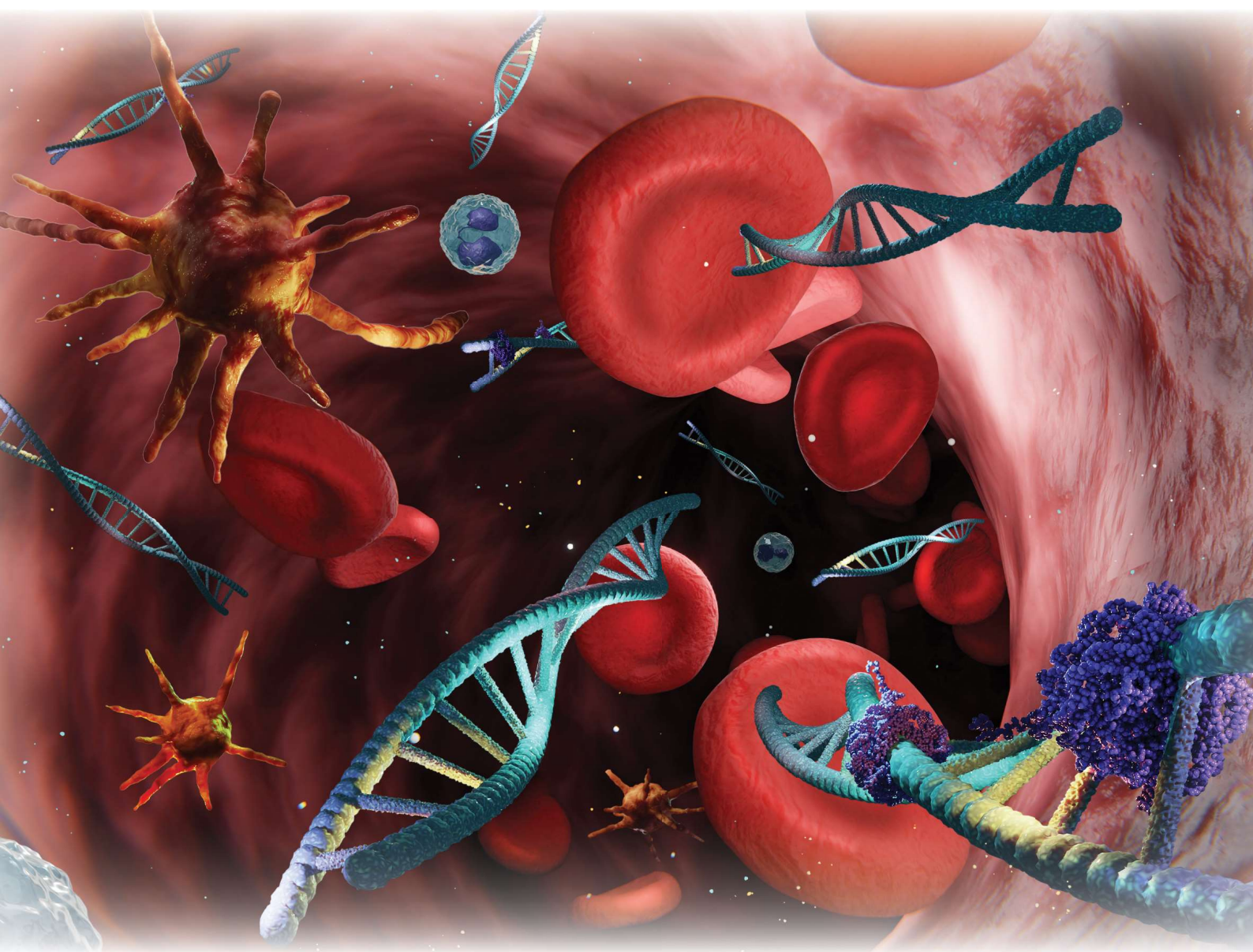


Oncology Insights

Official Journal of the Serbian Association for Cancer Research



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ONCOLOGY INSIGHTS

Aims and Scope

Oncology Insights is a yearly oncological open-access peer-reviewed journal that publishes new research from different areas of oncology. It strives to provide a platform for the exchange of cutting-edge research and knowledge in the field of oncology. This journal aims to advance the understanding, prevention, diagnosis and treatment through the dissemination of high-quality scientific discoveries.

The journal applies a fair and accurate peer review process, employing double-blind review methodologies. Acceptance of manuscripts is based on their scientific merit, originality, clarity, and contribution to the field.

Topics

Oncology Insights covers a wide spectrum of topics within the field of oncology, including but not limited to:

- Basic and Translational Research
- Clinical Oncology
- Radiation Oncology
- Surgical Oncology
- Pediatric Oncology
- Hematologic Oncology
- Palliative Care
- Epidemiology and Public Health
- Cancer Genetics
- Immunotherapy and Targeted Therapies
- Experimental Therapeutics
- Computational Biology and Artificial Intelligence

About/Information

Oncology Insights welcomes various types of contributions including original research articles, review articles, case reports, case studies, clinical trials, registered reports, comments, brief communications, editorials, letters to the editor, perspectives, and conference papers from a wide range of disciplines related to cancer research.

Through encouraging interdisciplinary collaborations, the journal welcomes contributions that integrate oncology with related fields such as immunology, genetics, biochemistry, radiology, and other relevant disciplines. The journal places a special emphasis on publishing research that highlights emerging trends, novel technologies, and innovative approaches in cancer research and clinical practice.

Oncology Insights is intended for a diverse readership, including oncologists, researchers, clinicians, nurses, allied healthcare professionals, patients, patient advocates, policymakers, and all stakeholders involved in the prevention, diagnosis, and treatment of cancer. It adopts a global perspective, encompassing research from diverse regions addressing oncological challenges that may vary across different populations.

The journal is committed to upholding the highest ethical standards in research and publication provided by established international guidelines.

Periodically, Oncology Insights may publish special issues focusing on specific topics to highlight particular areas of interest or emerging needs.

Authors are provided with clear and comprehensive guidelines for manuscript preparation, including structure, formatting, and other specific requirements.

AUTHOR GUIDELINES

Manuscript submission

By submitting a manuscript, authors affirm that their contribution to Oncology Insights journal is entirely original, has not been published before, is not under consideration for publication elsewhere, and has received approval for publication from all co-authors. Additionally, tacit or explicit consent must be obtained from the responsible authorities at the institution where the research was conducted.

Authors bear sole responsibility for the contents of their submissions, the accuracy of the experimental findings, and must ensure they possess proper permission from all involved parties to disseminate the data.

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Manuscripts undergo a preliminary assessment at the Editorial Office to ensure compliance with fundamental publishing criteria and quality benchmarks. Additionally, they are also screened for plagiarism.

Authors will receive an email confirmation upon receipt of their submission. Only contributions adhering to the provided guidelines can proceed for peer-review. Manuscripts not meeting these criteria will be returned to the authors with observations, comments and annotations.

Manuscript preparation

Authors are required to adhere strictly to the provided instructions.

The manuscripts should be written in English with clarity and conciseness. Both American and British English usage are accepted, but a combination of these is discouraged.

The manuscript should contain the title, authors' names and affiliations, abstract, keywords, the text of the manuscript:

- Introduction
- Materials and methods¹
- Results¹
- Discussion
- Conclusion
- Acknowledgments
- References

Mark the position of figures and tables in the text. Please, do not include tables and figures in the manuscript. They should be submitted as separate files.

Title

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Write the full name and surname of each author.

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The SI system should be used for all scientific and laboratory data.

Photos, drawings and other illustrations should be of good quality. The acceptable image formats include: TIFF and JPEG.

Acknowledgements

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References should be compiled at the conclusion of the article and numbered according to their appearance in the text. The list of references should exclusively comprise works that have been cited in the text, and they should be cited in the language of their original publication.

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Dear colleagues,

The second volume of Oncology Insights, the Official Journal of the Serbian Association of Cancer Research (Srpsko društvo istraživača raka, SDIR) has been shaped by three years of joint efforts of our scientific community through our new initiative SDIR Scientific Days, as well as excellent and up-to-date experimental cancer research and insights into hot and somewhat controversial topics. As in the first volume, we strived for excellence and integrity, aiming to exceed the expectations of our scientific community.

SDIR Scientific Days initiative outlined the strong potential for collaborative research in our country and united scientists in new networks. The abstracts cover three events held in Novi Pazar (2022), Kragujevac (2024) and Novi Sad (2024) and present diversity in scientific approaches and methodology. Two high-quality Original Research articles and one Review article dedicated to personalized approaches to the treatment of lung cancer and the application of liquid biopsy present modern directions in patient care and research globally as well as in Serbia.



As Oncology Insights pledged to evolve, adapt, reinvent, redefine, and reshape its content to serve its members and advances in the field, a new section named Personal-point-of-view has been launched to offer a platform for hot and controversial topics, which aim to challenge our comfort zone. In this volume, an intellectual discussion on the effect of artificial intelligence on scientific innovation guarantees to tickle your scientific senses.

We invite researchers, physicians, nurses, laboratory technicians, as well as patients, survivors, caregivers, and patient advocates to offer their valuable expert insights to our Journal, thus stimulating future progress of oncology. We hope you will join us in this exciting journey by providing evidence-based, unbiased multidisciplinary content depicting cancer research approaches and results, or insights into important topics from the broad field of oncology.

Please enjoy the second volume of our Journal. We look forward to your feedback and to shaping the future of Oncology Insights with you.

With kind regards,

A handwritten signature in blue ink, appearing to read 'M. Čavić'.

Milena Čavić, SDIR President

Editor-in-Chief

Oncology Insights

Official Journal of the Serbian Association for Cancer Research



The second number of Oncology Insights includes
**ABSTRACTS from
SDIR Scientific Days**



SAVE-THE-DATE

The first SDIR-HDIR-MOKAD regional congress

October 8-10, 2025. in Belgrade

with the support of EACR



Insights on the SDIR Scientific Days Experience and its Impact

Milica Pešić¹

¹*Department of Neurobiology, Institute for Biological Research “Siniša Stanković” – National Institute of the Republic of Serbia, University of Belgrade, Despota Stefana 142, 11108 Belgrade, Serbia*

The SDIR Scientific Days is an initiative launched by the SDIR Managing Board, aimed at becoming a recurring event held once or twice yearly to enhance collaboration in cancer research. This initiative facilitates interactions among researchers across multiple disciplines and universities throughout the Republic of Serbia. The event also includes online-streamed lectures that are accessible to registered members of SDIR and EACR, thereby broadening participation. During these gatherings, the SDIR Managing Board examines the cancer research resources available at the host institutes and seeks to establish collaborative initiatives among SDIR members affiliated with different universities in Serbia.

The inaugural SDIR Scientific Days took place on September 20, 2022, at the State University of Novi Pazar, supported by the Chemistry and Biology study program. Established in 2006 by the Government of the Republic of Serbia, the State University of Novi Pazar is among the newest higher education institutions in the country.

The second SDIR Scientific Days event was held on April 25, 2023, at the University of Kragujevac and organized by the Institute for Information Technologies Kragujevac (IIT). Founded in 2019, the IIT is a state scientific institute and the latest addition to the University of Kragujevac, which has a historical lineage tracing back to the establishment of the Lyceum of the Principality of Serbia in 1838. This event highlighted the IIT's research capabilities and its potential for collaboration with other Serbian institutions.

The third SDIR Scientific Days event took place on April 25, 2024, at the University of Novi Sad in Sremska Kamenica. With approximately 50,000 students and 5,000 employees, this university stands as a prominent educational and research entity with robust scientific infrastructure and diverse accredited medical programs through its Faculty of Medicine. Key healthcare institutions located in Sremska Kamenica, like the Institute for Pulmonary Diseases of Vojvodina and the Oncology Institute of Vojvodina, focus on the advanced treatment and research of respiratory disorders and oncology while emphasizing ongoing professional development and the integration of modern methodologies within their practices.

At the inaugural SDIR Scientific Days, Tanja Soldatović presented her research on novel anticancer metal-based drugs, highlighting the limitations of traditional platinum-based therapies due to toxicity and adverse effects. Her study introduced heterometallic complexes ([Pt-L1-Zn, Pt-L2-Zn, Zn-L1-Cu, Zn-L2-Cu]) that demonstrated enhanced cytotoxicity against human colorectal and MDA-MB-231 breast cancer cells, particularly after 72 h of exposure. The findings emphasized the significant influence of the unique reactivities of the metal centers and linker structures on the efficacy of these compounds.

At the second SDIR Scientific Days, Dragana Šeklić and Milena Jovanović marked a complementary approach by examining molecular markers associated with migration and invasion in colorectal cancer cell lines, specifically focusing on HCT-116 and SW-480. Their findings revealed that HCT-116, representing an aggressive stage IV carcinoma, had high migratory potential with decreased E-cadherin and elevated N-cadherin/vimentin levels. In contrast, SW-480, derived from stage II carcinoma, exhibited stronger intercellular connections due to higher E-cadherin levels, indicative of a less aggressive phenotype. Further advancing cancer research, Tijana Đukić's presentation detailed the application of numerical modeling to simulate cancer cell behavior and treatment responses. Using reaction-diffusion models and in vitro assessments, her work provided valuable insights into the dynamics of cell proliferation, death rates, and drug interactions. These validated models align closely with clinical data, enabling predictions in novel scenarios and offering a quantitative framework to aid experimental research.

The studies from the first and second SDIR Scientific Days showcase diverse angles in cancer research—from novel therapeutic compounds and molecular profiling to advanced modeling—highlighting the multifaceted approach necessary to enhance treatment strategies in oncology.



The studies presented at the third SDIR Scientific Days event cover critical aspects of cancer research, focusing on genetic factors, early detection, and treatment strategies. Karmen Stankov discussed genetic and epigenetic factors in cancer development, emphasizing how computational biology tools can analyze large datasets to identify driver mutations impacting patient survival and treatment. She highlighted the potential of epigenetic therapy in personalized medicine, supported by advancements in next-generation sequencing and artificial intelligence. Antoni Castells examined colorectal cancer (CRC) screening, noting its status as a leading cause of cancer deaths. He emphasized the need for early detection of asymptomatic CRC to improve outcomes and mentioned various screening strategies, including stool tests (FIT) and colonoscopy. The COLONPREV study, comparing colonoscopy and biennial FIT, aims to address current screening challenges like low sensitivity and high false-positive rates, while the development of non-invasive biomarkers offers promise for more effective detection. Nebojša Pavlović, Bojan Stanimirov, and Karmen Stankov explored the effects of antioxidants on the cytotoxicity of vorinostat, an HDAC inhibitor, in colon cancer treatment. They found that combining vorinostat with antioxidants N-acetyl-cysteine (NAC) and α -tocopherol (TOC) enhanced its effectiveness, indicating synergistic effects, particularly with NAC at lower concentrations. This suggests that antioxidant combination therapy could improve treatment responses, warranting further investigation.

The discussions at the third SDIR Scientific Days event on lung cancer in Serbia provide an insightful overview of the current situation and emphasize critical areas that need attention. Jelena Đekić Malbaša's presentation underscores the concerning trends in lung cancer epidemiology, particularly noting the rising incidence among women in Vojvodina despite a decrease in male mortality rates. This observation emphasizes the need for targeted smoking reduction initiatives and enhanced awareness, especially among female populations in regions like North Bačka, where the rates are notably high. In parallel, Goran Stojanović's insights into the National Program for Early Lung Cancer Detection reveal the efforts to decrease the alarming number of new diagnoses—over 6,500 annually. The pilot screening program's outcomes, particularly the 2.1% lung cancer detection rate in participants undergoing LDCT scans, present a promising start, though the challenge remains in improving engagement and adherence to screening protocols among the target demographic of smokers aged 50-74. Vanesa Sekeruš's focus on detecting EGFR mutations in non-small cell lung cancer (NSCLC) adds another layer to the discourse, illustrating the necessity of personalized treatment strategies. The efficacy of targeted therapies such as tyrosine kinase inhibitors (TKIs), which are contingent on the presence of specific mutations, is critical. Her emphasis on the role of both tissue and liquid biopsies offers a dual approach that aligns with contemporary diagnostic practices, as supported by the International Association for the Study of Lung Cancer (IASLC). Together, these presentations illuminate the multifaceted challenges of lung cancer management in Serbia and advocate for a multifactorial approach—combining prevention, early detection, and personalized treatment—essential for effectively combating this leading cause of cancer mortality. The integration of these strategies is vital for advancing national cancer control efforts and improving patient outcomes.

The following two presentations at the third Scientific Days event emphasize the importance of molecular testing in oncology, albeit in different contexts—solid tumors and hematopoietic disorders. Dimitar S. Jakimov's study focuses on identifying mutations in KRAS, NRAS, and BRAF among oncology patients, revealing significant mutation prevalence that can influence treatment strategies in solid tumors. On the other hand, the research conducted by Iva Barjaktarović, Marijana Marinkov, and Stanislava Nikolić addresses genetic mutations in myeloproliferative neoplasms (MPN), highlighting JAK2, MPL, and NPM1 mutations as crucial for risk assessment and personalized therapy in hematological cancers. Both studies utilize real-time PCR as a central methodology for mutation detection, showcasing its advantages in monitoring and rapid interpretation. However, they also acknowledge limitations in detecting specific mutations, such as the latter study's inability to identify CALR and FLT3 mutations. Collectively, these findings underscore the critical need for effective molecular diagnostics in solid and blood cancers to inform treatment decisions and improve patient outcomes. They also highlight the importance of addressing challenges in sample quality, reagent availability, and collaboration between labs and clinicians.

Vesna V. Kojić presented research on the antiproliferative effects of newly synthesized compounds, highlighting the challenges of drug discovery. Despite advances in technology, the process remains lengthy and costly. Initial drug testing occurs in vitro, utilizing cell lines derived from patient tumor samples, which are crucial for cancer research and drug discovery. The NCI-60 human tumor cell line panel is a valuable resource for identifying novel chemotherapeutics, offering insights into drug mechanisms and resistance patterns. Ultimately, the goal is to develop effective, affordable, and personalized therapies for patients.

The research presented at the SDIR Scientific Days highlights remarkable advancements in cancer therapies and deepens our understanding of cancer biology, specifically through the contributions of Serbian researchers. These studies underscore the importance of continuous and comprehensive exploration in diverse areas of cancer research. By

refining treatment strategies, such as personalized medicine approaches and novel drug development, and enhancing methods for early detection, we can significantly improve patient outcomes. Early detection methodologies, including advanced imaging techniques and biomarker identification, are crucial in identifying cancer at more treatable stages. These initiatives are expected to enhance patients' outcomes and improve their overall quality of life.

