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level I alterations in all patients presenting with advanced non-squamous NSCLC.
It has been shown in several clinical studies, that if patients whose lung cancers harbour driver mutation are not treated with adequate target therapy, their outcomes are worse than patients whose tumours do not harbour driver mutations. It is, therefore, necessary to utilise all molecular diagnostic tools of precision oncology at our disposal to diagnose, monitor and follow all our patients with NSCLC, in order to ensure the best possible outcomes.
Keywords: Precision oncology, Molecular testing, Non-small cell lung cancer, Targeted therapy

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High-throughput screening of multidrug-resistance markers in non-small cell lung carcinoma patient-derived cells – contribution to personalized treatment

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Introduction: Cancer remains one of the leading causes of death globally, despite significant advancements in cancer treatment over the past decades. A major challenge in cancer therapy is multidrug resistance (MDR), which is responsible for over 90% of deaths in cancer patients receiving both traditional chemotherapeutics and novel targeted

drugs. MDR arises from various mechanisms, including elevated metabolism of foreign substances (xenobiotics), enhanced drug efflux from cells, increased DNA repair capacity, and genetic factors such as gene mutations, amplifications, and epigenetic alterations. It is categorized into two types: primary resistance, which exists before initiating therapy, and acquired resistance, which develops after the initial treatment. The incidence of primary resistance to cancer treatment can be remarkably high (up to 60%) in certain cancer types. Furthermore, the majority of cancer patients are likely to develop resistance at some point during treatment. Although, the various underlying mechanism for drug resistance development in tumors have been highlighted in the past years, enhanced drug efflux, caused by increased expression of ATP-binding cassette (ABC) membrane transporters, is one of the major contributors to MDR. Among the known ABC transporters, three members, P-glycoprotein (P-gp, encoded by the MDR1 gene), Multidrug Resistance-Associated Protein 1 (MRP1), and Breast Cancer Resistance Protein – BCRP or Placenta ABC Protein – ABC-P), have been linked to chemoresistance to various drugs. P-gp and BCRP regulate various chemical compounds' distribution, absorption, and excretion. However, their overexpression can interfere with drug administration, reducing drug bioavailability and intracellular concentration. There is a significant correlation between increased expression of P-gp in cancer cells and enhanced resistance to drugs like paclitaxel, etoposide, DOX, and vinblastine. Overexpression of P-gp has been observed in approximately 50% of all human cancers. While in some tumor types, such as lung, liver, kidney, rectum, and colon, increased P-gp expression has been observed before chemotherapy treatment, in others, such as acute lymphoblastic leukemia and acute myeloid leukemia, it has been noticed after exposure to anticancer agents. Overexpression of P-gp and BCRP has been associated with poor clinical response and MDR in patients. Therefore, the pharmacological inhibition of the efflux function of these transporters was pursued as a strategy to overcome resistance to anticancer drugs in the clinic. However, despite showing high efficacy in preclinical studies, none of the P-gp inhibitors have been approved yet by the U.S. Food and Drug Administration (FDA) for clinical use in cancer treatment. Taking into account all the above-mentioned it is clear that screening and assessment of MDR markers in patient's cancer cells could play an important role in personalized treatment approaches. Expressing MDR markers in cancer cells could predict a patient's response to specific drugs or drug classes, allowing the selection of the most effective treatment regimen and avoiding using drugs that are likely ineffective due to resistance. Moreover, the presence of MDR markers associated with resistance to multiple drugs could guide the design of personalized treatment regimens with a combination of drugs that have a higher chance of overcoming the patient's specific drug resistance profile. Monitoring the expression level of MDR markers during the course of treatment could provide valuable insights into the development of drug resistance, and would allow healthcare professionals to adjust the treatment plan if drug resistance emerges, ensuring that the patient receives the most effective therapy. Our team established a promising method for high-throughput screening for MDR markers in non-small cell lung carcinoma (NSCLC) patient-derived cells, which implies pharmacological screening and an ex vivo experimental setting. It enables gaining valuable insights into patient characteristics and drug responses that may not be apparent through conventional sequencing or clinical trials. This strategy has the potential to improve personalized cancer treatment approaches, offering patients more effective and tailored therapies based on their individual characteristics and drug responses. **Methodology: Patient-derived NSCLC cell cultures.** Samples from NSCLC patients are collected from the Thoracic Surgery Clinic at the Clinical Center of Serbia. The histological grade is determined by histopathological analysis of the surgical specimen. Collected NSCLC samples are used to establish patient-derived NSCLC cell cultures comprising cancer and stromal cells (mainly fibroblasts). It is well known that the sensitivity of cancer cells depends on their interaction with the microenvironment including neighboring cells. The primary cultures obtained from the samples are grown for 1-2 weeks prior to drug testing. **Fluorescence immunoassay for high-throughput identification of cancers cells and MDR markers in NSCLC patient-derived cell cultures.** The fluorescence immunoassay utilizes antibodies against CK8 and CK18, which are expressed in nearly all carcinomas of epithelial origin, to identify epithelial cancer cells. Co-staining of CK8/18 with Hoechst 33342 allows the identification and quantification of two types of cells: CK8/18-negative (non-cancer cells) and CK8/18-positive (cancer cells). This immunoassay is also used to identify and quantify changes in the expression of MDR markers ABCB1, ABCC1, and ABCG2 both in cancer and non-cancer cells in primary NSCLC cultures that may occur during chemotherapy and tyrosine kinase inhibitors (TKIs) treatment. Co-staining of ABCB1, ABCC1, and ABCG2 with CK8/18 and Hoechst 33342 enables the identification of four types of cells in NSCLC primary cell cultures: drug-sensitive non-cancer cells, MDR non-cancer cells, drug-sensitive cancer cells, and MDR cancer cells. For validation of the immunoassay patient-derived cells are seeded in 384 well-plates and treated with 5 different concentrations of 8 chemotherapeutics known to induce overexpression of MDR markers (cisplatin, carboplatin, paclitaxel, docetaxel, gemcitabine, vinorelbine, etoposide, and pemetrexed), allowing the ex vivo evaluation of NSCLC MDR profile. Validated immunoassay is further used to evaluate the expression of MDR markers ABCB1, ABCC1, and ABCG2 (MDR profile) in patient-derived cell cultures after treatment with a panel of 10 TKIs (erlotinib, gefitinib, afatinib, osimertinibcrizotinib, alectinib, ceritinib, nintedanib, dabrafenib, and trametinib), allowing the evaluation of MDR profile in both cancer and stromal cells. The sensitivity of cancer and stromal cells for each individual NSCLC patient to a particular TKI is assessed using a discriminative immunoassay employing CK8/18 antibodies cocktail. **Whole Exome Sequencing (WES)**. Paired patient samples (normal and tumor)

