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Original article

The Most Common Methods Applied for *In Vitro* Research in Non-Small Cell Lung Cancer in the Last Decade

Jovana Todosijević Jovanović[1], Jasmina Obradović[2], Vladimir B. Jurišić[3]

[1]*University of Kragujevac, Faculty of Science, Institute of Biology and Ecology, Kragujevac, Serbia*
[2]*University of Kragujevac, Institute for Information Technologies, Department of Sciences, Kragujevac, Serbia*
[3]*University of Kragujevac, Faculty of Medical Sciences, Kragujevac, Serbia*

SUMMARY

Introduction/Aim. Epidermal growth factor receptor (EGFR) is one of the key tumor markers of non-small cell lung cancer (NSCLC) and a guideline for the choice of therapeutic procedures. The research aims to evaluate the methods used in the investigation of EGFR variants in NSCLC cell lines and combined research (NSCLC cell lines and patient samples) in the last decade in different geographical areas.
Methods. The study included 185 full-text articles in which EGFR mutations were examined on NSCLC cell lines and 37 full-text articles analyzed EGFR variants on combined research, published from January 1, 2010, to April 2020. A descriptive statistic was done using a pivot table in Microsoft Excel 2007 original software.
Results. The obtained results showed that Western blot, MTT Cell Viability Assay, and Polymerase Chain Reaction (PCR) were used the most in the evaluation of EGFR variants on NSCLC cell lines. In combined research of EGFR variants on cell lines and samples obtained from patients, sequencing, PCR, and Western blot are mostly used. The largest number of published articles of both groups research was published on the Asian continent.
Conclusion. There is a difference in the frequency of the most commonly used methods in testing EGFR variants in research on NSCLC cell lines and in combined studies, and the common feature of both types of research is that most of the published full-text articles on EGFR gene variation in NSCLC originate from the Asian continent in the last decade.

Keywords: **NSCLC, lung cancer cell line, EGFR, polymorphism, methods**

Corresponding author:
Vladimir B. Jurišić
e-mail: jurisicvladimir@gmail.com

INTRODUCTION

According to the World Health Organization (WHO) and the Global Cancer Observatory, in 2020, among cancer patients, the majority died from lung cancer (1). Active smoking and exposure to tobacco smoke is the main risk factor for lung cancer (2). Alcohol use, exposure to ionizing radiation, arsenic, chromium, nickel, cadmium and their compounds, pulmonary fibrosis, and human immunodeficiency virus increase the risk of developing lung tumors (2 - 4). Lung tumours are divide into epithelial tumours, lung neuroendocrine neoplasms, tumours of ectopic tissues, mesenchymal tumours specific to the lung, and hematolymphoid tumours (5). Globally, the most common type of epithelial lung tumour is non–small cell lung cancer (NSCLC) (80% - 85% of cases) (6). Adenocarcinoma and squamous cell carcinoma are histologically the most common types of NSCLC (7).

In NSCLC, one of the main tumor promoters is epidermal growth factor receptor (EGFR) (8). Epidermal growth factor receptor belongs to the transmembrane HER tyrosine kinase growth factor receptor family and has an important role in the differentiation, metabolism, proliferation, and survival of cells through the EGFR signal transduction network (8 - 11). Many different methods are used in studies of EGFR variants on NSCLC cell lines (preclinical, *in vitro* research) (12). Polymerase chain reaction (PCR) is a very important, semi-manual method in examining gene changes in NSCLC cell lines (13). Western blot is a method used to investigate proteins, their detection, and expression (14). Cell viability is assayed using the colorimetric MTT (-(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (15). Flow cytometry (FCM) is a method that can easily identify DNA content in a cell, cell volume, enzyme activity, surface antigens (16, 17). Point mutations, deletions, duplications, inversions, gene fusions are examined by sequencing and next-generation sequencing (NGS) of NSCLC cell genomes (18).

All the listed methods are combined in research *in vitro* conditions, giving valid results and guidelines for the characterization of cancer cell subtypes that will find application in clinical practice (19). Preclinical research is very important because it examines the evolution and progression of tumors and can predict the effects of potential biotherapeutics of natural or synthetic origin (20). In order to improve the quality of research, in addition to analyzes on cell lines, analyzes are additionally performed on samples obtained from patients (21, 22). The reason for conducting "combined research" is to obtain the most reliable, valid results (23). The aim of the study was to single out the most common, most important methods of EGFR variant research in NSCLC, on cell lines, and in clinical studies. Also, to examine whether the use of research methods differ by geographic area.