LEGACY INSIGHTS

SDIR Member Institution Presentations**Sector for Scientific Research and Education of the Institute of Oncology and Radiology of Serbia (IORS)**

Dr Siniša Radulović, Principal Research Fellow (retired)

Dr Radmila Janković, Principal Research Fellow
Institute for Oncology and Radiology of Serbia

The Sector for Scientific Research and Education (sr. Služba za naučno-istraživačku i obrazovnu delatnost, NIOD) has a long tradition and its beginnings are connected with the relocation of the Experimental Oncology Laboratory of the Faculty of Medicine in Belgrade to the first floor of the Institute in 1948. The first head of the Laboratory for Experimental Oncology was Prof. Dr. Xenophon Šahović, academician (1948-1956). Prof. Šahović was succeeded by Prof. Dr. Marija Višnjić-Frajnd (1956-1968). Scientific advisor, Blagoje Nešković, MD-PhD managed the Laboratory from 1968 to 1978. Until 1982, graduate physicist Đoka Polić was acting as a head of the Laboratory, and that year a major turning point happened in development of today's look of the NIOD. By merging the Laboratory for Experimental Oncology with the Laboratory for Immunology of the Institute and the laboratory of the Institute for Medical Research, the Department for Experimental Oncology (OEO) has been established. OEO represents the forerunner of the research part of the Institute, which still exists today under the name Service for Scientific Research and Educational Activity. Academician Professor Ivan Spužić, became a Head of the Department, and remained in that position until 1993. This period is significant for his intensive involvement in the writing and implementation of scientific projects in oncology. At the beginning of the nineties, with a change in the organizational structure, NIOD was transformed, in a way that OEO, Data Center and novel Department for Translational Research become parts of one organizational unit. Laboratories were formed in OEO. This organizational scheme of the Department, with minor changes, still functions today. The first director of the sector was Labuda Mitrović, MD, PhD, Research Associate (1994-2000). In that period, the Scientific Council of the IORS was established. One of the most important moments in the history of the NIOD Service occurred in 1994, when the Institute of Oncology and Radiology of Serbia, as the first health institution in the country, received the accreditation of a Scientific institute. Through repeated accreditations, the institute has maintained this status until today. At the end of 2000, Ljiljana Vučković-Dekić, MD, PhD, Senior scientific advisor, took over the position of director of the NIOD Sector. After the departure of Dr. Dekić to the position of scientific adviser to the director of the IORS, since 2006, the management of the Department was taken over by Siniša Radulović, MD, PhD, Senior scientific adviser. In 2007, by adapting the organization of the Institute to the Rulebook on the Organization of Tertiary Health Care Institutions, the NIOD Sector became the NIOD Service. The Service includes the Department for Experimental Oncology with 5 laboratories/departments, as well as the Department for Translational Research. Dr. Radulović remained in the position of director of the Service until his retirement in 2018. During that period, the number of employees in the Service increased, especially through participation in projects financed by the Ministry of Education, science and technological development. Also, during the leadership of Dr. Radulović, health activities were intensified, especially through the field of clinical trials, molecular diagnostics, among others, genetic profiling of tumors in order to select patients for treatment with targeted therapies. Dr. sc med Mirjana Branković Magić, Senior scientific advisor acted as a head of the Service for NIOD from 2018-2019. Dr. Branković Magić was responsible for the establishment of genetic testing for hereditary predisposition to breast, ovarian and colon cancers, which resulted in the establishment of the Department of Genetic Counseling for Hereditary Cancer in 2008. Since 2019, the service has been managed by PhD Radmila Janković, Senior scientific advisor, responsible for the introduction of a large number of new diagnostic methods based on qPCR, NGS and dPCR methods, as well as for accreditation within the European Molecular Genetics Quality Network (EMQN).



Organizational structure

Within NIOD and OEO, there are 5 laboratories: Department of Genetic Counseling for Hereditary Cancer, Laboratory for Molecular Genetics, Laboratory for Radiobiology and Experimental Oncology, Laboratory for Immunology, Laboratory for Pharmacology. NIOD employs 51 staff members, of whom 39 have a university degree, 9 are laboratory technicians with higher education, 2 laboratory technicians with secondary education and 1 is a service worker. All employees with a university degree have a research or scientific titles. Twenty-six colleagues with a university degree are fully funded by the Ministry of Science, Technological Development and Innovation through the Scientific Research Plan and Program. According to the educational profile, the majority are health associates, primarily molecular biologists and biochemists, but there are also three MDs, - two specialist in immunology and pathology, one dentist and four pharmacists.

Equipment

NIOD has all the modern equipment necessary for extensive healthcare activities as well as scientific research work: 2 new generation sequencers (MiSeq and NextSeq 550DX, Illumina) Digital PCR apparatus (Absolute Q Digital PCR, ThermoFisher) 4 Real-Time PCR apparatus (QuantStudio (ThermoFisher), COBAS Z 480 real time iLight Cyclor 480 II (Roche), Rotor-Gene Q (Qiagen) Agilent microarray Scanner Agilent Technologies2100 Flow cytometer Becton Dickinson FACS Calibur Carl Zeiss PALM MicroBeam microscope The Service has a modern equipped sterile room with 3 chambers, CO2 incubators and 6 workstations. There are also two laminar chambers, several PCR machines, one ultracentrifuge, more centrifuge with cooling, centrifuge and minifuge, and a plate reader. The service has modern devices for water distillation, sterilizer, containers for liquid nitrogen, freezers at -80C and -20C, and laboratory refrigerators.

Healthcare activities

With its organizational structure, NIOD service implements a multidisciplinary approach to oncology, and therefore keeps pace with modern achievements in medicine. An example of this approach is the introduction of modern technologies of molecular biology in diagnostics, primarily within the Department of Genetic Counseling for Hereditary Cancer and the Laboratory for Molecular Genetics. In the Genetic Counseling Center, genetic testing of a panel of genes whose harmful changes contribute to the hereditary predisposition to cancer is carried out. Testing has been done using new generation sequencing technology since 2016, and at the expense of RFZO (Health fund), for a limited number of patients, since 2018. Hereditary predisposition testing is done within hereditary breast and ovarian cancer syndromes as well as Lynch syndrome (hereditary non-polyposis colon cancer). In addition to analysis, patients are provided with adequate genetic counseling before and after testing. Analysis, biological and clinical interpretation with the new sequencing technology is certified by the European Quality Control Network (EMQN). The Institute is the only institution in Serbia that offers a unified service of genetic testing, processing the results and further monitoring of patients in whom the existence of a hereditary predisposition is determined. Testing of germline BRCA1/2 mutations in breast and ovarian cancer as predictive biomarkers for poly (ADP) ribose polymerase (PARP) inhibitor therapy is also being performed. The molecular genetics laboratory performs testing from tumor samples (paraffin tissue preparations or liquid biopsy) in order to select patients for specific targeted therapies. Modern therapeutic approaches in oncology are focused on targeting precisely defined molecular targets, which achieves greater therapeutic selectivity and consequently reduces the frequency of side effects. The methods developed in this area ensure that patients whose tumor cells contain certain somatic mutations receive specific therapy that targets the affected gene or signaling pathway. The results of diagnostic tests carried out in the Laboratory of Molecular Genetics allow for the selection of the most adequate therapy for the patient based on the presence or absence of a specific drug target or an altered signaling pathway that makes tumor cells sensitive or resistant to therapy. The pharmacogenomic service in the Molecular Genetics Laboratory was established in 2008 when testing began. The Laboratory performs: KRAS and NRAS gene testing in patients with metastatic colorectal cancer, EGFR testing in patients with metastatic lung adenocarcinoma (from paraffin samples and liquid biopsy), BRAF testing in patients with metastatic melanoma, testing for the presence of mutations in the BRCA1/2 genes in patients with serous ovarian cancer, who are candidates for PARP inhibitor therapy. The laboratory is multiple certified through the EMQN, and, according to the number of tested patients, it is the largest laboratory of this type in Serbia. In the Laboratory of Immunology, the analysis of immunophenotypic and functional characteristics of different populations of leukocytes in peripheral blood, bone marrow, and cerebrospinal fluid is carried out, primarily in patients suffering from hematological malignancies, but also solid tumors, autoimmune diseases, immunodeficiencies, and febrile conditions of unknown cause by the method of immunophenotyping using a flow cytometer.

Scientific and Research Activities

The IORS Program of scientific and research work defines the appropriate scientific areas and is in accordance with the relevant programs and strategy of the Ministry of Science, Technological Development and Innovation. In the period 2020-2024. Several national projects approved by the Science Fund of the Republic of Serbia were implemented. IORS researchers are engaged in the implementation of projects supported by the Institute of Oncology and Radiology of Serbia, then projects supported by other scientific research organizations, as well as projects supported by other scientific research organizations, the European Commission program and international projects.

National projects of the Science Fund of the Republic of Serbia in which the Institute is the coordinator:

Drug repurposing in pancreatic ductal adenocarcinoma, REPANCAN (PROMIS 2020-2022 program). Project manager: Dr. Jelena Grahovac Senior Research Associate

Tracking systemic therapy resistance of lung and colorectal cancer through targeted NGS analysis of genetic and epigenetic variants in liquid biopsies, TRACEPIGEN, (PROMIS 2020-2022 program). Project leader: Dr. Miljana Tanić, Senior Research Associate.

International projects of the framework program of the European Commission:

Blood test for clinical therapy guidance of non-small cell lung cancer patients, LungCARD (H2020-MSCA-RISE-2016 - Research and Innovation Staff Exchange, n°: 734790). PI from IORS Dr. Radmila Janković, Principal Research Fellow

Drug repurposing in pancreatic ductal adenocarcinoma (H2020-MSCA-IF-2019, REPANCAN - 891135). PI from IORS Dr. Jelena Grahovac, Senior Research Associate

Twinning for a European Consortium of Rectal Cancer Research Institutions through Stepping Up Scientific, Technological and Innovation Excellence of IORS (HORIZON-WIDERA-2021-ACCESS-03, STEPUP IORS – 101079217). Coordinator Dr. Milena Čavić, Principal Research Fellow

Twinning to skyrocket scientific excellence towards individual radiosensitivity prediction by raising the bar in knowledge transfer, networking, and technological innovation in radiobiology, RadEx IORSBoost (HORIZON-WIDERA-2023-ACCESS-02, RadEx IORSBoost – 101158832). Coordinator Dr. Ivana Matić, Senior Research Associate.

EXPANDING the value of Extracellular Vesicles as carriers of biomarker and therapy in precision healthcare, EXPAND-EV (HORIZON-WIDERA-2021-ACCESS-03, STEPUP IORS – 101079217). PI from IORS Dr. Milena Čavić, Principal Research Fellow



NIOD staff members in 2024. Image courtesy of the Institute for Oncology and Radiology of Serbia, source: Monography "85 Years of the Institute for Oncology and Radiology of Serbia", ISBN 978-86-80401-49-2, 2024, p.163.

At IORS, in the next period, basic, applied and clinical research in the field of oncology will be performed. Research will continue to be carried out within NIOD and OEO, focusing on several key topics. Primarily, the topics that will be in focus are from the field of experimental pharmacology, immunology, molecular genetics, as well as radiobiology. Other health workers/associates, as well as new doctoral students, all in research and teaching positions, will be involved in the research. In the following period, it is expected to continue and further strengthen the cooperation between research and the clinics. Excellent cooperation with the Institute's clinics, where there are comprehensive databases of clinical data, will also contribute to the improvement of the quality of translational research. As a result of this type of research, many doctoral dissertations by both researchers and clinicians have been completed or are in progress. Since its establishment, the IORS has been the teaching base of the Faculty of Medicine in the fields of surgery, radiology, oncology, as well as other complementary disciplines for undergraduate and postgraduate teaching. Since the academic year 2013/2014, the Service for NIOD has been teaching doctoral studies in biology within the Cellular and Molecular Oncology module of the Faculty of Biology, University of Belgrade, Serbia. From the academic year 2025/26, the NIOD Service will participate in the Master's program Bioinformatics 4.0 of the Faculty of Medicine of the University of Belgrade, Serbia and the Faculty of Biology of the University of Belgrade, Serbia.

FUTURE HORIZONS IN CANCER

Review/Perspective

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CtDNA: What We Know and What We Are Looking For

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Abstract

In recent years, circulating tumor DNA (ctDNA) has become an essential analyte in both scientific research and the treatment of oncology patients. While many questions remain unanswered, the information contained in ctDNA molecules is crucial for understanding the biological characteristics of malignant tumors. Currently, plasma is the standard biological source of ctDNA, although other sources are being explored as potential alternatives. The kinetics of ctDNA is influenced by various factors, all of which must be considered when determining the timing and volume of body fluid samples. CtDNA has applications in oncology, particularly in selecting the most appropriate targeted therapy based on the mutations present in tumors. Its role in the early detection of cancer or minimal residual disease is still under investigation.

Our aim was to highlight outstanding challenges for the applications of ctDNA in research and molecular diagnostics. To expand the use of ctDNA in clinical practice it is necessary to establish clear and standardized protocols for the isolation and detection of ctDNA. Until then, we must continue to summarize existing literature, highlighting the issues that we are eager to resolve.

Introduction

As our understanding of the biology of malignant tumors grows, it is increasingly evident that significant inter- and intra-tumor heterogeneity poses a major challenge in treating cancer patients. The advancement of therapeutic strategies and the more frequent customization of treatment pathways for individual patients have led to the development of personalized treatment approaches (1). Such treatments require ongoing monitoring to predict therapy response, the development of resistance, disease progression, and potential side effects. Ideally, valuable information can be obtained from a tumor biopsy. However, the tumor's location and the patient's overall health often make rebiopsy at various time points impractical. This challenge has driven the intensive development of liquid biopsy methods aimed at identifying suitable biological markers in a more accessible and less invasive manner. Components of the tumor and its microenvironment can be isolated from body fluids, including circulating tumor cells (CTCs), extracellular vesicles, tumor-educated platelets, cell-free RNA (cfRNA), and circulating tumor DNA (ctDNA) (2, 3).

The presence of circulating fragments of DNA was first described in 1948, although significant findings were scarce until 1977 (4). At that point, Leon et al. reported that patients with various malignancies exhibited increased levels of circulating free cellular DNA (cfDNA) compared to healthy individuals (5). In 1989, Strawn et al. concluded that the elevated cfDNA levels in cancer patients were due to DNA fragments released into circulation by cancer cells (6). Today, we recognize that ctDNA, which originates specifically from malignant cells, constitutes a fraction of the total cfDNA found in body fluids.

The insights provided by ctDNA have made it an essential analyte in cancer treatment. This review aims to summarize the current literature on ctDNA, identify the associated challenges and issues, and predict the future directions for research in this field.

What is ctDNA?

Several mechanisms contribute to the release of ctDNA. Most commonly, it results from tumor cell death, but it can also arise from cellular senescence, the active secretion of extracellular vesicles, or the egestion of mitochondrial DNA (2).

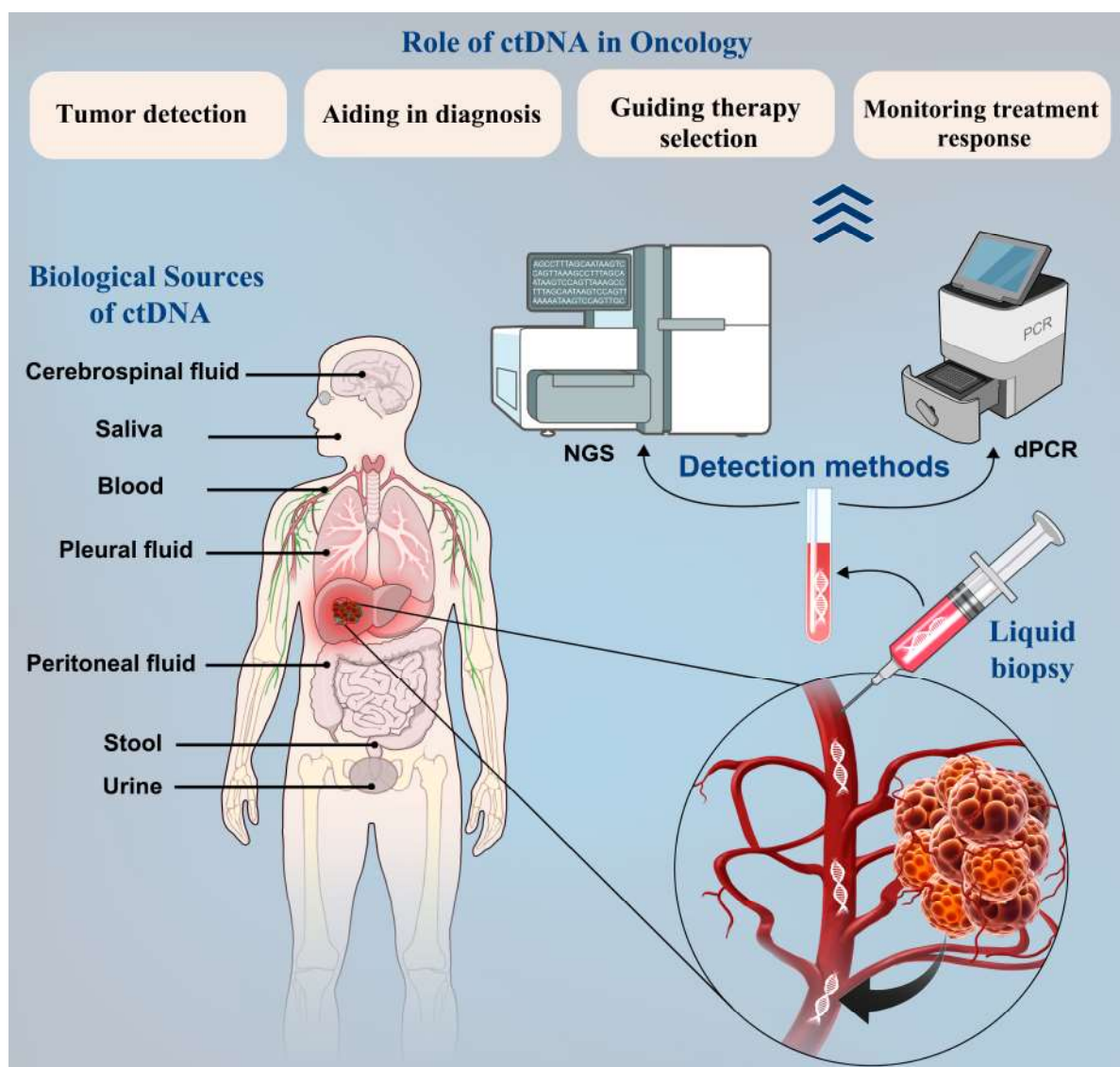
CfDNA consists of fragments typically ~167 base pairs (bp) in size, which corresponds with nucleosome-associated fragments of DNA, primarily originating from hematopoietic cells. (2, 7). However, it is believed that cells from other organ systems may also release cfDNA under both physiological and pathological conditions, which warrants further investigation (7). The size of ctDNA fragments can vary significantly, depending on the biological source from which the

ctDNA is isolated; generally, ctDNA is smaller than 150 bp (8). It is hypothesized that cfDNA mainly comes from cells undergoing apoptosis, which are subsequently phagocytosed by macrophages via apoptotic blebs, leading to their release into circulation. The role of necrosis in the release of total cfDNA is still not fully understood (9).

