3, which acts as an anti-apoptotic agent, is implicated in the development of resistance to chemotherapeutics in breast cancer and in the development of acquired resistance in malignant melanomas. Furthermore, Enolase 2 (Gamma-enolase) and CapG were expressed in at least two cell lines and the expression of these proteins varied greatly between cell lines. Interestingly, Enolase 2 and CapG are used as diagnostic tumour markers in the diagnosis and prognosis of many cancer types, and both proteins have been associated with cell proliferation, invasion, migration, and metastatic capacity in several types of cancer, including melanoma. The high variability of protein expression between the resistant cell lines indicates the necessity for more personalized (patient-specific) treatment.

Several pre-clinical studies have shown that discontinuation of treatment after the development of resistance may lead to tumour regression and, in many cases, can

restore sensitivity to therapy. This phenomenon is based on the concept that drug addiction in the absence of the drug causes a selection disadvantage in the resistant population. Recent clinical results, however, tend to show that intermittent BRAF inhibitor therapy is inferior to continuous targeted therapy, emphasizing the difficulty of translating dose and treatment regimens from animal model to patients. To explore the molecular changes associated with intermittent drug treatment, we first determined the viability of resistant cells removing the inhibitor combination, and analysed the protein expression alterations. Drug withdrawal resulted in protein expression changes in all three cell lines. Proteome profiler analyses revealed seven proteins (CapG, enolase 2, galectin-3, HO-1/HMOX1, OPN, survivin, and vimentin) with altered expression after the removal of BRAFi/MEKi, some of those are well-known players in drug resistance. However, this is the first study to shed light on the co-expression changes of proteins related to "drug holiday" in BRAFi/MEKi resistant melanoma cells.

Over the last decade, a number of studies have reported differentially expressed genes between drug-sensitive and drug-resistant cells after monotherapy using BRAF inhibitors. Nevertheless, relatively little is known about molecular mechanisms involved in the development of acquired resistance to BRAFi/MEKi combinatorial treatment. Our RNA-seq analysis revealed almost 1600 differentially expressed genes that are probably linked to the development of BRAFi/MEKi resistance. Majority of the transcripts (n=1024) were overexpressed, the downregulated genes included 567 genes. Our pathway analysis has revealed significant differently expressed were linked to biological pathways such as the epithelial-mesenchymal transition, the TNF-alpha signalling, KRAS and IL-2/STAT5 signalling. Within the top 10 overexpressed genes, the *COL5A1* gene (an ECM component) has been already reported to contribute to BRAFi resistance in melanoma cells during the treatment with PLX4720 BRAF inhibitor, and probably has a dominant role in the development

of drug resistance. The other overexpressed genes are also associated with tumour progression and metastasis formation through different signalling pathways. For example, *ALPK2* is known to influence cell cycle as well as DNA repair mechanisms and acts as a tumour promoter in malignant cells, while *HHIPL2* is linked to gastric malignancies via hedgehog signalling. The *ABCC3* (ATP Binding Cassette Subfamily C Member 3) was characterized as one of the significantly upregulated gene in our resistant cells compared to their sensitive counterparts. However, until now, these upregulated genes have not been linked to BRAFi/MEKi resistance, which enhances the novelty of the present study.

The downregulated genes were associated with KRAS and IL-2/STAT5 signalling. Previously it was reported that some of the downregulated genes play a significant role in cell migration and tumour metastasis initiation and progression, and all have been involved in tumorigenesis through different pathways; for example, *DMRT* and *FABP7* play important role in epithelial-mesenchymal transition, while *VEPH1* suppresses vascularization by inhibiting AKT activation.

In agreement with published data, we found novel long noncoding RNA transcripts (lncRNA), including *RP11-326A19.5*, *RP11-459E5.1*, and *RP4-718J7*, which support that lncRNA expression is associated with cancer in humans. Through various molecular mechanisms, lncRNAs regulate a variety of biological processes in cancers and may serve as biomarkers of response to treatment. However, these transcripts are not well documented in BRAFi resistance at the field of melanoma. The present study is the first to suggest that several long non-coding gene transcripts might play a role in the development of BRAFi/MEKi resistance.

Even a large amount of data is published, it is hard to describe how to prevent drug resistance at this moment. Many different studies are going on to explore the most effective possible treatment combinations. We believe that the sequence of

immunotherapy and combination of BRAF/MEK inhibitors would be the most effective option. One of the future directions is to explore the underlying mechanisms of acquired drug resistance and strategies for the different drug combinations. Probably the most beneficial approach will be to discover key molecules and molecular pathways that are differentially expressed between sensitive and resistant cells to prevent drug resistance and develop targeted drugs against the functionally relevant molecules.

In summary, our data provide the first insight into differently expressed genes that might be involved in spheroid formation in BRAFi sensitive and resistant melanoma cells. Generally, the results underline the molecular background of spheroid formation and highlight important molecular pathways that are different between traditional monolayer/adherent and 3D cell culture. The current data on the development of acquired resistance using combination of BRAF and MEK inhibitors, offer the first understanding into differentially expressed genes and provide protein expression patterns associated with a BRAFi/MEKi-resistant phenotype in melanoma cells. Our findings contribute to a better understanding of the complex mechanisms leading to acquired resistance during combined treatment of *BRAF*-mutant melanoma. However, further studies are needed to identify the key molecules and signalling pathways responsible for therapeutic escape during BRAFi/MEKi treatment and to prevent the initiation of acquired drug resistance in melanoma.