MATERIALS AND METHODS

Literature data review and interpretation

This review analyzes the representation of certain methods about EGFR gene variants (mutations and polymorphisms) on cell lines and patients' samples in the NSCLC, published from January 1, 2010 to April 2020. Here was used a broader term "variants", referring to both mutations and polymorphisms, since the patient samples were included in this search. Usually in the research papers examining cell lines, EGFR mutations were concerned. This study continued to a previous comprehensive search that was performed on three databases: Pub Med (n = 861), ISI WOS (n = 1514) and Scopus (n = 3272) (24). The search methodology, inclusion, and exclusion criteria were similar to previous search, and here it was presented in brief (24). The key words for the inclusion criteria of the published papers were: "receptor", "epidermal growth factor", "EGFR gene", "polymorphism", "cancer", "non-small cell lung cancer", "NSCLC", "drug therapy", "treatment", "gefitinib", "erlotinib hydrochloride", "TKI", "TK inhibitors", "tyrosine kinase inhibitors", "response", "prognosis", "toxic", "toxicity", "side effect", "humans" (24). The meta-analyzes, reviews, reports, editorials, letters, unavailable papers, papers not written in English, and research that does not support search terms were excluded from the search. After removing duplicates and papers that did not meet the mentioned criteria, the full-text articles (n = 222) were selected for analysis. Finally, in this study were analyzed 185 full text articles in which EGFR mutations were examined on NSCLC cell lines referred as preclinical *in vitro* research. Similarly, 37 full text articles analyzed EGFR variants on both NSCLC cell lines and patients' samples. They were termed as combined research, since those papers could not be separated to either basic or clinical research. The

search criteria implicated the period from 2010 - 2020, but it is important to emphasize that the analysis included papers published in the first three months of 2020. The selected studies were investigated according either to the year of publication or the country where the research was performed. As highlighted previously, the results under the term "consortium" included the papers where a team of researchers was from different countries, for example Russia and Turkey - the results were grouped into "Euro-Asia". A descriptive statistic was done using a pivot table in Microsoft Excel 2007 original software (24).

RESULTS

The purpose of this research was to present the methods used in testing the EGFR variants in non-small cell lung cancer, in *in vitro,* and clinical studies. A detection of EGFR variant in NSCLC cell line studies was evident with a notable number of different methods (Figure 1). The most commonly used methods in basic (*in vitro*) research are western blot, MTT cell viability assay, and PCR (Figure 1). Slightly less presented were the methods of sequencing and flow cytometry. Contrary to the immunostaining techniques, for the western blot, PCR and sequencing methods, as well as recently developed methods were evident increasing trend over the years, thus the largest number of papers were published in 2019. Among them, the western blot and PCR were the most used methods in 2019. Next generation sequencing was evident in 2015 and mostly applied in recent years.

The largest number of papers investigating EGFR in the NSCLC cell line in the examined ten years has been published on the Asian continent (Figure 2). The three countries with the highest number of published papers on EGFR gene variants in NSCLC cell lines are China, Japan, and Korea (data not shown). A similar order of the most commonly used methods *in vitro* is observed on the Asian and European continents. The western blot method is the most widely used, followed by PCR and MTT tests, on both continents.