In patients with malignancies, particularly in advanced disease stages, the amount of cfDNA tends to increase. It is assumed that this increase in cfDNA, specifically ctDNA, is largely derived from necrotic malignant cells. Additionally, autophagy may play a critical role, serving as an alternative energy source for tumors (10). Regardless of whether the mechanism of cell death is apoptosis, necrosis, or another type, ctDNA is also phagocytosed by immune cells and subsequently released as smaller fragments into the tumor microenvironment or circulation. It is also important to note that ctDNA can originate from living tumor cells through their extracellular vesicles (2).

The levels of cfDNA and ctDNA in circulation depend directly on the balance between the release of DNA fragments and their clearance. Studies have shown that the half-life of cfDNA ranges from 16 minutes to 2.5 hours (2). The breakdown and excretion of DNA fragments involve mechanisms such as DNases present in circulation, active clearance of nucleosomes, and filtration through organs like the kidneys and lymph nodes (11). The association of ctDNA with macromolecular complexes, as well as its size, also influences the rate of clearance (12). Further research is needed to better understand the kinetics of ctDNA.

Moreover, a significant challenge remains in distinguishing ctDNA from cfDNA. Current studies are focusing on three main approaches to analyze the structure of ctDNA: examining tail motifs, nucleosome positioning, and methylation patterns (13). By enhancing these techniques, researchers strive to establish methods for quantifying ctDNA, a challenge that remains unsolved. Thus far, ctDNA levels are often described numerically through variant allele frequency (VAF), which is the only way to quantify ctDNA within the total cfDNA present (2).



The primary biological sources for isolating circulating tumor DNA (ctDNA) have been identified. After detection using next-generation sequencing (NGS) or digital polymerase chain reaction (dPCR) methods, ctDNA plays a role in various stages of personalized treatment for oncology patients.

What Affects ctDNA Levels?

Several important factors influence the levels of ctDNA in the body beyond its kinetic characteristics.

Currently, it is understood that the level of ctDNA primarily depends on the type of malignant tumor present. For instance, tumors located in the central nervous system (CNS) tend to release undetectable amounts of ctDNA into the bloodstream (14). This is why it is crucial to consider the biological source from which ctDNA is isolated, tailoring this approach to the specific type of tumor and its stage of progression. In addition to CNS tumors, low levels of ctDNA are also observed in conditions such as medulloblastoma, kidney cancer, prostate cancer, and thyroid cancer. Conversely, detecting ctDNA is significantly more straightforward in cancers of the ovary, liver, pancreas, bladder, lung, stomach, breast, and colon, among others (14). Furthermore, the histological characteristics of the tumor are also important. Research indicates that cancers with a pronounced necrotic profile release larger amounts of ctDNA. This phenomenon is particularly evident in squamous cell lung cancer and triple-negative breast cancer. These findings further emphasize the role of necrosis in influencing ctDNA levels (15, 16).

The level of ctDNA is influenced by the stage of the disease. Numerous studies have demonstrated that patients with more advanced stages of the disease have significantly higher amounts of ctDNA. For example, in the early stages of non-small cell lung cancer (NSCLC), the ctDNA constitutes only 0.01% of cfDNA, whereas in the later stages, this percentage can rise to as much as 10% (14, 17).

Research has also shown a strong correlation between tumor size and volume with ctDNA levels. Patients with larger tumors tend to have a significantly higher VAF value (18).

When sampling, detecting, and analyzing ctDNA, it is crucial to consider the impact of therapy on these measurements. After surgical removal of a tumor, the ctDNA level in the body drops significantly. However, tissue damage caused during the procedure can lead to a substantial increase in total cfDNA levels immediately afterward. This surge in cfDNA may mask the presence of ctDNA, potentially leading to false negative results that suggest the disease is absent when it may not be (19). Additionally, treatments like radiotherapy and chemotherapy, which have cytotoxic effects, can further elevate cfDNA levels, diminishing the detectability of ctDNA. To avoid receiving misleading negative results, it is essential to collect cfDNA samples at carefully chosen time points, considering the clearance rate of DNA fragments (2, 19).

What Are the Biological Sources of ctDNA?

Currently, ctDNA isolated from plasma has the most significant application in clinical practice. Plasma is generally a better biological source than serum due to the smaller amounts of cfDNA found in serum (2, 3). However, as mentioned in the previous chapter, certain tumors release ctDNA in insufficient quantities into the bloodstream, complicating the detection process. Consequently, recent research has focused on exploring other biological sources within the body from which ctDNA can be isolated. These alternative sources often yield a significantly higher proportion of ctDNA compared to the total cfDNA present, particularly due to the reduced number of the hematopoietic system cells. In some instances, ctDNA from these sources may more accurately reflect the clonal heterogeneity of the tumor itself than ctDNA obtained from plasma (8).

Urinary ctDNA can originate from plasma that has undergone glomerular filtration or from tumor cells located directly within the urinary tract (20, 21). Isolating ctDNA from urine presents several advantages: it is readily accessible and convenient for patients. Additionally, the level of cfDNA in urine is significantly lower than in plasma because there is no lysis of blood cells, although cfDNA from epithelial cells in the urinary tract may still be present (8). However, a challenge with urine as a biological source is the variability in the size of the ctDNA molecules due to glomerular filtration. Furthermore, the larger volume of urine samples compared to plasma samples can pose difficulties in the isolation process, while also leading to excessive dilution of ctDNA. The amount of ctDNA found in urine can fluctuate throughout the day, especially if the patient is undergoing systemic anticancer therapy. There are also concerns regarding the optimal methods for storing and transporting urine samples, as well as the ideal time frames for analysis, given the short half-life of insufficiently protected ctDNA (22, 23). Despite these challenges, research has demonstrated that urinary ctDNA provides valuable information about mutations in the *EGFR* and *KRAS* genes in patients with NSCLC and enhances the accuracy of results when combined with plasma ctDNA analysis (24). Promising results have also been reported in the context of breast cancer and predicting relapses in hepatocellular carcinoma (8). Undoubtedly, ctDNA isolated from urine offers crucial insights regarding malignant tumors of the urinary system.

For tumors located in CNS, which typically do not release ctDNA into the bloodstream, *cerebrospinal fluid* (CSF) can serve as an alternative source for diagnosis and predicting disease relapse. CSF contains a lower level of circulating immune cells compared to blood, leading to lower levels of cfDNA (8). CtDNA can provide genetic information about primary CNS cancers or metastases from distant sites, such as melanoma. In the case of gliomas, several molecular markers are well-established. Their detection in ctDNA using a digital polymerase chain reaction (dPCR) approach allows for the classification of tumors into different molecular subtypes, positively influencing the choice of optimal therapy (25, 26). However, CSF is less readily available than other biological sources and its sampling is associated

with high risks, making routine or repeated sampling nearly impossible. Further research is needed to standardize the processes for isolating and storing CSF, and this research is complicated by the necessity of obtaining special ethical approvals (8).

Pleural and peritoneal fluids are in close contact with tumors and may contain ctDNA even when plasma results are negative. The overall levels of cfDNA in these fluids are lower than in blood since they generally lack blood cells. These fluids have been utilized in clinical analyses for NSCLC and colon cancer. Studies have indicated that target mutations in pleural and peritoneal effusion can be detected with higher VAFs than those detectable in plasma (27, 28). However, the sampling procedures for these fluids can be invasive and are only pursued when they offer significant benefits for the patient, which complicates their use in monitoring oncology patients.

Other biological sources for ctDNA isolation, such as *saliva* and *stool*, have shown potential as well. While salivary ctDNA provides useful information concerning malignant tumors of the head and neck, the size of the DNA fragments, which measures 40-60 bp, complicates analysis (29). In stool samples, human DNA constitutes only 0.01% of the total, as the sample is predominately composed of the gastrointestinal microbiome, complicating the isolation and detection of ctDNA (30). Ongoing research will likely reveal the importance of additional biological sources for ctDNA, including *seminal fluid*, *bile*, *uterine lavage fluid*, *vaginal fluid*, and more (8).

What are the methods for isolation and detection of ctDNA?

Research has focused significant attention on the development of techniques for the isolation and detection of ctDNA in recent years, alongside advancing knowledge about ctDNA and liquid biopsies in general. CtDNA represents a portion of the total cfDNA regardless of the biological source from which it is isolated.

Currently, isolation methods have been standardized primarily for blood or plasma samples. When using standard tubes with K2 EDTA, the entire analysis must be completed within six hours of blood collection, with careful maintenance of the cold chain during transport. Alternatively, specialized collection tubes with stabilizing agents that can keep cfDNA stable at room temperature for up to 14 days can be used (31). Effective isolation requires the complete removal of all cellular components from the sample. For this reason, it is recommended to perform a two-step centrifugation at 1600g for 10 minutes when isolating plasma. To ensure the integrity of cfDNA during storage, samples should ideally be kept at -80°C and should avoid multiple freeze-thaw cycles (32).

There are three primary techniques used for isolation: phase separation, silica membrane-based isolation, and magnetic bead-based isolation. The chosen isolation method is crucial for the success of the subsequent detection, as the quantity and preservation of ctDNA fragments depend heavily on it (2).

The initial platform for ctDNA detection was quantitative polymerase chain reaction (qPCR). However, due to its reduced sensitivity, dPCR and next-generation sequencing (NGS) have gained prominence. While dPCR offers high sensitivity for detection, it is limited by the finite number of mutations that can be analyzed in a single assay. One advantage of dPCR over NGS is that it does not require bioinformatic analysis, allowing for faster results, which is beneficial for patients (3).

Nevertheless, improvements in NGS, such as the incorporation of molecular tags (barcoding) for individual DNA molecules and advancements in bioinformatic analyses, have addressed many of the previous limitations, making NGS increasingly user-friendly. Today, NGS provides comprehensive information regarding the identification of genetic alterations, making it an indispensable method for ctDNA analysis and detection (3, 33).

What is the Role of ctDNA in Oncology?

CtDNA is emerging as a valuable analyte in oncology, offering potential for early disease detection, aiding in diagnosis, guiding therapy selection, and monitoring treatment response. It may also play a role in predicting resistance or relapse. Currently, ctDNA is primarily utilized to select the most appropriate therapy for patients based on target mutations identified within the ctDNA, which represents the tumor's complete genetic landscape. This is in contrast to traditional biopsy samples, which usually represent only a small fraction of the tumor.

The first major approval for the clinical use of ctDNA was the Cobas® EGFR Mutation test for detecting mutations in the *EGFR* gene (specifically, exon 19 deletions and the exon 21 L858R mutation) using the qPCR method. This allows for the identification of patients eligible for tyrosine kinase inhibitor therapy (34). Additionally, a kit for detecting 11 *PIK3CA* mutations in ctDNA through PCR has been used to select breast cancer patients for treatment with alpelisib (3, 34). As the list of targetable mutations has expanded to include the exon 20 T790M mutation in the *EGFR* gene, *KRAS* G12C, *ALK*, and *MET* exon 14 single-nucleotide variants (SNVs), the detection methods have advanced as well. This led to the development of the first gene panels for NGS testing. Today, there are NGS panels available for various cancers, including NSCLC and panels for prostate, breast, and ovarian cancers that also test for *BRCA1* and *BRCA2* mutations (34).

One notable limitation of ctDNA is its low concentration during the early stages of cancer, which can hinder early detection. Research has explored the use of ctDNA for the early detection of urothelial cancers from urine samples

and pancreatic cancer from stool samples (8). These alternative biological samples were chosen under the assumption that they would contain higher levels of ctDNA compared to blood samples. Furthermore, a study evaluated whether a targeted methylation-based test could be used for screening multiple cancers in individuals over 50 years of age. While the test yielded promising results, it also resulted in 57 false positives, along with reduced sensitivity in detecting early-stage disease (35).

Moreover, ctDNA can indicate the presence of minimal residual disease after surgical intervention or completion of therapy. It can also be used to monitor the patient's response to treatment and to detect early resistance before it becomes apparent through standard diagnostic methods. Currently, the most significant applications of ctDNA monitoring are found in clinical studies (2, 34). These studies define specific time points to monitor ctDNA levels before and after the administration of neoadjuvant or adjuvant therapy. From this data, it is possible to predict the development of resistance to the treatment or the likelihood of disease relapse (34). The combination of these findings with research from other studies will pave the way for more widespread use of ctDNA in patient monitoring throughout the treatment process.

Conclusion

Based on the current literature and the findings summarized in our review paper, we can conclude that two key challenges must be addressed for ctDNA to be widely used in clinical practice.

The first challenge is to gain a better understanding of the kinetics of ctDNA. This involves defining the characteristics of different malignant tumors and identifying the factors that influence the release of ctDNA into the bloodstream. This knowledge will help in selecting the most appropriate biological source for each type of cancer. Additionally, when determining the timing and quantity of sample collection, it is crucial to consider all factors that may impact ctDNA kinetics, such as surgical interventions and cytotoxic therapies. Establishing a protocol for ctDNA sampling can help minimize the occurrence of false-negative results due to a low proportion of ctDNA in the total cfDNA.

The second challenge is the standardization of methods for isolating and detecting ctDNA from various biological sources, as well as improving technology to enhance sensitivity. This advancement will allow for the detection and analysis of ctDNA present in small quantities.

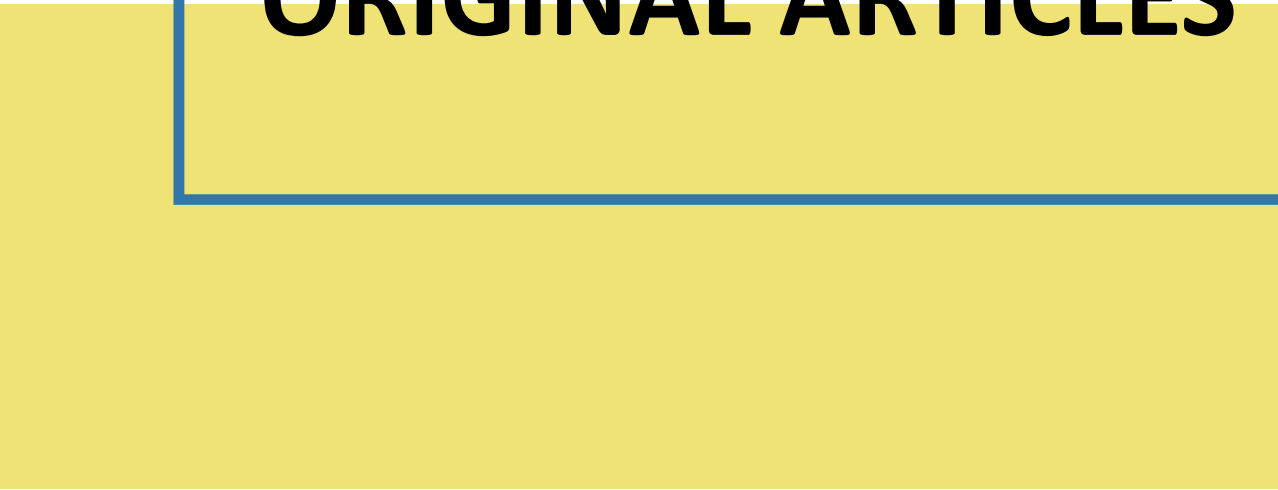
Research in the coming years will provide answers to these challenges. It is clear that ctDNA holds the potential to significantly improve the personalized treatment of oncology patients, potentially making many malignant tumors manageable and even curable, even in the later stages of the disease.

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ORIGINAL ARTICLES

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Optimizing Treatment Response in Lung Squamous Cell Carcinoma by Cell-Based Functional Sensitivity Screening

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Abstract

Traditional chemotherapy is the standard of care for lung squamous cell carcinoma (LSCC). However, recent studies have suggested that precision medicine platforms could help identify better treatment options. Our approach involves testing various chemotherapeutics in cells isolated from LSCC patients' biopsies to make more informed treatment decisions. Five primary LSCC cultures from patient-derived cells were established and exposed to eight different chemotherapeutics and erlotinib 72 h after culturing, with treatments lasting for 7 days. An immunofluorescence assay with a CK8/18 antibody mixture was utilized to distinguish cancer cells from stromal cells, while a scoring method involving 11 weighted parameters was applied to calculate the drug response score for LSCC patients' cell responses. Our results suggest that erlotinib could be an effective treatment for LSCC patients and that the dosage of erlotinib might be reduced without compromising its effectiveness. Our findings show that gemcitabine, in addition to platinum drugs, could be beneficial for LSCC patients. The results underscore the potential of functional screening methods to identify effective treatments for LSCC.

Introduction

Lung cancer is the primary cause of cancer-related deaths in Serbia, accounting for 16.4% of all cancer diagnoses and around 24.4% of all cancer-related deaths (GLOBOCAN Cancer Today (iarc.fr)). The morbidity and mortality rates of lung cancer in Serbia are among the highest globally. According to the GLOBOCAN estimates for 2022, Serbia ranks in Europe for both incidence and mortality rates, right after Hungary, Poland, and Romania. In comparison to Western Europe and the USA, where a decline in mortality rates has been observed in recent decades, especially for males, mortality rates in Serbia have been continuously rising in both genders, particularly in females.

Non-small-cell lung cancer (NSCLC) accounts for 80%–85% of all lung cancer cases and consists of two major subtypes: lung adenocarcinoma (LADC) and lung squamous cell carcinoma (LSCC) (1). LADC and LSCC have distinct histologies and patterns of occurrence within the lung. Multiple significant oncogenic drivers in LADC have been recognized, such as activating mutations in KRAS, EML4-ALK gene fusions, and activating mutations and/or amplification of the EGFR. The most prevalent somatic mutations in LSCC involve the inactivation of the tumor suppressors LKB1, PTEN, and TP53, while oncogenic mutations present in LADC are rare (2).