MAIN FINDING AND RESULTS

This study aimed to investigate the effect of BRAFi (PLX4720) and BRAFi/MEKi (ENCO/BINI) on human melanoma cell lines.

I. Development and characterization of BRAF inhibitor resistant melanoma cell lines growing under traditional and 3D cell culture conditions

Generation of PLX4720 resistant cell lines and spheroid culture

- We successfully developed reproducible three-dimensional melanoma spheroid models from *BRAF*^{V600E} mutant melanoma cell lines that are sensitive and resistant to BRAFi.

Gene expression patterns of *BRAFi-sensitive cells* cultured in 2D and 3D conditions

- We found a total of 1049 significantly differentially expressed genes (562 upregulated and 487 downregulated) between 2D and 3D cultured BRAFi sensitive cells.
- These differentially expressed genes were involved in a variety of biological pathways, mainly in the regulation of cell cycle, G2/M checkpoints, p53 signalling pathway, cellular responses to external stimuli and stress, interleukin-4 and interleukin-13 signalling and other cancer-related signalling pathways.

Gene expression patterns of *BRAFi-resistant cells* cultured in 2D and 3D conditions

- We found the drug-resistant cells grown under 2D and 3D cell culture conditions exhibited 297 differentially expressed genes (72 upregulated and 225 downregulated).
- These differentially expressed genes were mainly involved in cellular and mitochondrial translation, axon guidance pathway, ROBO receptor regulation and signalling.

Gene expression patterns of *BRAFi sensitive-and resistant spheroid*

- We found that only 1% of genes expressing upregulations and 5.6% of genes expressing downregulations were commonly expressed between drug-sensitive and drug-resistant cells cultured in 3D environment.
- Furthermore, 13 genes (*HIST1H2BB, CENPF, LOXL2, BNIP3, DCUN1D1, CMSS1, SMC3, ZNF639, IKBIP, IFT57, SCN8A, RING1*, and *ABHD4*) with

functional relevance in anoikis resistance and cell cycle regulation exhibited inverse expression between sensitive and resistant spheroid.

II. Molecular alterations associated with acquired resistance during combined treatment of BRAF and MEK inhibitors on BRAFV600E mutated melanoma cell lines

Characterisation of BRAFi/MEKi (ENCO/BINI) resistance cells

- We successfully developed the ENCO/BINI resistant melanoma cell lines.
- Drug-resistant melanoma cells had a higher invasive potential and acquired a spindle-like structure.

The effect of ENCO/BINI inhibitor treatment on viability of melanoma cell lines

- ENCO/BINI combination significantly inhibit the cell viability in only *BRAF*-mutant cell lines, but not in wild type cell lines.
- We also observed that the resistant cells behaved differently after the withdrawal of the inhibitors; five out of six were not drug addicted at all and did not exhibit significantly increased lethality. Additionally, one resistant cell line ($WM1617^{E+BRes}$) showed significantly decreased viability, indicating drug addiction.

Gene expression patterns of ENCO/BINI sensitive and resistant cell lines

- We found 1591 significantly differentially expressed genes (1024 upregulated and 567 downregulated) with functional in role cancer related pathways including ATF-2 and AP-1 transcription factor networks, extra cellular matrix, TNF-alpha signalling via NF-kβ, epithelial mesenchymal transition, KRAS signalling.

Protein array analysis of the ENCO/BINI sensitive and resistant melanoma cell lines

- We found several differentially expressed proteins, with no similar pattern among the cell lines. Furthermore, high expression of Galectin was observed in four resistant cell lines (WM983A, WM983B, WM1617, and WM902B) and decreased in counterpart drug-sensitive cells.
- Following drug withdrawal, Enolase 2, CapG, and OPN showed a consistent increase in expression following the 10 days of drug holiday.

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List of publications related to the dissertation

1. **Patel, V.**, Szász, I., Koroknai, V., Kiss, T., Balázs, M.: Molecular Alterations Associated with Acquired Drug Resistance during Combined Treatment with Encorafenib and Binimetinib in Melanoma Cell Lines.
   Cancers (Basel). 13 (23), 1-22, 2021.
   DOI: http://dx.doi.org/10.3390/cancers13236058
   IF: 6.639 (2020)

2. Koroknai, V., **Patel, V.**, Szász, I., Ádány, R., Balázs, M.: Gene Expression Signature of BRAF Inhibitor Resistant Melanoma Spheroids.
   Pathol. Oncol. Res. 26 (4), 2557-2566, 2020.
   DOI: http://dx.doi.org/10.1007/s12253-020-00837-9
   IF: 3.201

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List of other publications

3. Szász, I., Koroknai, V., **Patel, V.**, Hajdú, T., Kiss, T., Ádány, R., Balázs, M.: Cell Proliferation Is Strongly Associated with the Treatment Conditions of an ER Stress Inducer New Anti-Melanoma Drug in Melanoma Cell Lines.
Biomedicines. 9 (2), 1-19, 2021.
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