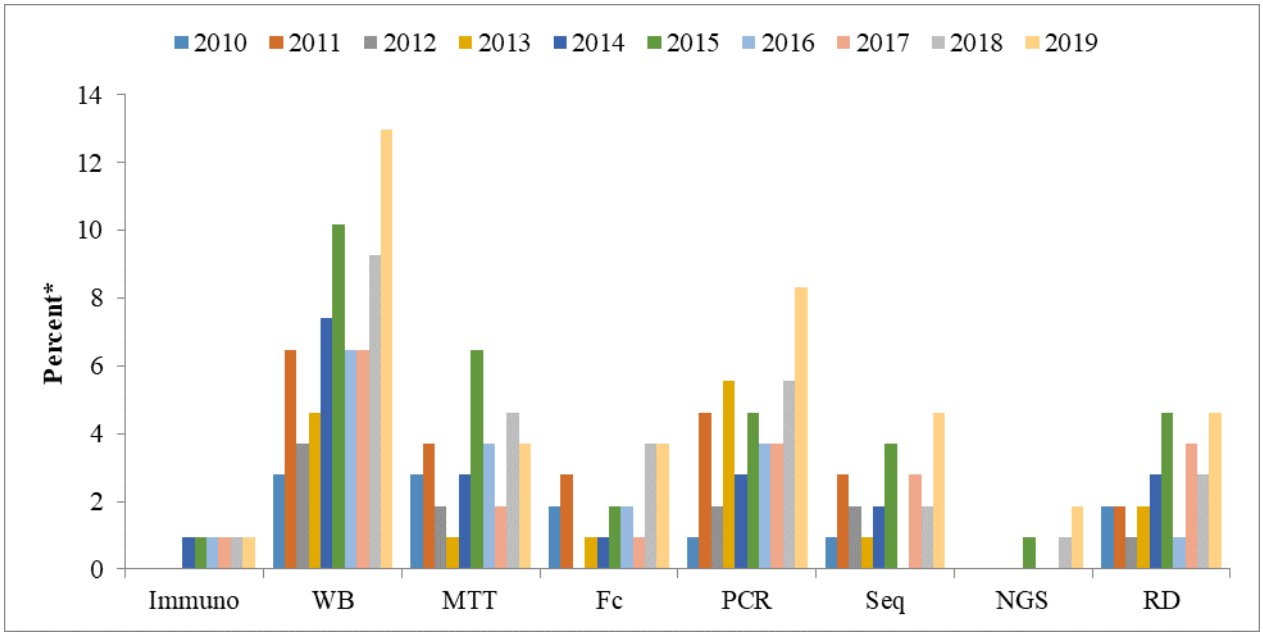

Figure 1. *Percent of methods in in vitro research over the years for EGFR variants in NSCLC*

Legend: *counted in relation to total number of 185 full text articles for in vitro research
Abbreviations: Immuno-Immunostaining techniques; WB-Western blot; MTT-(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and MTS (3- (4,5-dimethylthiazole-2-yl) -5- (3-carboxymethoxyphenyl) -2- (4-sulfophenyl) -2H-tetrazolium); Fc-Flow cytometry; PCR-Polymerase chain reaction; Seq-Direct sequencing; NGS-Next generation sequencing; RD-Recently developed

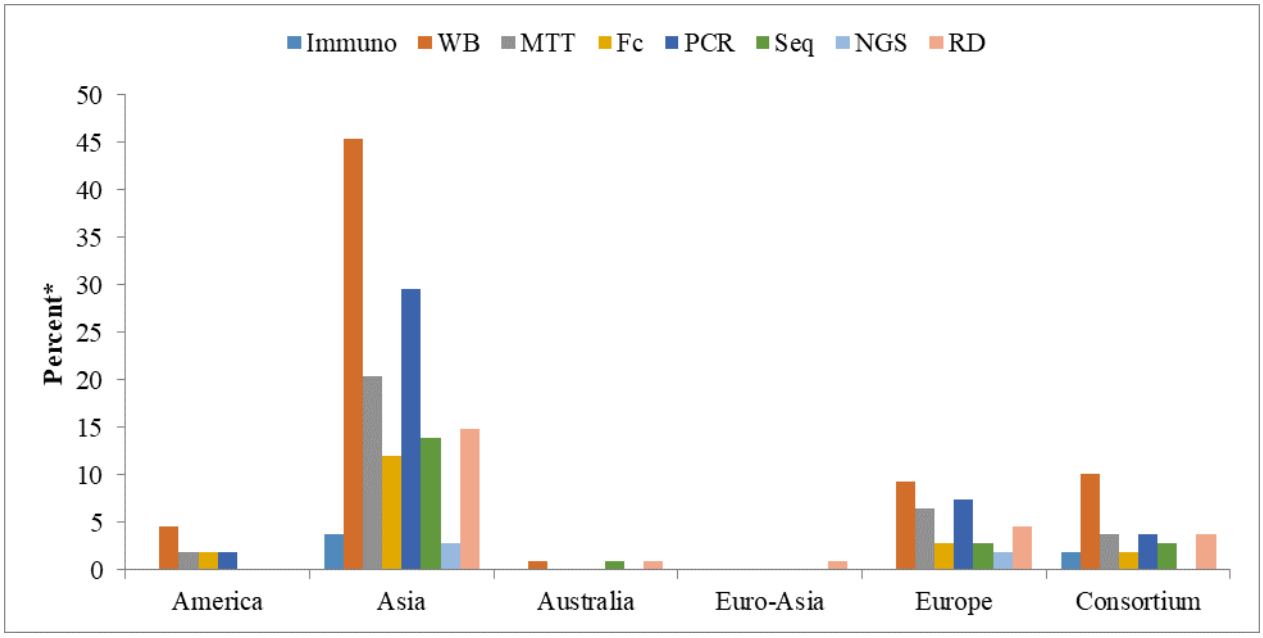

Figure 2. *Percent of methods in in vitro research through the regions for EGFR variants in NSCLC*