Although LSCC incidence has dropped over the years due to a decrease in tobacco smoking, the primary causative agent of LSCC, its prevalence is still high. Unlike LADC, the majority of patients diagnosed with LSCC are former or current heavy smokers. Tobacco smoking leads to a high somatic mutational burden in LSCC (3). However, the DNA mutations targeted for LADC therapy are essentially absent in LSCC. Therefore, the standard of care for metastatic LSCC is chemotherapy, traditionally using a dual platinum/taxane or platinum/etoposide approach (4).

The FDA-approved non-traditional chemotherapy compounds for LSCC include the antiangiogenic VEGFR inhibitor ramucirumab (for second-line treatment), necitumumab (a monoclonal antibody inhibitor of EGFR for first-line treatment when combined with chemotherapy), afatinib (an EGFR/HER2 inhibitor for second-line treatment), and immune checkpoint inhibitors such as nivolumab, pembrolizumab, atezolizumab, and durvalumab (3). While there have

been some advancements in the development of targeted therapies for LSCC, the response rate to these treatments has been limited. Only 7% and 17% of patients have responded positively to targeted therapies or immune checkpoint inhibitors, respectively (5).

Recent studies indicate that precision medicine platforms could potentially act as surrogate biomarkers for cancer types lacking druggable targets. These platforms encompass diverse patient-derived cellular models and screening techniques. Notably, a significant proportion of clinical trials across various cancer types, including lung cancer, have demonstrated correlations with clinicopathological parameters, pointing to the most efficacious therapies for cancer patients (6). Our approach includes screening for different chemotherapeutics in patient-derived cells to discern optimal treatment options for patients with LSCC and address the scarcity of easily targetable treatments. Through this method, we can categorize responses of LSCC patient cells based on anticancer activity, selectivity towards cancer cells, and the nature of the response, whether it is dose-dependent or biphasic. These evaluations are carried out using short-term primary cell cultures comprising cancer and stromal cells (7). In addition, our recent findings indicate an excellent response of both LADC and LSCC patient-derived cells to erlotinib (8). Therefore, this study compared the responses of LSCC primary cell cultures among 8 chemotherapeutics and erlotinib.

Materials and methods

Establishment of LSCC primary cell cultures

Tissue samples from LSCC patients were obtained from the Clinic for Thoracic Surgery at the University Clinical Center of Serbia after informed consent from the patients and approval from the Ethics Committee of the University Clinical Center of Serbia (approval reference number 623/4). After surgical removal, the samples were placed in a sterile tube containing an antibiotic-antimycotic solution (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) and immediately transferred to the research laboratory for further processing.

To establish primary cultures, the tissue was manually minced with a surgical blade in a sterile Petri dish upon arrival at the laboratory. The samples were cut into 3 – 5 mm pieces and dissociated using the Tumor Dissociation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The tissue pieces were incubated in an orbital shaker (KS 4000 ic control, IKA, Königswinter, Germany) at 37°C and 300 rpm for 90 minutes. After incubation, the dissociated tissue was placed in DMEM/Ham's F12 (1:3) growth medium supplemented with 5% fetal bovine serum (Corning, NY, USA), antibiotic-antimycotic solution, 4 µg/mL hydrocortisone (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany), 1 µg/mL insulin (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany), 10 ng/mL epidermal growth factor (BioLegend, San Diego, CA, USA), and 24 µg/mL adenine (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). The DMEM and Ham's F12 growth media were purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). The dissociated tissue was cultured in T-25 cell culture flasks until cell attachment was observed before the medium was replaced. Five successfully established primary LSCC cultures (stage II: TR36 and TR87, and stage III: TR33, TR34, and TR107) were maintained at 37°C in a humidified atmosphere with 5% CO₂ and grown to confluence before further experiments.

Treatments with chemotherapeutics and erlotinib

Vinorelbine and pemetrexed were purchased from Selleckchem (Houston, TX, USA). Carboplatin, cisplatin, docetaxel, etoposide, gemcitabine, and paclitaxel were purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). Erlotinib was purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). Cisplatin, carboplatin, and gemcitabine were dissolved in sterile water, while etoposide, docetaxel, vinorelbine, paclitaxel, pemetrexed, and erlotinib were dissolved in DMSO and stored at –20°C. Before treatment, all stock solutions were freshly diluted in sterile water. The following concentrations were used for the treatments: vinorelbine (100, 250, 500, 750, and 1000 nM); pemetrexed (50, 75, 100, 200, and 300 µM); carboplatin (10, 25, 50, 75, and 100 µM); cisplatin (5, 7.5, 10, 12.5, and 15 µM); docetaxel (1, 2, 3, 4, and 5 µM); etoposide (10, 15, 20, 25, and 30 µM); gemcitabine (10, 25, 50, 75, and 100 µM); paclitaxel (1, 2, 3, 4, and 5 µM); and erlotinib (0.5, 1, 2, 3, and 4 µM).

Patient-derived cells were seeded in black, clear-bottom 384-well cell culture microplates (Thermo Fisher Scientific, Waltham, MA, USA) at a density of 1000 cells per well in 50 µL of cell growth medium. The drugs were administered 72 h after seeding, and the treatment lasted for 7 days.

Immunofluorescence assay

The immunofluorescence assay was used to distinguish cancer cells from stromal cells using a cytokeratin 8/18 (CK8/18) antibody mixture as previously described (7). CK8/18-positive cells were considered cancer cells, while CK8/18-negative cells were considered stromal cells. Cells were fixed in 4% paraformaldehyde for 20 min at RT and washed using the Wellwash™ Versa microplate washer (Thermo Fisher Scientific, Waltham, MA, USA). Cells were then blocked with 2% bovine serum albumin (BSA) in PBS for 1 h at RT. Cells were then incubated overnight at 4°C with a primary rabbit CK8/18 antibody cocktail (clone SU0338, #MA5-32118, Thermo Fisher Scientific, Waltham, MA, USA). Cells were then

incubated with secondary Alexa Fluor 488 goat anti-rabbit antibody (#A-11008, Thermo Fisher Scientific, Waltham, MA, USA) at RT for 2 h under photoprotective conditions. Cell nuclei were counterstained with 1 µg/mL Hoechst 33342 at RT for 2 h. Cells were stored at 4°C in the dark before imaging.

Fluorescently labeled cells were imaged using the ImageXpress® Pico Automated Cell Imaging System (Molecular Devices®, San Jose, CA, USA) with a 4x objective after determining the appropriate exposure time for each illumination filter. The obtained images were analysed using CellReporterXpress® software v. 2.8.2.669 (Molecular Devices®, San Jose, CA, USA). The Cell Scoring Analysis Protocol was used to assess the cytotoxicity of drugs as previously described (7).

Scoring of chemotherapeutics and erlotinib responses

LSCC patients' cell responses were scored using a modified scoring method (9). We generated an overall drug response score by considering various weighted parameters. This score was determined through the comparison of drug effects on cancer and stromal cells using our immunoassay.

- 1) The inhibitory effect (inhibition percentage) at the maximum dose is multiplied by 0.1. If the inhibitory effect is greater with stromal cells, it is multiplied by -0.1.
- 2) The dose required for 10% inhibition is multiplied by 0.05. If the dose is higher than that for stromal cells, it is multiplied by -0.05.
- 3) The dose required for 25% inhibition is multiplied by 0.05. If the dose is higher than that for stromal cells, it is multiplied by -0.05.
- 4) The dose required for 50% inhibition is multiplied by 0.1 (if this dose is above 100 µM, it is multiplied by 0). If the dose is higher than that for stromal cells, it is multiplied by -0.1 (if this dose is above 100 µM, it is multiplied by -10).
- 5) The dose required for 75% inhibition is multiplied by 0.05 (if this dose is above 100 µM, it is multiplied by 0). If the dose is higher than that for stromal cells, it is multiplied by -0.05 (if this dose is above 100 µM, it is multiplied by -5).
- 6) The dose required for 90% inhibition is multiplied by 0.05 (if this dose is above 100 µM, it is multiplied by 0). If the dose is higher than that for stromal cells, it is multiplied by -0.05 (if this dose is above 100 µM, it is multiplied by -5).
- 7) Slope
- 8) The area under the curve (AUC) is multiplied by 0.35
- 9) If none of the applied doses stimulates the growth of cancer cells, the lowest dose is multiplied by 0.05, and if the dose stimulates the growth of cancer cells up to 125%, it is also multiplied by 0.05. If the dose stimulates the growth of cancer cells from 125% to 150%, it is multiplied by 0; if it stimulates over 150%, it is multiplied by -0.05.
- 10) If the drug acts rapidly at lower doses and has a limited effect at higher doses, the breakthrough dose is multiplied by -0.05. If there is a dose-dependent effect, the dose that inhibits 50% is multiplied by 0.05.
- 11) If the drug incompletely inhibits tumor growth at the highest dose¹: if more than 25% of cancer cells remain, the highest dose is multiplied by -0.05, if 10% – 25% of cancer cells remain, the highest dose is multiplied by 0, and if less than 10% of cancer cells remain, the highest dose is multiplied by 0.05.

All individually weighed parameters were combined to calculate the drug response score. A score from 0 to 60 indicates that the treatment is more effective on cancer cells, while a score from 0 to -60 suggests that the treatment has a greater impact on stromal cells. The R package PharmacGx was utilized to determine dose-response values, slope, and area under the curve (AUC). Specifically, we used the computeAUC function from the PharmacGx package to calculate the AUC for the drug dose viability curve. This function takes drug concentration and cell viability as inputs and normalizes the AUC by the concentration range. The AUC, representing the response area (1 - Viability), is calculated as the area under the curve on a log₁₀ concentration scale. A higher AUC indicates greater sensitivity to the drug.

Results

Two LSCC primary cell cultures (TR36 and TR87) were derived from patients with stage II disease, while three (TR33, TR34, and TR107) were derived from patients with stage III. A month after surgical resection, three LSCC patients (TR87, TR33, and TR107) were scheduled to undergo three cycles of cisplatin-etoposide chemotherapy, but TR36 did not attend the follow-up examination after surgery, and TR34 experienced disease progression immediately after surgery and passed away three months later. The LSCC patients who were supposed to receive chemotherapy did not receive it at the hospital where they were enrolled in this study.

Five primary cell cultures derived from LSCC patients were exposed to eight chemotherapeutics (cisplatin, carboplatin, etoposide, paclitaxel, docetaxel, gemcitabine, pemetrexed, and vinorelbine) and an EGFR inhibitor (erlotinib).

¹The concentrations used were chosen based on clinically relevant levels of anticancer drugs, with the upper limit concentration (the highest dose) set as the maximum concentration reached in human plasma during therapy (C_{max})(10).

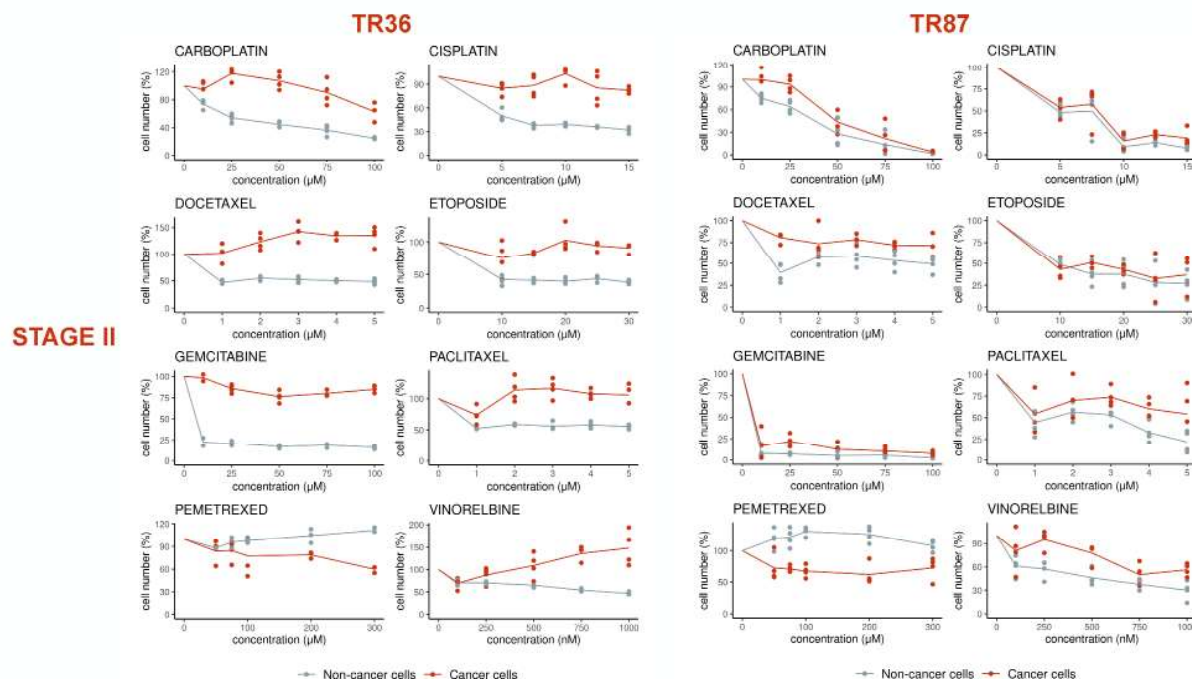


Figure 1. Cytotoxicity of 8 chemotherapeutics evaluated in two patient-derived lung squamous cell carcinoma (LSCC) cultures stage II. The CK8/18 antibody was used to discriminate between cancer and non-cancer (stromal) cells in a mixed culture. Treatments with chemotherapeutics lasted for 7 days. Values are expressed as the mean $\pm$ standard error of the mean (SEM) (n = 4).

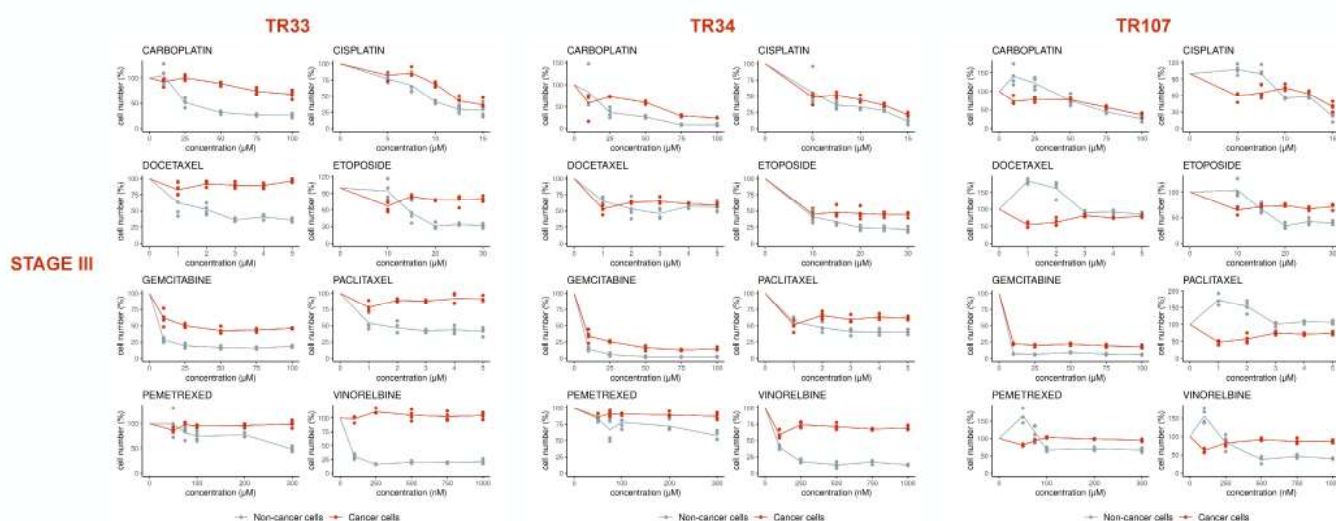


Figure 2. Cytotoxicity of 8 chemotherapeutics evaluated in three patient-derived lung squamous cell carcinoma (LSCC) cultures stage III. The CK8/18 antibody was used to discriminate between cancer and non-cancer (stromal) cells in a mixed culture. Treatments with chemotherapeutics lasted for 7 days. Values are expressed as the mean $\pm$ standard error of the mean (SEM) (n = 4).

Stage II LSCC samples (TR36 and TR87) exhibited a similar selectivity pattern towards cancer cells, with only pemetrexed showing a more pronounced effect on cancer cells than on stromal cells (Figure 1). TR87 demonstrated a superior response to all chemotherapeutics compared to TR36 (Figure 1). TR36 displayed significant resistance to all chemotherapeutics, with none of them reaching 50% inhibition in the case of cancer cells within the given concentration range (Figure 1). On the other hand, carboplatin, cisplatin, and gemcitabine exhibited noteworthy anticancer activity against TR87 but with a similar effect on stromal cells (Figure 1).

Stage III LSCC samples (TR33, TR34, and TR107) exhibited varied responses without following a consistent pattern (Figure 2). TR33 exhibited a positive response only to cisplatin, whereas TR34 responded well to cisplatin and gemcitabine (Figure 2). TR107 displayed the most favorable selectivity pattern, demonstrating a preference for cancer cells, particularly at lower concentrations of carboplatin, cisplatin, docetaxel, and paclitaxel. Nevertheless, only carboplatin and gemcitabine showed significant anticancer effects in the TR107 sample (Figure 2).

All LSCC samples exhibited a consistent response to erlotinib, as shown in Figure 3. Erlotinib demonstrated selectivity towards cancer cells across all five LSCC patients' samples and exhibited biphasic activity, achieving 50% inhibition of cancer cell growth at the lowest concentration of 0.5 μ M (Figure 3). However, the anticancer effect of erlotinib was not further enhanced with higher concentrations (Figure 3).