were subjected to a DNA isolation procedure using Qiagen genomic DNA extraction kit, recommended for NGS applications. Isolated DNA samples underwent WES analyses by Novogene Company. Bioinformatics and statistics tools were employed to define clinically relevant gene alterations in MDR markers ABCB1, ABCC1, and ABCG2. **Results:** In order to understand how NSCLC patient cells respond to chemotherapy and targeted therapy, ex vivo testing was performed. The maximum concentration of drugs in human plasma that the patient is exposed to during therapy (Cmax) was used as an upper limit for drug concentration during testing, with four lower concentrations also used. The results showed that patient-derived cells display individual differences in sensitivity to both chemo and targeted therapeutics. IC50 values, which indicate sensitivity, fell within the concentration range for most chemotherapeutics. Only some chemotherapeutics (cisplatin, etoposide, docetaxel, gemcitabine, and pemetrexed) showed selectivity towards cancer cells with lower IC50 values in cancer than in stromal cells. Among TKIs, only erlotinib was efficient with IC50 below Cmax, showing selectivity towards cancer cells in all investigated patient-derived cell cultures. A number of chemotherapeutics increased the expression of ABCB1, ABCC1, and ABCG2, while TKIs afatinib, alectinib, ceritinib, osimertinib, and trametinib did not affect these transporters. Some TKIs increased the expression of ABC transporters, with nintedanib showing the potential to select cancer cells with higher MDR marker expression. WES showed significant ABCC1 gene instability, while ABCB1 had many SNPs with clinical relevance for drug response. ABCG2 had the lowest number of SNPs, but intron deletions were still identified. However, the clinical significance of these changes is currently unknown. **Conclusion:** Screening for multidrug-resistance markers through a high-throughput process provides valuable information about how a patient will respond to therapy. This process can identify if the MDR phenotype is already present or if it can be induced with targeted or chemotherapy. Based on this information, it can provide recommendations for a patient's mono- and combined therapy. This methodology has the potential to greatly impact cancer treatment strategies and improve patient outcomes by tailoring therapies to individual patient profiles. Ultimately, this will benefit a wider range of patients with non-small cell lung carcinoma and other cancers, as it leads to more precise and targeted treatment selections.

Keywords: Multidrug-Resistance Markers, NSCLC, Patient-Derived Cell Cultures, Personalized Therapy,

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