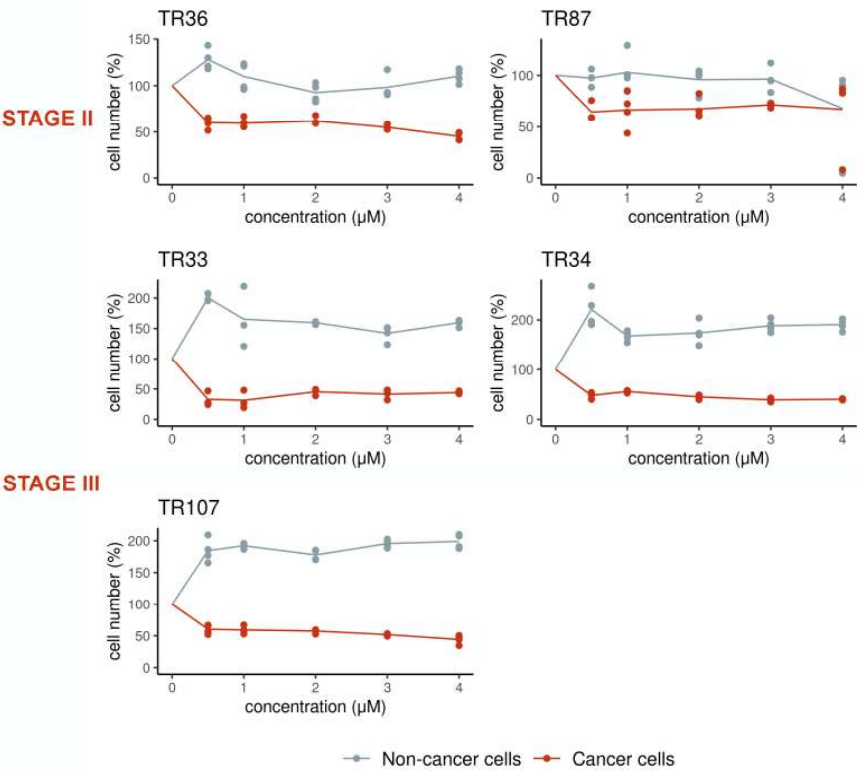


Figure 3. Cytotoxicity of erlotinib evaluated in five patient-derived lung squamous cell carcinoma (LSCC) cultures. The CK8/18 antibody was used to discriminate between cancer and non-cancer (stromal) cells in a mixed culture. Treatments with chemotherapeutics lasted for 7 days. Values are expressed as the mean ± standard error of the mean (SEM) (n = 4).

Scoring results have validated these findings and have provided a comprehensive overview of the responses observed in LSCC patients (Figure 4). A score above 10 is indicative of a good response. Notably, TR36 and TR33 exhibited positive responses exclusively to erlotinib, TR87 responded well to erlotinib, cisplatin, and carboplatin, with the most significant response to gemcitabine, TR34 responded favorably to erlotinib, cisplatin, and gemcitabine, while TR107 showed positive responses to erlotinib, carboplatin, gemcitabine, paclitaxel, and docetaxel (Figure 4).

Discussion

The treatment landscape for NSCLC is rapidly evolving, especially in EGFR inhibitors and immunotherapy, with many ongoing clinical trials. Advances in molecular characterization have led to improved treatment options and increased survival rates for LADC. However, targeted molecular therapies still need to make significant progress for LSCC (11). An earlier study demonstrated the benefits of erlotinib as a second-line therapy for advanced LSCC (BR.21 trial). It showed increased median overall survival and reduced tumor-related symptoms such as dyspnea, pain, and cough compared to the placebo group (12). In LSCC, identifying predictive markers for responsiveness to EGFR inhibitors like erlotinib is a critical area of research.

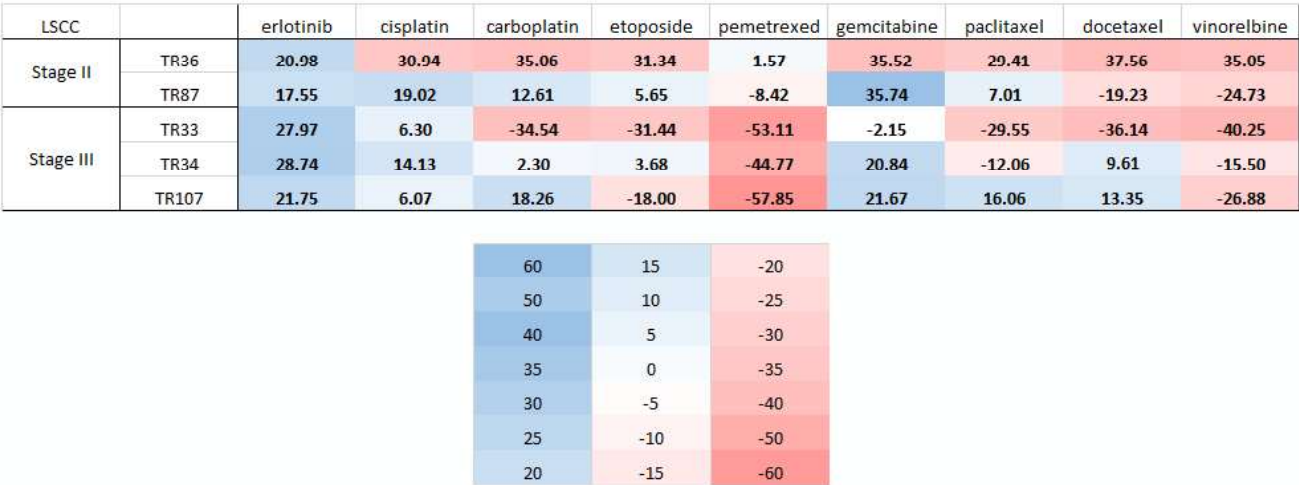


Figure 4. Drug sensitivity scoring array for five patient-derived lung squamous cell carcinoma (LSCC) cultures against 8 chemotherapeutics and erlotinib. Scores from 0 to 60 indicate increasing efficacy in toxicity towards cancer cells, while scores from 0 to -60 reflect cases in which non-cancer (stromal) cells are more sensitive than cancer cells. Responses with a score above 10 are considered beneficial.

High levels of EGFR protein expression may correlate with a better response to erlotinib (13). While gene amplification tends to be more common in LADC, it can also play a role in a subset of LSCC. Although activating mutations in the EGFR gene are less frequent in LSCC than in LADC, their presence can significantly influence sensitivity to erlotinib. Mutations such as L858R in exon 21 or deletions in exon 19 enhance the response to erlotinib (14).

Erlotinib explicitly targets the ATP-binding site of the EGFR, leading to multiple downstream effects on signaling pathways that promote cell proliferation and survival, including the MAPK and PI3K/AKT pathways. Altered expression of downstream signaling molecules, such as PI3K/AKT, may change the response to EGFR inhibition. Furthermore, co-alterations in other pathways, such as RAS mutations or loss of PTEN, may influence the response to erlotinib (15).

Treatment with erlotinib can lead to cell cycle arrest, particularly at the G1 phase, and promote apoptosis in cancer cells (16). In vitro studies have shown that NSCLC cell lines exhibit decreased proliferation rates and increased markers of apoptosis (e.g., cleaved PARP and caspases) in response to erlotinib (17).

While the efficacy of EGFR inhibitors in treating LSCC after previous treatment failures (second and further-line therapy) is established, there is ongoing debate regarding their effectiveness in first-line and combination therapies for LSCC. Despite concerns about potential toxicity, EGFR inhibitors continue to be considered a viable option due to their oral administration, tolerability, and positive impact on patients' quality of life (13).

Our functional screening approach demonstrated that all five LSCC samples responded favorably to erlotinib, outperforming platinum drugs. Three of the five LSCC patients (TR87, TR34, and TR107) could benefit from platinum-based treatment, the standard of care for LSCC patients. TR36 and TR33 showed insensitivity to cisplatin and carboplatin. None of the samples responded to etoposide, and only one LSCC sample (TR107) exhibited sensitivity to paclitaxel and docetaxel, suggesting that the standard approach of combining platinum drugs with either etoposide or paclitaxel may offer little to no benefit to LSCC patients. Notably, three out of five LSCC samples (TR87, TR34, and TR107) positively responded to gemcitabine. Recent findings indicate that platinum-gemcitabine combination therapy is highly effective among platinum doublet regimens and holds promise as a treatment for advanced LSCC (18).

Among five LSCC patients, TR33 has shown the highest tumor mutation burden (TMB) according to our earlier publication (8). TR34, TR36, and TR107 had a considerable TMB, while TR87 had the lowest TMB in the examined NSCLC patients' cohort (8). In a recent study, it was observed that NSCLC patients in the low TMB group demonstrated a significantly longer progression-free survival (PFS) when treated with chemotherapy (19). Conversely, NSCLC patients in the high TMB group exhibited extended PFS when administered immunotherapy subsequent to unsatisfactory chemotherapy (19). In line with these findings is the unresponsiveness of TR33, which has the highest TMB (1476 mutations), to the eight tested chemotherapeutics. Mutations in TP53, which are characteristic of LSCC patients, were identified in four out of five samples: in TR36 and TR107 (missense mutations), TR33 (splice site), and TR34 (a nonsense mutation) (8). The additional mutation that can influence drug response identified in TR36 is a PIK3CA missense mutation (8), a common LSCC driver (20). Concomitant TP53 and PIK3CA mutations may cause TR36 resistance to all tested chemotherapeutics. A recent study has shown that both mutated genes are associated with poorer therapeutic responses compared to the TP53 mutation alone in breast cancer (21).

Conclusion

Our research suggests that conducting functional sensitivity screening is essential for determining the optimal treatment strategies for each LSCC patient. Our previous study found that erlotinib was equally effective in patient samples with low and high TMB, regardless of their EGFR mutational status (8). The concern that many LSCC patients may lack well-defined biomarkers to predict their response to anticancer drugs can be addressed by the functional approach we utilized. Our scoring method, which considers selectivity, efficacy, and inhibitory pattern (dose-dependency), is particularly valuable for guiding treatment decisions. Furthermore, we observed that erlotinib exhibited anticancer effects in all patients at the lowest tested concentration, and this effect did not increase with higher concentrations. This suggests that the dose of erlotinib for LSCC patients could be reduced without compromising its efficacy.

Acknowledgments

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Original article

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Improved detection of T790M Mutation in the *EGFR* gene at the Institute for Oncology and Radiology of Serbia: A comparative study of dPCR and qPCR technologies over two yearsVanja Stevanović¹, Miodrag Vuković¹, Jelena Kijac¹, Aleksandra Stanojević¹, Valentina Karadžić¹, Anđela Obradović², Ana Damjanović¹, Milena Čavić¹, Radmila Janković¹¹Department of Experimental Oncology, Institute for Oncology and Radiology of Serbia²University of Belgrade, Faculty of Pharmacy, Belgrade, Serbia**Abstract**

The T790M mutation in the *EGFR* gene is a critical biomarker of resistance in patients undergoing EGFR tyrosine kinase inhibitor (TKI) therapy. Accurate and efficient detection of this mutation is essential for guiding treatment decisions in non-small cell lung cancer (NSCLC) patients. This study, conducted over two years at the Institute for Oncology and Radiology of Serbia, compared the effectiveness of digital PCR (dPCR) and quantitative PCR (qPCR) in detecting the *EGFR* T790M mutation in liquid biopsies from patients with disease progression on EGFR TKIs. Our findings reveal higher detection rate using dPCR (30.48%) compared to qPCR (19.44%). The improved sensitivity of dPCR supports its implementation as a preferred technique for *EGFR* T790M mutation detection, enhancing clinical decision-making and personalized treatment options for patients with NSCLC.

Introduction

Lung cancer is one of the leading causes of cancer-related deaths (18.7%) with the highest incidence worldwide (12.4% of all cancer cases) (1). In recent years, the incidence of lung cancer in Serbia has been steadily increasing, with a rate of 22.4 per 100,000 in women and 57 per 100,000 in men (2).

Molecular targeted therapies with the presence of corresponding alterations including the *EGFR*, *ALK*, *ROS1* and *KRAS* genes are already part of routine clinical practice. Screening for activating *EGFR*-TK (tyrosine-kinase) mutations in non-small cell lung cancer (NSCLC) is critical for several reasons, particularly for molecular profiling to tailor therapy to the patient, as well as to monitor therapy outcomes and determine prognosis. Certain *EGFR*-TK mutations and overall tumor heterogeneity may be the causes for the development of resistance to therapy (3).

Tyrosine-kinase inhibitors (TKI) are a type of targeted therapy designed to bind directly to the TK domain and inhibit further signaling. First-generation TKIs prevent ATP binding through their reversible interaction with the tyrosine kinase domain of *EGFR*, while second-generation inhibitors are characterized by their irreversible binding, which covalently bind to the TK domain (4). Osimertinib, a third-generation EGFR-TKI, was originally approved for the treatment of patients with EGFR T790M mutations after disease progression on first- or second-generation EGFR-TKIs and is now emerging as the standard first-line treatment for advanced *EGFR*-mutated NSCLC. This shift is supported by the results of the FLAURA trial, which showed a progression-free survival (PFS) of 18.9 months and an overall survival (OS) of 38.6 months (5). In Serbia, osimertinib was officially approved for first-line therapy in 2024. Therefore, it is important to improve the ability to monitor disease progression in order to select appropriate treatment options and strive for personalized patient care at each stage of disease evolution.

As repeating tissue biopsies in patients with progressive disease is often very difficult and uncomfortable for patients, liquid biopsies sometimes remain the only viable option for detecting mutations in the *EGFR* gene. Therefore, liquid biopsies allow the quantification of circulating cell-free DNA (cfDNA) to detect circulating tumor DNA (ctDNA), which contains specific tumor-associated genetic aberrations. However, the analysis of ctDNA poses several challenges and limitations due to its fragmentation as well as its low and variable concentration (6).

In Serbia, *EGFR* mutation testing from liquid biopsies for patients with tumor progression under EGFR-TKIs was introduced in 2016 and was performed on the Cobas qPCR instrument until 2023. To increase the sensitivity and specificity of mutation detection from liquid biopsy samples with a very low concentration of ctDNA, it was necessary to improve the technology. Therefore, dPCR technology was implemented in our laboratory from 2023 as a method that offers unparalleled sensitivity and precision in nucleic acid quantification and mutation detection. The technology is based on the application of optimal PCR conditions for the amplification of a single copy of the PCR template, dividing the sample into thousands of individual reactions, which allows precise quantification of the target sequences even

when they are present at an extremely low level (low concentration of ctDNA). This partitioning, combined with the use of fluorescent probes, enables dPCR to detect and quantify rare mutations with much higher accuracy compared to conventional PCR techniques.

The aim of the study is to compare the detection rates of the T790M mutation in the *EGFR* gene in NSCLC patients at the Institute for Oncology and Radiology of Serbia over the past two years, using two different PCR platforms.

Patients and methods

Study cohort

This study included patients with lung adenocarcinoma (stage IIIb/IV, ECOG performance status 0, 1 or 2) who were diagnosed and treated at the Institute for Oncology and Radiology of Serbia over a two-year period. All patients had tumors with *EGFR* mutations and received first- or second-generation *EGFR*-TKIs, in whom the tumors progressed, making them candidates for genetic testing for the presence of the resistant *EGFR* T790M mutation. The study included two cohorts of patients tested at the Department for Experimental Oncology, Laboratory for Molecular Genetics from March 2022 to February 2024. The first cohort was tested on the Cobas® platform from March 2022 to February 2023 and included 72 patients (39 females, 33 males, age range 65.8, median 67.5), while the second cohort was tested on Absolute Q™ DNA Digital PCR (dPCR) from March 2023 to February 2024 and included 76 patients (52 females, 24 males, age range 67.01, median 68).

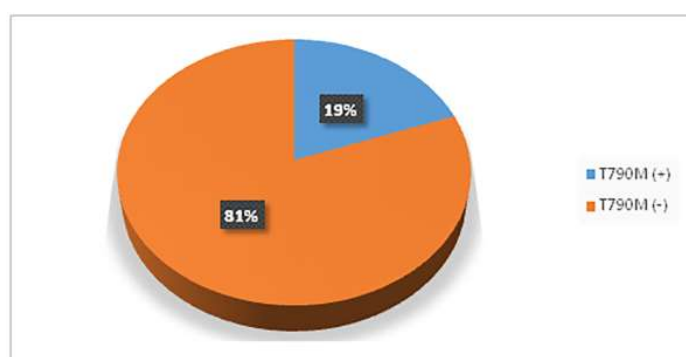


Figure 1. Percentage of T790M mutation detection rates using traditional qPCR technology on Cobas® platform

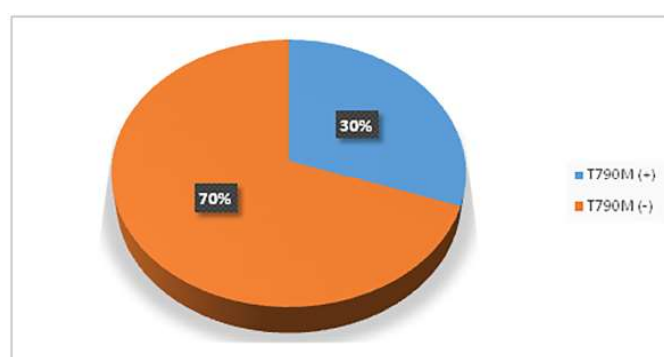


Figure 2. Percentage of T790M mutation detection rates using dPCR technology

DNA extraction

For quantitative PCR analysis, cfDNA was isolated from plasma, pleural effusion (PE) or cerebrospinal fluid using the Cobas® cfDNA Sample Preparation Kit (Roche Molecular Systems, Inc., Pleasanton, CA, USA). Circulating cell-free DNA for digital PCR was extracted from plasma and PE using the QIAamp® MinElute® ccfdNA Mini Kit (QIAGEN GmbH, Hilden, Germany). For cfDNA isolation QIAamp MinElute ccfdNA Mini Kit (QIAGEN, Hilden, Germany) isolation kit was used, which uses magnetic bead-based technology to enable the isolation of maximum amount of cfDNA. Concentration of cfDNA was measured using Qubit™ dsDNA HS Assay Kit (Invitrogen Thermo Fisher Scientific, Waltham, United States). In both cfDNA extraction methods, extraction was performed by trained laboratory technicians in the Laboratory for Molecular Genetics at the Department of Experimental Oncology, following the manufacturer's instructions.

PCR amplification and detection

The Cobas® *EGFR* Mutation Test v2 was utilized on the Cobas® 4800 platform. It is a test that provides mutation detection in exons 18-21 of the *EGFR* gene, including the T790M resistance mutation. *EGFR* dPCR mutation testing was performed using Absolute Q™ DNA Digital PCR MasterMix (5X), Taqman probe Hs000000029_rm for *EGFR* p. T790M and QuantStudio™ Absolute Q™ Isolation Buffer on QuantStudio™ Absolute Q™ MAP16 Digital PCR Kit 12-pack plates. The detection was performed on Applied Biosystems QuantStudio Absolute Q Digital PCR System (Thermo Fisher Scientific, Waltham, Massachusetts, SAD) and all samples were collected exclusively for the purpose of performing diagnostic procedures in order to adequately select the patients' therapy and to implement the analysis success statistics. The Laboratory for Molecular Genetics is annually certified by the European Molecular Genetics Quality Network. All analyses in this study are part of routine diagnostic procedures, approved by the institutional Ethics Committee (approval no. 5665-01, dated December 17, 2014). All patients signed an informed consent.

Statistical analysis

Descriptive statistics were used to summarize the sample data. Fisher's exact test was used for statistical analysis. Two-sided p-values <0.05 were considered statistically significant.

Results

Across 108 analyses conducted on the Cobas® platform, 21 mutations were successfully detected, yielding a detection success rate of 19.44% (Figure 1). In some patients, detection was performed multiple times, bringing the total number of analyses to 72 patients. The detected mutations included 17 identified in plasma and 4 in PE. In some patients, the same mutation was found in both plasma and PE, resulting in 19 patients overall with detected mutations (Table 1).

Table 1. The number (percentage) of detected T790M mutations using qPCR and dPCR technology

	qPCR		dPCR	
	T790M (+)	T790M (-)	T790M (+)	T790M (-)
Total analyses performed	21 (19.44%)	87 (80.56%)	32 (30.48%)	72 (69.52%)
Plasma	17 (17.35%)	81 (82.65%)	23 (25.27%)	68 (74.73%)
Pleural effusion	4 (40%)	6 (60%)	8 (66.67%)	4 (33.33%)

Among these 19 patients, 12 were females (63.16%) and 7 were males (36.84%). In 105 tests performed on the Applied Biosystems QuantStudio Absolute Q Digital PCR System, 32 mutations were identified, achieving a detection rate of 30.48% (Figure 2). The analyses included a total of 76 patients.

Of these detected mutations, 23 were found in plasma samples, whereas 8 were found in PE. One female patient had the same mutation in both types of samples, bringing the total number of patients with detected mutations to 31 (Table 1). Of these 31 patients, 19 were females and 12 were males, corresponding to a mutation detection rate of 71.88% in females and 37.5% in males. There was no significant difference between dPCR and qPCR *EGFR* T790M mutation detection rate ($p=0.08$, Fisher exact test). The result is not significant at $p<0.05$, but shows a trend towards statistical significance. The statistical significance could potentially be reached with more tested patients.

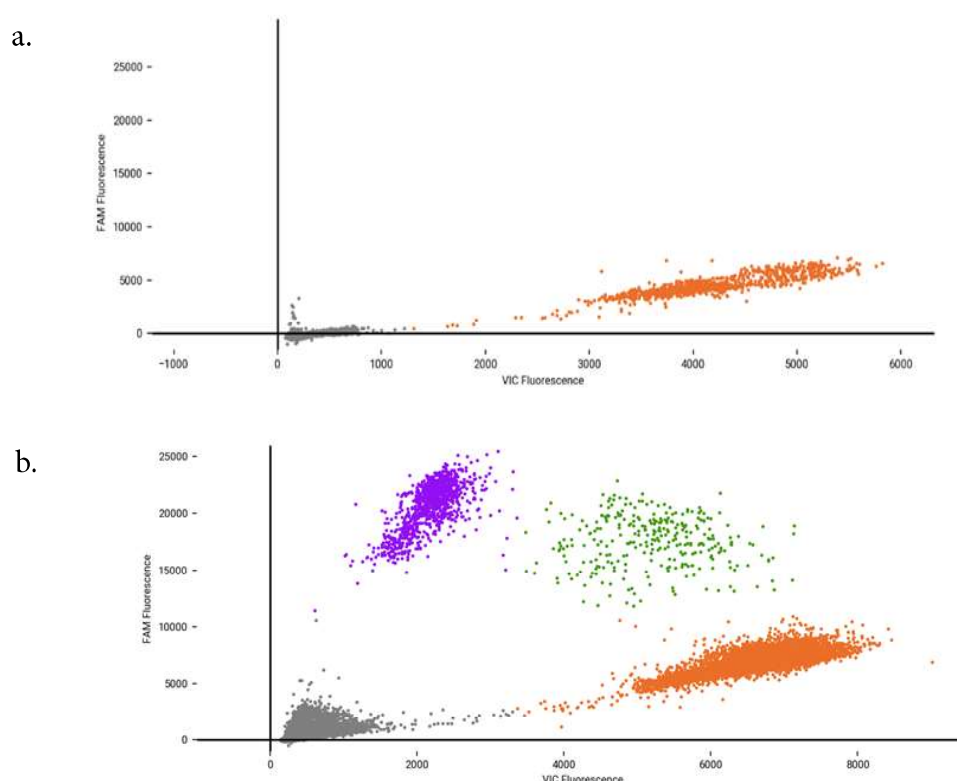


Figure 3. *EGFR* T790M mutation dPCR results: a. *EGFR* T790M mutation-negative and b. *EGFR* T790M mutation-positive result.

Concentrations of DNA extracted from the plasma/PE ranged from 0,065 to 31.8 ng/μL (the highest DNA concentration which was extracted from plasma samples was 15.4 ng/μL). Examples of samples with and without the present mutation are shown in Figures 3a and 3b, respectively.

Discussion

In non-squamous NSCLC there are numerous targetable mutations (*EGFR*, *ALK*, *ROS1*, *BRAF*, *KRAS*, *MET*, *HER2*, *RET*, *NRG1*, *NTRK1/2/3...*), therefore there are different testing strategies for the identification of driver mutations. The most common strategies are sequential or exclusionary single gene testing and NGS testing (7). Although the only model currently used in Serbia is the sequential testing of single genes, several studies have shown that this approach

is more expensive and less sensitive compared to NGS (7–9). In addition, it takes longer to obtain the results of the genetic test than with NGS analysis. The sensitivity of sequential testing of individual genes is lower, resulting in a lower number of patients being prescribed appropriate targeted therapy if the targeted mutations are present, which in turn leads to reduced OS (7,10). Sequential testing of individual genes (*EGFR*, *ALK*) from tissue and liquid biopsy samples is currently being applied in Serbia. Taking into account the above-mentioned results, it is necessary to perform an analysis of the price evaluation of NGS tests compared to the prices of sequential tests, considering that fewer genes are currently approved for testing in Serbia than in the previously mentioned studies.

In Serbia, where the sequential approach was used at the time, patients were prescribed first- or second-generation *EGFR*-TKIs following the detection of a mutation in the *EGFR* gene. Although most patients initially respond to *EGFR*-TKI therapy, acquired resistance to *EGFR*-TKI develops in almost all patients after a progression-free period of 9 to 14 months, and a common cause of resistance is the occurrence of the T790M mutation in exon 20 of the *EGFR* gene (approximately 50% of cases) (11). When cancer progression is suspected, PCR testing for the T790M mutation is recommended to determine whether third-generation *EGFR*-TKIs are indicated. Analysis of ctDNA in plasma and pleural effusion samples, a minimally invasive test, has been used as a valid and cost-effective alternative to tissue biopsies and identifies a large proportion of *EGFR* T790M mutations responsible for therapy resistance (12). If the mutation was not detected in patients in whom the disease progression was suspected, some of them were advised to return for regular testing approximately every one to three months (patients with detected positive events on dPCR but below the established threshold) to repeat the PCR analysis, with the aim of subsequently attempting to detect the T790M mutation. If the resistance mutation is not detected, patients are referred to treatment with chemotherapy. Due to the low concentrations of ctDNA in the samples, the dPCR method was preferred because its higher sensitivity (13) increases the probability of detecting mutations even at minimal DNA concentrations (14,15). It is important to emphasize that in addition to the higher sensitivity of the dPCR method compared to qPCR, the use of different kits for the isolation of cfDNA, which work according to different principles, can also play an important role. There are several commercially available DNA isolation techniques, each utilizing different binding chemistries. These chemistries can impact both the efficiency and purity of DNA extraction, with each method having its own specific binding capacity. Common techniques include ethanol precipitation, anion-exchange resin (combined with ethanol precipitation), silica gel membrane binding, and magnetic silica particle binding. Among these, magnetic particle-based methods are more cost-effective, faster, and easier to scale and automate, while membrane binding methods tend to offer higher yields (16).

Comparing the percentage of dPCR analyses that were positive for the T790M mutation from March 2023 to February 2024 (30.48%) with the percentage of positive results from tests performed on the Cobas platform from March 2022 to February 2023 (19.44%), greater success in detecting the desired mutation was observed with dPCR technology. This study is significant, but has certain limitations. It is important because it shows that the more sensitive dPCR method achieves a higher detection rate of the resistant *EGFR* T790M mutation compared to the qPCR method, thus increasing the number of patients in whom third-generation *EGFR*-TKIs are indicated. On the other hand, the main limitation of this study is that the presence of this resistant mutation is only one of many mechanisms through which tumor resistance to therapy develops, and that some of the other mutations contributing to this resistance can only be detected by multiplex PCR for a larger number of mutations/genes or NGS sequencing.

For all the aforementioned reasons, the use of the most sensitive methods available for mutation detection is crucial. At the Institute for Oncology and Radiology of Serbia, dPCR technology is preferred, as it has shown higher sensitivity for the detection of point mutations compared to the qPCR Cobas platform. Although the dPCR method is more sensitive, it is not without limitations. Despite the fact that the cost per sample analysis is lower compared to qPCR, the main challenge/limitation is the lower possibility of multiplex PCR reactions. Given the fact that the focus of this method is currently on the detection of single mutations with higher sensitivity, the choice of this method is justified.

Conclusion

In comparison with conventional qPCR, which uses relative quantification, dPCR enables absolute quantification, offering significantly higher sensitivity and accuracy for detecting mutations. The study confirmed that dPCR technology demonstrated a higher detection rate of the T790M mutation over a one-year period, compared to the conventional qPCR method. Therefore, the detection of the T790M mutation in the *EGFR* gene in patients with NSCLC at the Institute for Oncology and Radiology of Serbia was significantly improved by the introduction of dPCR technology, which has led to enhanced diagnostic accuracy and efficiency in selecting appropriate therapies for patients with NSCLC.

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PERSONAL POINT OF VIEW

Personal Point of View

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The Point of View: Will Artificial Intelligence Soon Become the Main Driver of Scientific Innovation?

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Abstract

In the pre-AI world, scientific progress was incremental, with gradual discoveries leaving many questions unanswered. Is rapidly advancing AI positioned to solve all the remaining questions within the next several decades? Only two years ago, writing an original essay seemed uniquely human. Then ChatGPT emerged, demonstrating that AI can produce human-like essays. Now, we wonder: How far will this go? What will AI be able to accomplish in the future?

This perspective article discusses a fundamental and imminent shift in the way science is conducted, as research is set to become the first intellectual activity largely transitioned from humans to machines. This is because all intellectual activities require reasoning, creativity, and imagination, while science uniquely demands a comprehensive knowledge of the vast and ever-growing body of scientific data as a foundation for effective experiment planning. Humans, however, are inherently limited in their capacity to acquire and retain such extensive knowledge. This is precisely where AI is expected to gain a decisive advantage over humans, as it can rapidly assimilate information.

However, the full advantage of future AI's ability to brainstorm through the entire scientific knowledge can only be realized when its reasoning abilities at least much human intellect. Such a convergence of comprehensive scientific knowledge and more advanced reasoning is projected to create a superior AI scientist, likely by the end of this decade, when AI is expected to match human-level reasoning. Moreover, AI is even expected to surpass human reasoning ability in the following decade, further emphasizing its superiority in scientific discovery and innovation.

As AI is poised to rapidly permeate scientific research, this article not only presents a point of view but also introduces the fundamental concepts of artificial intelligence that all scientists, regardless of their field, urgently need to master.

What is Artificial Intelligence?

Understanding the current stage of AI development is crucial for getting a clear grasp of its present capabilities and future potential. AI evolution is typically conceptualized in three major stages [1]:

- 1) Weak AI: Performs only some tasks better and faster than humans. This type of AI is already available (Gemini, ChatGPT, Copilot, xAI, Grok).

- 2) Strong AI: Capable of performing intellectual tasks as well as a human, including reasoning, critical thinking, creativity, adaptability, and understanding complex concepts, this stage has not yet been achieved but is expected by the end of this decade.
- 3) Super AI: Surpasses human intelligence in all areas and capable of experiencing emotions and desires. This is a more futuristic concept projected to emerge within next decade or beyond.

Currently, narrow or weak AI is the only available form of artificial intelligence. Despite being in its early stages, AI is becoming a clinically valuable tool [2, 3] with the U.S. Food and Drug Administration (FDA) having already authorized 950 AI/ML-enabled medical devices, mainly in radiology (<https://www.fda.gov/medical-devices/software-medical-device-samd/artificial-intelligence-and-machine-learning-aiml-enabled-medical-devices>).

What are Artificial Neural Networks and what is behind their rapid advancement?

Artificial Neural Networks (ANNs) are computational models inspired by the neural circuitry of the brain. Although the renewed interest in ANNs is very recent, this concept has been in development since Frank Rosenblatt's work in 1958 [5]. In 1989, Yann LeCun et al. introduced the first commercially applied convolutional neural network (CNN), named LeNet, for use in recognizing handwritten postal codes on envelopes [6].

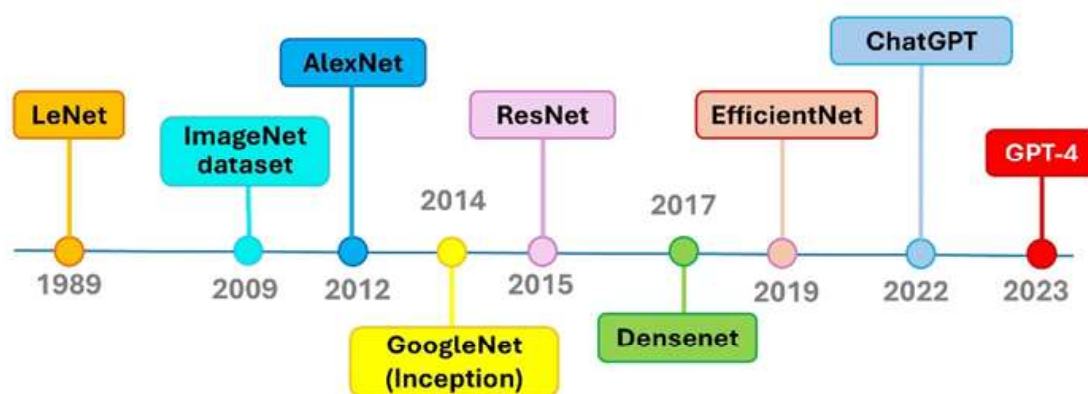


Figure 1. A brief history of ANNs.

The release of the ImageNet dataset, containing 15 million high-resolution images for CNN training, in 2009 further fuelled the advancement of CNNs [7]. AlexNet, which won the 2012 ImageNet competition, demonstrated the power of deep CNNs and GPU acceleration, sparking widespread interest in deep learning [8]. Despite consisting of only eight layers, five convolutional and three fully connected, it greatly outperformed other image recognition models (Figure 1). GoogleNet (Inception), introduced in 2014, featured novel Inception modules that optimized the computational efficiency of deep networks. In 2015, ResNet introduced residual learning, which allowed for the training of extremely deep networks by addressing the vanishing gradient problem (Figure 1). DenseNet (2017) further improved this architecture by connecting each layer to every other layer, enhancing feature reuse and reducing the number of parameters. EfficientNet (2019) utilized a compound scaling method to balance network depth, width and resolution, achieving state-of-the-art performance with less resources (Figure 1). ChatGPT (2022) and GPT-4 (2023), based on the novel transformer architecture, revolutionized natural language processing (NLP), enabling more sophisticated conversational AI and pushing the boundaries of what AI can achieve in understanding and generating human language (Figure 1).

The rapid development of ANNs was primarily driven by advances in graphical processing unit (GPU) hardware, which was originally designed for efficient processing of computer graphics and images. GPUs conveniently provided the massive computing power required for ANN training due to their architecture, which is optimized for parallel processing. Unlike CPUs, which have only several large cores and are thus better suited for sequential processing, GPUs feature thousands of smaller cores that are capable of handling multiple tasks simultaneously. This parallel processing capability makes GPUs ideal for large-scale matrix operations and tensor calculations in deep learning. Additionally, GPUs have higher memory bandwidth, enabling them to process vast amounts of data more effectively. Before the use of GPUs, training on large image datasets was prohibitively time-consuming and limited to low-resolution images, and large language models (LLMs), such as ChatGPT, were not feasible [9]. AI has primarily focused on text and image inputs, though applications in sound processing are also emerging. LLMs, like ChatGPT, are trained on billions of words from sources such as articles, books, and internet-based content.

What are the main ANN architectures?

Artificial Neural Networks (ANNs) consist of multiple layers, each containing numerous nodes that mimic biological neurons. These nodes are connected to input features and other nodes through weights mimicking neuron's dendrites and synapses. ANNs typically include an input layer, one or more hidden layers, and an output layer (Figure 2).

Input features are passed from the input layer to the hidden layers (Figure 2), where they are modified by weights, biases, and activation functions (Figure 3).

Each node functions as a simple processing unit, receiving inputs, modifying them and producing an output. The number of hidden layers and neurons can vary, resulting in different architectures, ranging from shallow networks with a single hidden layer to deep learning models with many hidden layers [10].

The dominant contemporary ANN architectures are:

- **Transformer:** used by LLMs, such as ChatGPT
- **Convolutional:** used for image analysis and classification
- **Diffusion:** used for image generation

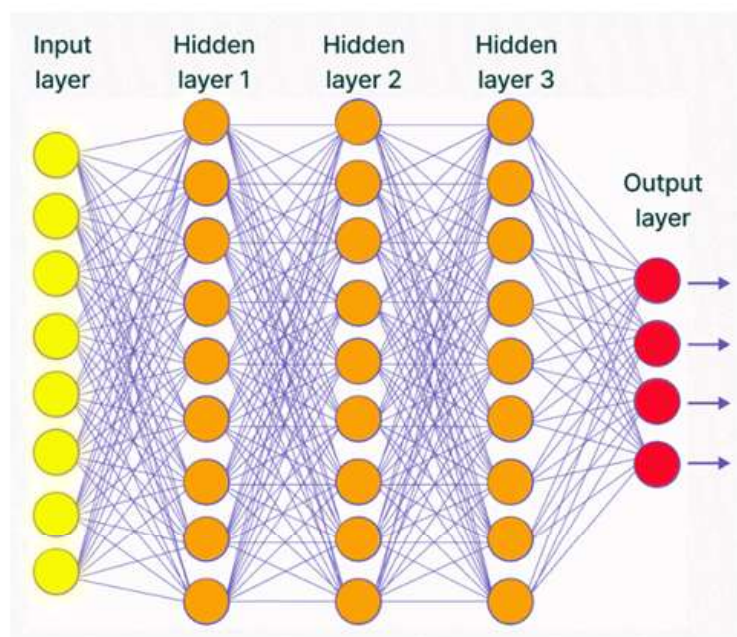


Figure 2. General ANN architecture

Transformers are designed for sequential data processing, particularly for NLP which is a subfield of artificial intelligence that enables computers to understand and communicate in human language. Sequential data processing is crucial for NLP because natural language itself is inherently sequential. Another essential component of transformer-based models is the self-attention mechanism which determines the relative importance of different words in a sentence. The transformer architecture consists of multiple layers of encoders and decoders. GPT-4, for instance, has a total of 120 layers. First introduced in 2017, transformers gained prominence with the release of ChatGPT by OpenAI in November 2022[11].

Convolutional Neural Networks are primarily applied to image-related tasks such as object detection, image classification, segmentation and generation. They have a hierarchical architecture composed of three types of layers, starting with convolutional layers, which extract and learn local features from the input image; pooling layers, which reduce dimensionality; and fully connected layers, which integrate the features to produce the final output [12]. This architecture enables CNNs to learn increasingly abstract features at each layer, from simple edges in the initial layers to more complex patterns in the deeper layers.

Diffusion models gained prominence in image generation with models like DALL-E in 2021. They typically use U-Net architectures with encoder-decoder structures for the denoising process generating high-quality images from random noise. Their clinical application includes tumour segmentation in medical images [13].

Vision-Language Models (VLM) allow users to upload an image and engage in textual conversations with the model, giving instructions or asking questions based on the image. VLMs typically combine CNN architectures for processing images and transformer architectures for text processing [14].

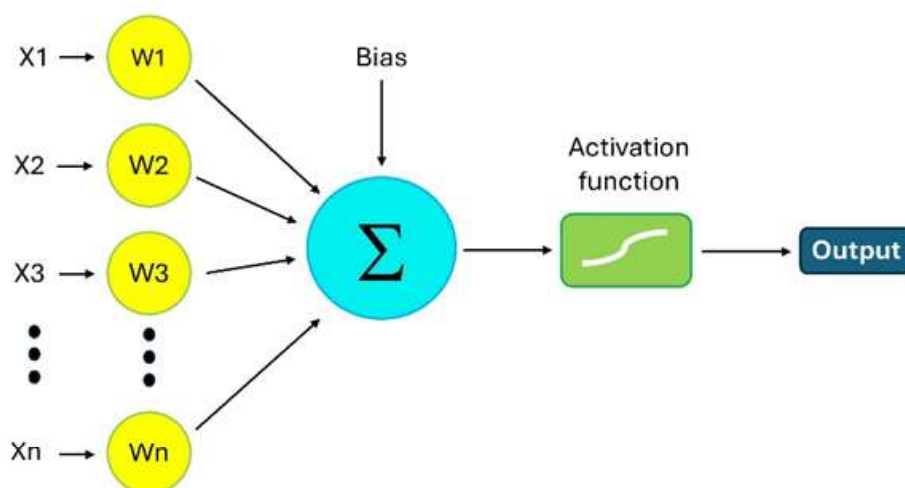


Figure 3. Schematic representation of a Neuron in a Neural Network: This diagram illustrates the functioning of a neuron, depicting inputs x , weights w , bias b , and activation function. The inputs x are multiplied by corresponding weights w and summed together along with the bias b . The resultant weighted sum is then passed through the activation function, which introduces non-linearity and produces the neuron's output. This process enables the neuron to learn and model complex patterns from the data.

How does ANN learn?

Learning in neural networks is based on weights and biases assigned to each node, following this formula [15]:

$$z = x_1 w_1 + x_2 w_2 + \dots x_n w_n + b \quad (1)$$

z = weighted sum

x = input values

w = weights or “parameters”

The weighted sum (z) depends on input values (x_1 and x_2), which are either features from the dataset or outputs from neurons in the previous layer. The weights w_1 and w_2 are parameters that the model learns during training, while the bias b , is added to the weighted sum of inputs for each node. This bias allows the node to activate even when the weighted sum of the inputs is zero. Further downstream, the activation function introduces non-linearity to the output signal, enabling the network to learn complex patterns. This non-linearity is essential because linear models can only capture linear relationships, which are insufficient for solving real-world problems that predominantly involve non-linear patterns. Learning in ANNs is achieved through the training process, which begins with small random values for weights and biases—the key trainable parameters. These values are gradually adjusted to minimize the error between the predicted and desired outputs, using optimization algorithms that involve backpropagation and gradient descent [16].

What can ANNs learn?

ANNs can be trained with text, images or sound [16].

- 1) Text: remarkably, “labelled” text, structured as pairs of questions and answers allows for most efficient training, while human-like learning of unlabelled text is less efficient for ANNs. This is because the question-answer format allows the network to compare its predicted answer with the correct answer, calculate its error and adjust its parameters during several epochs to minimize that error. Once trained, the network should generalize to new, unseen data, enabling it to respond accurately to questions formulated differently.
- 2) Images: Training with images also involves using pairs of images and their descriptions, allowing the network to learn to recognize and interpret image content.

The initial training of a large ANN requires immense hardware resources. For example, GPT-4 was trained on 25,000 Nvidia A100 GPUs simultaneously for approximately 100 days. Each A100 GPU has 80 GB of VRAM and costs \$15,000. GPUs were used instead of CPUs because the A100 GPU has 6,912 cores, whereas the most powerful CPU in 2024 (Xeon) has only 288 cores. Although GPU cores are smaller, their large quantity results in significantly higher performance for AI training and inference compared to CPUs, especially when the trained model is used to make predictions based on new data. Inference refers to the process of using a trained machine learning model to make predictions or decisions based on unseen data.

ANNs in Science: Transfer learning using Pretrained networks

Because ANNs need thousands or millions of examples to efficiently learn, they are mainly applied in science through “transfer learning” [17]. This technique uses the knowledge a model has learned from one task as a starting point for a second, related task. Transfer learning provides a significant head start, enabling the application of ANNs in experiments with small sample sizes by transferring pre-acquired knowledge to new tasks. For example, a CNN pre-trained on the ImageNet dataset, which contains millions of general images, can be further trained with only 100+ specific medical images. Transfer learning is crucial in fields like cancer research, where assembling patient cohorts with thousands of cases is difficult.

Expansion of a network knowledge can be achieved by learning through:

- 1) **Fine-tuning**
- 2) **Embeddings**

Pre-trained networks are typically trained on broad, general knowledge, while researchers often need detailed, specialized knowledge for specific fields, such as oncology. The ability to expand a network's knowledge is of critical practical importance because it allows scientists to take a pre-trained language model (LLM) like GPT-4, or freely available models like LLAMA, MISTRAL, FALCON, or BERT, and feed it with expertise in their specific area of interest. Such acquisition of a large amount of specialised knowledge by LLMs has a potential to finally integrate the scientific knowledge, which is of crucial importance because the scientific knowledge is highly fragmented due to the fact that the rate of scientific publication is expanding at a high speed while the human ability of information intake rate cannot be improved. The expertise of a pre-trained network can be expanded by inputting PDFs of research articles, either through fine-tuning the model on this data or by using embeddings:

Fine-tuning is a technique within transfer learning which takes a pre-trained model and continues its training process on a new dataset. Rather than training the entire model from scratch, it starts with the pre-trained weights that already incorporate general reasoning, logic, knowledge, language and conversational skills, and adjusts them to fit the new data. This process typically involves unfreezing some layers of the pre-trained model and training them alongside any newly added layers. Fine-tuning is computationally very intensive, requiring large hardware resources, especially for deep learning models with many layers and parameters, as it involves updating the neural network's weights [18].

Embeddings provide a simple, fast method to expand a neural network's pre-trained knowledge without extensive re-training and massive hardware resources [19]. More than a basic database, embeddings capture semantic relationships, allowing for easy retrieval or clustering of similar data based on vector representations. They transform high-dimensional data, such as words, into lower-dimensional vectors that still retain semantic meaning and relationships between words. For example, words are considered high-dimensional because if a vocabulary contains 10,000 words, each word would be represented as a 10,000-dimensional vector, with only one value being 1 and the rest 0. Embeddings reduce this to 50-300 dimensions, making text analysis more efficient for machine learning models. Consequently, embeddings serve as an efficient contextual database for machine learning applications and can also represent images as continuous numerical vectors that capture key image features.

Continuous Learning

Contrary to popular belief, even the latest models like GPT-4, do not have the ability for continuous learning. Once trained and deployed, these models do not update their knowledge or learn from new inputs in real-time. Any updates or improvements require a separate fine-tuning process on new data in an additional training phase.

Applications of ANNs in Oncology Research

The most common use of AI in science over the past decade has been in supervised learning, where a model is “trained” on retrospective data that has been annotated with the correct answers and is then expected to generalize by providing accurate predictions on new, unseen data. The main goal is to use available data to capture prognostic clues that can reliably predict future events. Specifically, in cancer research, AI is primarily applied to tumour detection and the prognosis of chemotherapy response and disease outcomes [20]. These tasks are typically achieved through computational AI analysis of medical images, including tumour histopathology, MRI, CT scans, X-rays, and PET scans [17, 21]. Improving disease outcome prognosis and therapy response prediction is expected to enhance patient survival by personalizing treatment plans. Additionally, AI may assist surgeons in planning complex oncological surgeries by providing detailed 3D models of tumours. AI's role could also extend to drug discovery and development through “virtual compound screening,” which predicts the biological activity of chemical compounds against cancer targets [22]. Another application is molecular docking, where AI models drug-target interactions to optimize drug design [23]. Monitoring data from wearable devices using historical and real-time activity/vital metrics to detect early signs of cancer progression is an obvious application of AI [24]. Wearables have been successfully used for early detection of

conditions such as COVID-19 [25], heart failure [26] and seizures in epilepsy patients [27]. Common physiological metrics measured include blood pressure, oxygen saturation, body temperature, heart rate, and respiratory rate. Additionally, devices can monitor galvanic skin response, blood glucose levels, electromyography, electroencephalography and electrocardiography. Physical activity and sleep patterns are often tracked using accelerometer-derived data, with several studies indicating that low physical activity correlates with worsening cancer symptoms [28]. When combined with gyroscopes and magnetometers, accelerometers form an inertial motion unit (IMU), which can detect changes in walking style and posture, both valuable for identifying cancer-related deterioration. IMUs also assist LED optical photoplethysmography sensors in heart rate monitoring, which detect blood volume changes in the microvascular bed of tissues. AI could thus support decision-making by flagging potential early signs of cancer during routine check-ups.

The Point of View - Will AI Soon Become the Main Driver of Scientific Innovation?

The AI applications in clinical practice and medical research mentioned above are anticipated, as they merely enhance existing computational methods that did not use AI. However, a truly surprising and groundbreaking application would be AI's independent role in the most creative aspects of research—planning experiments and formulating novel approaches that could enable and accelerate scientific breakthroughs.

Effective and innovative scientific experiment planning depends on several key factors: robust reasoning and analytical skills, combined with a comprehensive understanding of the scientific literature. Although the common sense and logical reasoning abilities of even the most advanced AI models, like GPT-4, currently fall short of those of an average human, this gap is expected to disappear in the coming years. With each new generation, AI models are trained on exponentially larger datasets and demonstrate progressive improvements in reasoning capabilities [29].

Even more staggering is the fact that AI will surpass humans by billions-fold in the above crucial aspect: reading and memorizing of scientific articles. AI can already learn from scientific text millions of times faster than humans, the only problem is that scientific articles are not available in the form of clean text but PDF files which contain scientific text mixed with information about authors, publisher, legal formulations, page numbers, acknowledgements, footnotes, tables and figures. Extracting clean scientific text from this mixed format remains surprisingly difficult, even for powerful AI models.

This challenge in comprehending scientific articles is further compounded by AI's limited ability to interpret data in tables and even more so in figures. Additionally, ANNs struggle with unsupervised learning directly from raw text; instead, text often needs to be converted into a “question-answer” format for supervised learning [30]. Although AI can perform this transformation quickly, there is a risk that the original meaning may be altered in the process, requiring human verification to ensure accuracy.

In unsupervised learning, the input data does not include the desired output, requiring the network to independently identify meaningful patterns [31]. Advances in self-supervised learning, where models learn from raw data, will greatly improve AI's ability to learn scientific information efficiently [32]. Once AI becomes proficient in unsupervised learning, including extracting scientific content from research paper PDFs and interpreting tables and figures, it will inevitably surpass humans in the amount of scientific knowledge it can acquire by several additional orders of magnitude, ultimately exceeding human capacity for creative scientific thinking [33].

This shift in science away from humans may come as a surprise to most scientists who were not aware of the impact of comprehensive literature knowledge as a basis for scientific innovation.

Conclusion

AI is positioned to enhance both scientific research and the medical treatment of cancer patients. The main current and potential applications of AI in cancer research include:

- Improving early tumour detection and diagnosis through the analysis of tumour imaging, genomic data, real-time body activity monitoring, and comprehensive integration of medical data.
- Providing more accurate cancer prognosis and predicting chemotherapy outcomes, enabling personalized treatment plans and thus improving patient care and survival.
- Advancing scientific understanding far beyond human capabilities, based on AI's ability to quickly and efficiently learn from the vast and growing body of published scientific literature. This is crucial as the rate of scientific publication continues to exponentially accelerate, while human information processing capacity remains limited.
- Acceleration of scientific breakthroughs by planning innovative experimental strategies based on AI's broad and detailed knowledge of scientific literature, combined with its analytical and reasoning skills.

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ABSTRACTS

Novel perspective of anticancer metal-based drugs: Characteristics of heterometallic complexes and their potential applications

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Background: Platinum-based drugs are widely used in medical treatments, but their effectiveness is limited by toxicity. The mechanism of action is well understood, while the side effects are thought to arise from interactions between platinum (a soft acid) and biomolecules containing sulfur as a donor atom (soft bases), such as thiols and thioethers. One potential approach to overcome these limitations is to design novel heterometallic complexes with metal centers that have different coordination geometries, kinetic properties, affinities, and reactivities towards biologically relevant nucleophiles.

Material and Methods: The novel heterometallic complexes: $[\{cis-PtCl(NH_3)(\mu-pyrazine)ZnCl(terpy)\}](ClO_4)_2$ (Pt-L1-Zn), $[\{cis-PtCl(NH_3)(\mu-4,4'-bipyridyl)ZnCl(terpy)\}](ClO_4)_2$ (Pt-L2-Zn), $[\{ZnCl(terpy)(\mu-pyrazine)CuCl(terpy)\}](ClO_4)_2$ (Zn-L1-Cu), $[\{ZnCl(terpy)(\mu-4,4'-bipyridyl)CuCl(terpy)\}](ClO_4)_2$ (Zn-L2-Cu) (terpy = 2,2':6',2''-terpyridine, L1 = pyrazine, L2 = 4,4'-bipyridyl) were synthesized and characterized by elemental analyses, UV-Vis, IR and 1H NMR spectroscopy. Cell viability was assessed using the MTT assay.

Results: The study of interactions between the complexes and biomolecules under physiological conditions revealed that heterometallic complexes with two different metal centers significantly influence the reactivity order and coordination modes of biomolecules. Cytotoxicity assays showed that all complexes substantially reduced cell viability in human colorectal cancer cells (HCT-116) and human breast cancer cells (MDA-MB-231). Notably, these complexes exhibited significant cytotoxic effects, with improved efficacy against HCT-116 cells compared to cisplatin, especially after 72 hours.

Conclusions: The differing reactivities of the metal centers result in various coordination modes of biomolecules and increased cytotoxicity. Additionally, the nature of the linker between the metal centers significantly impacts reactivity.

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Keywords: copper(II); heterometallic complexes; platinum(II); zinc(II);

Application of numerical models in the behavior of cancer cells

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Background: The aim of this paper is to present diverse numerical models that were applied to model the behavior of cancer cells on various levels.

Material and Methods: A numerical model based on reaction-diffusion equations was applied to analyze the behavior of cancer cells over time on tissue level and the effect of prescribed drug therapy on the tumor was also considered [1]. A similar numerical model was used to model the behavior of cancer cells at the cellular level, *in-vitro* following drug treatment [2]. Another model was applied to predict the behavior of cancer cells after electroporation treatment [3]. The numerical simulations of the WNT signalling process in cells are presented in [4].

Results: In all considered numerical models, the results of numerical simulations are compared with experimental findings. The proposed method for modeling the behavior of cancer cells at the tissue level was tested using patient-specific data that was used to calculate tumour volumes at specific moments in time. The comparison and good agreement of numerical results with the clinical data showed the efficiency and accuracy of the proposed method. In the case of other mentioned numerical models, the experimental data was used to estimate the values of parameters of the model. The analysis of the parameter changes for different treatments helped better elucidate the effect of the considered treatments have on some aspects of the behavior of cells, i.e. decrease cell proliferation, intensify cell death, and/or increase oxygen consumption, among others.

Conclusions: The main benefit of using numerical simulations is that they enable a quantitative description of the behavior of cells, with a set of parameters, that then makes it possible to analyze how the applied treatments influence the change of these parameters and hence the behavior of cells. Also, numerical models can be used to predict the behavior of cells in other conditions and estimate the number of viable cells after other treatments that were not studied experimentally, thus reducing the time and money needed to perform many experiments.

Keywords: cancer cell progression; finite element method; multi-scale models; parameter estimation; WNT signaling

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Molecular markers of migration and invasion in colorectal cancer cell lines and tissues

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Background: The aim of this paper is to present investigation of migratory/invasive potential of two different colorectal cancer cell lines HCT-116 and SW-480, and expression of markers of epithelial-mesenchymal transition (EMT), responsible for cell behavior allowing an epithelial cell to gain greater motility as a key point in triggering metastasis [1]. **Material and Methods:** individual cell migration of colorectal cancer cell lines was detected via Transwell and RTCA assays. For the determination of collective cell migration, we used Scratch assay, and migratory potential was tracked within 12 and 24 h [2, 3]. Detection and quantification of proteins responsible for EMT: promigratory (vimentin, N-cadherin and nuclear β -catenin), antimigratory (E-cadherin and cytoplasmic β -catenin), and markers of invasion (Snail, Slug, MMP-7 and MMP-9) was done, were performed using immunofluorescence and flow cytometry [1].

Results: HCT-116 cells showed strong migratory potential detected in both individual and collective migration. Furthermore, low expression of E-cadherin and β -catenin was observed in intercellular connections, as well as high expression of N-cadherin, vimentin, MMP-7 and MMP-9. Also, these cells are characterized by elevated expression of the regulatory markers: nuclear β -catenin, Slug and Snail. On the other side, we observed a weaker migratory potential of SW-480 cells, probably due to a strong expression of antimigratory markers E-cadherin and cytoplasmic β -catenin, which are responsible for establishment of intercellular junctions. Moreover, promigratory markers vimentin, N-cadherin and Snail were observed as highly expressed. Lower concentrations of MMP-7, MMP-9, and nuclear β -catenin were detected in SW-480 cells, indicating that these are clearly important markers responsible for the migratory/invasive potential of these cells.

Conclusions: Based on the obtained results, it can be concluded that these two cell lines are completely different in terms of migratory and invasive potential. This can be explained by the origin of the investigated cells. Namely, HCT-116 is an aggressive cell line isolated from stage IV colorectal carcinoma, poorly differentiated and resistant to chemotherapeutics. These cells are characterized with advanced EMT that enables it to have an epithelial-like phenotype and to migrate individually. As for SW-480 cells, they are less aggressive, isolated from stage II colorectal carcinoma, well differentiated with less advanced EMT. Stronger intercellular connections between these cells enable them to form clusters and to retain the collective type of migration. Based on everything presented, we can conclude that these two cell lines will have completely different responses to therapeutic treatments in terms of cell behavior in migration and invasion processes.

Keywords: cancer cell migration, EMT, invasion, colorectal cancer, protein expression

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Detection of mutations in *JAK2*, *MPL* and *NPM1* genes in myeloproliferative neoplasms

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Background: Myeloproliferative neoplasms (MPN) are clonal hematopoietic stem cell disorders, characterized by the abnormal proliferation of one or more lineages and an increased number of mature cells (erythrocytes, leukocytes, and platelets) in the blood. The genetic basis of MPN includes the *JAK2* (Janus kinase 2), *MPL* (myeloproliferative leukemia virus oncogene), *NPM1* (nucleophosmin 1), *CALR* (calreticulin) and *FLT3* (fms-like tyrosine kinase 3) gene mutations. **Material and methods:** *JAK2* V617F, *MPL* W515L, *MPL* W515K, *NPM1* type A, B and D, *CALR* type I and II, and *FLT3* ITD mutation testing was performed on whole blood samples of patients with MPN, using the real-time PCR technique for *JAK2*, *MPL* and *NPM1* mutations and gel electrophoresis for *CALR* and *FLT3* mutations.

Results: *JAK2* V617F mutation was detected in 78 (58,21%), *MPL* W515L in 12 (8,96%), *MPL* W515K in 4 (2,99%), *NPM1* type A in 3 (2,24%) and *FLT3* ITD in 19 (14,2%) of 134 samples. *CALR* type I mutation was detected in 6 (15,0%) and *CALR* type II in 1 (2,5%) of 40 samples.

Conclusion: The real-time PCR technique provides the possibility of monitoring the amplification of the target gene at any time, as well as a faster and more precise software interpretation of the results compared to gel electrophoresis-however, it is not applicable for the detection of *CALR* and *FLT3* mutations. Determining the exact mutation present in patients with MPN is crucial for assessing the risk of developing severe disease or complications, as well as providing adequate personalized therapy.

Keywords: electrophoresis, *JAK2*, Myeloproliferative, *MPL*, *NPM1*, Real-Time PCR

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Colorectal cancer screening: great opportunities and new challenges

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Colorectal cancer (CRC) is the third most common cancer worldwide and the second leading cause of cancer-related death in Western countries. Evidence from several studies have shown that CRC screening is effective and cost-effective in average-risk population. The aim of population-based screening is to discover asymptomatic disease, thus favoring its detection at early stages and, consequently, enabling its adequate treatment. Implementation of organized programs is recommended because they include a structure responsible for service delivery, quality assurance and evaluation. Recommended CRC screening strategies fall into two broad categories: stool tests that primarily detect cancer, which include detection of occult blood or exfoliated DNA; and structural exams, that are effective in detecting both cancer and premalignant lesions, which include flexible sigmoidoscopy, colonoscopy, endoscopic capsule, and CT-colonography. Among these strategies, testing for occult blood in stool using the fecal immunochemical test (FIT) is predominant in Europe, Canada and Australia, while colonoscopy is the dominant screening modality in the US.

The COLONPREV study, an ongoing pragmatic multicenter, nation-wide, randomized controlled trial, will allow us to evaluate the efficacy of once-only colonoscopy and biennial FIT with respect to the reduction of CRC-related mortality in average-risk individuals in a programmatic screening setting. This study, along with two additional ongoing projects –the NORDICC and the CONFIRM studies–, may contribute to demonstrating the usefulness of these approaches, as well as establishing the most cost-effective strategy in a population-based scenario.

Finally, it is important to recognize that current screening strategies are limited by low sensitivity for advanced adenomas, a high false-positive rate resulting in unnecessary colonoscopies, and insufficient compliance. The use of biomarkers, others than blood in feces, may overcome these limitations. In that sense, genetic and epigenetic changes can constitute non-invasive biomarkers if they are measurable in different biological fluids. In that sense, exfoliation of neoplastic cells in feces is a continuous process in patients with CRC, whereas cancer cells and other tumor-associated markers may reach systemic circulation facilitated by the angiogenic process.

Keywords: colorectal neoplasms, screening, prevention

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Lung cancer in Vojvodina: progress and prioritiesJelena Đekić Malbaša¹¹Faculty of Medicine, University of Novi Sad, Serbia²Institute for Pulmonary Diseases of Vojvodina, Sremska Kamenica, Serbia

Background: Lung cancer (LC) is the most frequently diagnosed cancer and the leading cause of cancer mortality in both sexes in Serbia and worldwide. The aim of this presentation was to describe LC incidence and mortality in Vojvodina, Serbia, and to analyze Institute for Pulmonary Diseases (IPBV) LC hospital registry data from 2010 to 2020.

Material and methods: Institute for Public Health of Vojvodina and Serbian cancer registry data for trend analysis, and IPBV LC hospital registry data for patient characteristics from 2011 to 2020 were used.

Results: A decrease in LC mortality rates among males in Vojvodina was observed, from 73.7 per 100 000 in 2010 to 48.1 per 100 000 in 2021. The average LC incidence showed differences by Districts of Vojvodina. The highest LC incidence for males was reported in Central Banat (80.2/100 000), and for females in North Bačka District (38.1/100 000). The highest LC mortality among males was found in North Banat (70.4/100 000), while for females in North Bačka District (26.0/100 000).

According to the IPBV LC hospital registry from a total of 12,055 LC patients, 30.4% were females. The percentage of female LC patients significantly ($p < 0.001$) increased from 26.9% (306/1139) in 2011 to 35.9% (379/1056) in 2020. Male LC patients were older compared to females (64.56 ± 8.4 vs. 63.43 ± 9.0 years; $p < 0.001$). Most of the patients at the time of LC confirmation were active smokers (61.9%), without significant differences by gender (62.1% vs. 61.4%, respectively, $p = 0.466$).

The most common histological LC type was adenocarcinoma (41.8%), followed by squamous cell carcinoma (30.0%) and SCLC (15.4%). A significantly ($p < 0.001$) higher percentages of adenocarcinoma (49.9% vs. 38.4%), SCLC (17.6% vs. 14.5%) and carcinoid tumors (1.1% vs. 0.4%) were observed in females compared to males, while a percentage of squamous cell carcinoma was significantly higher in males than in females (34.8% vs. 19.0%, respectively; $p < 0.001$).

During the study period, the total number of patients with adenocarcinoma, squamous cell carcinoma and SCLC decreased in males, while among females the linear trends for adenocarcinoma and squamous cell carcinoma is increased.

Conclusion: LC mortality rates among males showed a decreasing trend, while among females incidence rates are still increasing. Due to increasing trends in the incidence and mortality of LC among women, it is essential to reduce the prevalence of smoking in the female population and to promote LC screening in Serbia.

Key words: lung cancer, rates, gender, histological types, Vojvodina

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Detection of KRAS, NRAS and BRAF mutations in oncology patients

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The aim of this study was to present method and statistical results of RAS and BRAF gene mutation testing in CRC and melanoma patients, respectively, from the Autonomous Province of Vojvodina, Republic of Serbia. DNA mutations in related genes were analyzed from FFPE samples by Real-Time PCR. RAS mutation was detected in 1073 of 2161 patients (49.65%). The most common mutations of KRAS gene was in exon 2 with slightly increased incidence in women. Mutation rates in KRAS exons 2, 3 and 4 were 41.2%, 4.8% and 4.7%, respectively. Mutation rates in NRAS exons 2, 3 and 4 were 3.9%, 4.1% and 2.4%, respectively. BRAF mutation was detected in 226 of 474 patients (47.68%). The biggest challenges of RAS/RAF molecular testing are: the availability and quality of tissue samples and reagents for molecular tests, as well as the speed of testing to obtain valid findings in a timely manner. The validation of detection methods and the communication of clinicians/therapists with laboratory personnel who perform these and other tests are of great importance, as well as the exchange of experience with other health centers and laboratories.

Keywords: KRAS; NRAS; BRAF; mutation, colorectal cancer; melanoma.

In vitro investigation of the antiproliferative effects of newly synthesized compounds

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In practice, finding new drugs is very challenging. Despite advances in the understanding of biological systems and the development of state-of-the-art technologies, the process is still long and expensive. These drugs are tested at different stages, all with the goal of bringing that drug to the market and to be intended for the treatment of future patients. Medicines are first tested *in vitro*. Cell lines form the backbone of cancer research. These individual groups of cells, typically collected from patients' tumor samples and cultured to grow indefinitely in the laboratory, enable everything from basic genetic research to drug discovery. The NCI-60 human tumor cell line panel has proved to be a useful tool for the global cancer research community in the search for novel chemotherapeutics. The publicly available cell line characterization and compound screening data from the NCI-60 assay have significantly contributed to the understanding of cellular mechanisms targeted by new oncology agents. Signature sensitivity/resistance patterns generated for a given chemotherapeutic agent against the NCI-60 panel provide target information and the insights into the mechanism of action associated with the tested agent. We hope that one day it will become possible to quickly design cheap, more specific, effective, non-toxic and personalized drugs.

Keywords: Antiproliferative effect, Cell Lines, Cytotoxicity, *In Vitro*.

Potential of epigenetic therapies in the management of colon cancer: the impact of antioxidant pretreatment on vorinostat *in vitro* cytotoxicity

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Background: Vorinostat is a histone deacetylases (HDAC) inhibitor that promotes apoptosis of malignant cells by several mechanisms. Multiple studies have failed to show efficacy of vorinostat as a monotherapy against solid tumors, but it has shown a great potential to act synergistically with various chemotherapeutics. Conflicting results were obtained regarding the role of oxidative stress in antitumor effects of HDAC inhibitors, and therefore the aim of our study was to analyze the influence of antioxidants on cytotoxic activity of vorinostat towards colon cancer cells.

Material and Methods: Human colon adenocarcinoma HT-29 cells were used to assess the cytotoxicity of vorinostat, alone or in combination with antioxidant agents N-acetyl-cysteine (NAC) and α -tocopherol (TOC), using the colorimetric MTT assay. Multiple drug effects were examined by calculating the combination index (CI) as described by Chou and Talalay, using CompuSyn software. $CI < 1$ is evidence for synergism, whereas $CI > 1$ is evidence of antagonism.

Results: Vorinostat exhibited a modest cytotoxic activity against HT-29 cells, in a concentration dependent manner. IC₅₀ value of vorinostat was 5.1 μ M, while the clinically relevant concentrations are between 1 and 2 μ M. In combination studies, HT-29 cells were treated with 1 μ M and 2 μ M vorinostat, 30 minutes after being treated with 10 mM NAC and 3 μ M TOC that displayed negligible antiproliferative effects. Both NAC and TOC managed to sensitize cells towards the activity of vorinostat, especially in a concentration of 2 μ M. Calculated CIs of 0.1981 and 0.0803 for NAC, and CIs of 0.3566 and 0.0774 for TOC, in combination with 1 μ M and 2 μ M vorinostat respectively, suggest that their synergistic effects in concentrations that can be achieved *in vivo*. The effect of NAC pretreatment was more pronounced than that of TOC for a lower concentration of vorinostat, while it was similar for a higher concentration of vorinostat.

Conclusion: The response to vorinostat could be improved by combining it with antioxidants. The mechanisms responsible for this synergistic effect should be investigated in more depth.

Key words: solid tumor; epigenetics; histone deacetylase; combination index.

Detection of *EGFR* mutations in lung cancer – the role of tissue and liquid biopsy

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Over the past two decades, the identification of oncogenic genomic alterations in non-small cell lung cancer (NSCLC) and the development of targeted therapies designed to block the oncogenic driver has enabled personalized treatment and improved outcomes. Activating mutations in the gene encoding the epidermal growth factor receptor (EGFR) tyrosine kinase are present in about 15% of Caucasian and 50% of Asian NSCLC patients. The most common activating *EGFR* mutations are deletions of exon 19 and L858R point mutation within exon 21, representing 90% of all *EGFR* mutations. Despite the success of EGFR tyrosine kinase inhibitors (TKIs) in the first line treatment setting in NSCLC patients with *EGFR* mutations, acquired resistance inevitably occurs and can be caused through EGFR-dependent or EGFR-independent mechanisms. Since the initial therapeutic choice depends on the genetic profiling of tumours, tissue biopsy has remained the gold standard for molecular analysis. Commercial real-time polymerase chain reaction (PCR) assays were developed, followed by the clinical application of next generation sequencing (NGS) panels. Given that many patients with NSCLC are diagnosed by either a cytology specimen or a small tissue biopsy, the appropriate management of specimens is critical in attempting to maximize the amount of diagnostic, prognostic, and predictive information obtained from molecular testing. Several recommendations state that cell blocks, smears, or other cytological preparations, which contain sufficient tumor cells can be suitable specimens for lung cancer biomarker

molecular testing, including *EGFR* testing. In cases in which the tumour is inaccessible or tissue sample is inadequate, liquid biopsy represents an alternative source for investigating genetic alterations in cancer. Liquid biopsy is the analysis of tumour-derived material (e.g cell-free tumour DNA, ctDNA) from body fluids. Liquid biopsy is a minimally invasive procedure which can be serially repeated to monitor disease evolution, including the development of resistance, and can demonstrate tumour heterogeneity. However, this procedure has several limitations, such as unknown potential of tumour cells to shed ctDNA, lower test sensitivity and inability to monitor histological transformation. The International Association for the Study of Lung Cancer (IASLC) highlights the complementary nature of tissue and liquid biopsies in the therapeutic decision-making for advanced NSCLC.

Keywords: Biomarkers, Biopsy, Cell-Free Nucleic Acids, EGFR protein, Lung Neoplasms

Genetic and epigenetic factors in cancerogenesis

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Basic and translational cancer research have advanced at an impressive pace in recent years. Molecular mechanisms of cancer initiation, promotion and progression have been identified and described in detail, directing the discovery and development of innovative cancer therapeutic agents. Cancerogenesis is the multistage clonal process of mutation accumulation, enabling the progression into a more malignant stage. The genetic mutations that contribute to the transformation of healthy cells into cancerous cells have been the subject of extensive research. The molecular aberrations that lead to cancer development are often characterized by gain-of-function (GOF) and loss-of-function (LOF) mutations in a variety of oncogenes and tumor suppressor genes. One of the key examples is the LOF mutation of p53, after which the mutant p53 serves as a dominant negative inhibitor of wild-type p53. Numerous computational biology and bioinformatics groups developed the tools to analyze these large datasets and distinguish mutations in the genes that contribute to tumor progression (cancer driver mutations) from those that are neutral (passenger mutations). Mutations in cancer-driver genes qualitatively or quantitatively alter the function of genes and proteins, consequently affecting the cellular processes in which these proteins participate. Oncogene mutations are linked with differences in patient survival, clinical outcomes, metastatic or recurrent tumors, and serve as predictors of tumor responsiveness to anti-cancer drugs. The complexity of cancer biology can be explained as the interplay between genetic and epigenetic abnormalities that are mutually beneficial in order to drive cancer initiation and progression. The main objective of epigenetic therapy in the era of personalized precision medicine is to detect cancer biomarkers to improve risk assessment, diagnosis, and targeted treatment interventions. The emergence of next-generation sequencing technology and artificial intelligence has advanced our understanding of carcinogenesis, enabling the multi-omics analyzes of the genome, transcriptome, and epigenetic panels to guide the further discoveries and developments in precision medicine.

Key words: carcinogenesis; genetics; epigenomics; precision medicine

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Lung cancer screening in Vojvodina, is it beginning of achieving the goal?

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Lung cancer (LC) is one of the leading public health problems in the Republic of Serbia. More than 6,500 new cases of lung cancer are diagnosed annually. Unfortunately, almost a similar number of patients die each year from this deadly disease. Five-year survival from lung cancer in the world is about 20%. At the time of diagnosis, about 70% of patients with non-small cell lung cancer have advanced disease (stages IIIB, IIIC and IV). The introduction of the National Program for screening and early detection of lung cancer would enable diagnosis in the earlier stages of the disease, which would increase the possibilities of radical treatment, improve overall survival and reduce overall mortality. There are several current guidelines for lung cancer screening. Most guidelines recommend the use of low-dose computed tomography (LDCT) for the population at a high risk for lung cancer, such as smokers and individuals over 50 years old. The first lung cancer screening pilot program in Serbia, Vojvodina started in September 2020 at the Institute for Lung Diseases of Vojvodina, and from March 2023 in the general hospital Subotica. Subjects in this program were people without symptoms, aged 50-74 years with a history of smoking. Active smokers > 30 cigarettes/year or ex-smokers who have stopped smoking within the previous 10 years. The selection of respondents included doctors from the Novi Sad Health Center, the Subotica Health Center, and the LDCT imaging was performed at the Institute for Pulmonary Diseases of Vojvodina and general hospital Subotica. During the three-year period, the program faced difficulties and challenges that needed to be overcome in order to improve and increase the number of subjects screened. During that period, a total of 6,589 LDCT scans were performed on 4,005 people who showed up for screening. Positive results for pulmonary RADS score were found in 9.7% (389/4005). The detection rate of lung cancer was 2.1% (86/4005). More than 50% of lung cancers detected in screening were stage I or II disease. 65% of lung cancer cases are diagnosed in the surgically resectable disease stage (I-III A). After analyzing the first screening results, we can conclude that innovative approaches, education and a more recognizable campaign are needed to increase the response rate among participants with a negative baseline LDCT.

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