Legend: *counted in relation to the total number of 185 full text articles for in vitro research. Abbreviations: Immuno-Immunostaining techniques; WB-Western blot; MTT-(3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide) assay and MTS (3- (4,5-dimethylthiazole-2-yl) -5- (3-carboxymethoxyphenyl) -2- (4-sulfophenyl) -2H-tetrazolium); Fc-Flow cytometry; PCR-Polymerase chain reaction; Seq-Direct sequencing; NGS-Next generation sequencing; RD-Recently developed

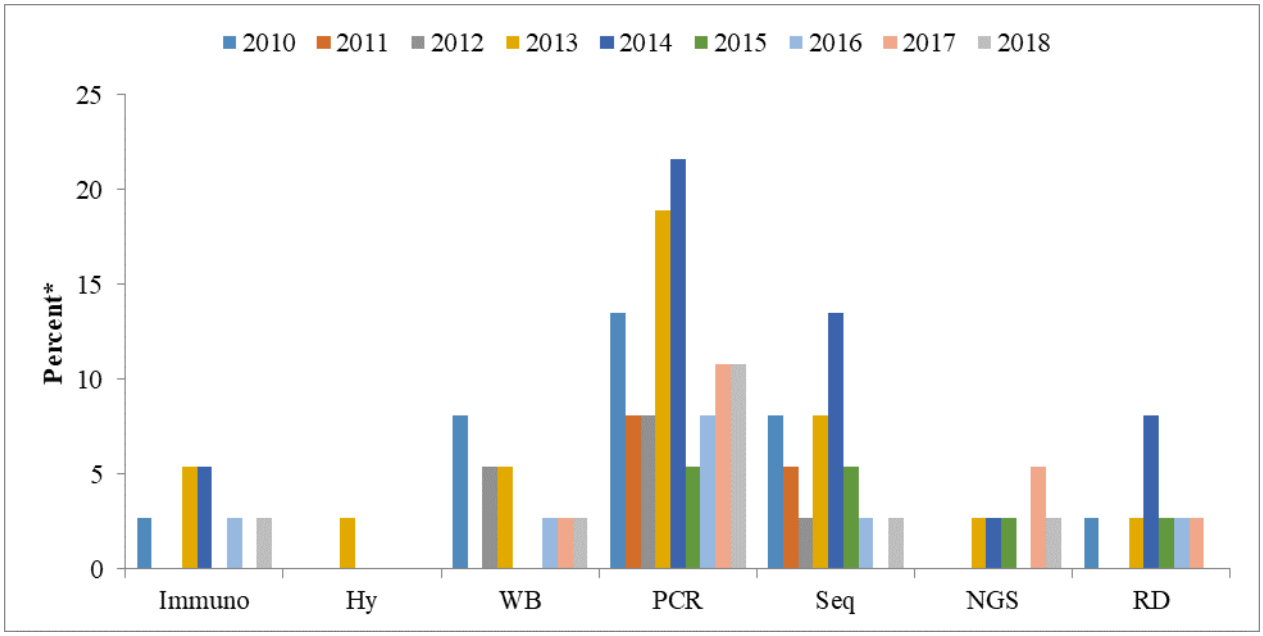

Figure 3. *Percent of methods in in vitro and clinical research over the years for EGFR variants in NSCLC*

Legend: *counted in relation to the total number of 37 full text articles for in vitro and clinical research. Abbreviations: Immuno-Immunostaining techniques; Hy-Hybridization techniques; WB-Western blot; ARMS- Amplification refractory mutation system, or allele specific PCR; HRM- High-resolution melting analysis; PCR-Polymerase chain reaction; Seq-direct sequencing; NGS-Next generation sequencing; RD-Recently developed

In studies concerning both *in vitro* and clinical research, over the years, it was evidenced that PCR and sequencing were the most commonly used methods (Figure 3), followed by the western blot methods. Immunostaining techniques, as well as hybridization techniques, were applied in smaller percents in general, compared to the PCR, sequencing or western blot methods. A similar result was applied for the next generation sequencing techniques compared to direct sequencing.

In addition to the most commonly used methods, the aim was also to investigate the geographical distribution of combined studies examining EGFR gene variants in NSCLC. Geographical distribution was similar across continents in *in vitro* research, as well as in combined research. The largest number of pa-pers examining EGFR gene variants in the NSCLC cell line and NSCLC patients has been published on the Asian continent (Figure 4).

The key findings show that the most commonly used methods in *in vitro* research are western blot, MTT assay and PCR, and in studies concerning both *in vitro* and clinical research, PCR followed by sequencing and western blot. The largest number of papers dealing with EGFR testing in NSCLC cell lines, as well as in combined research, in the examined ten years were published on the Asian continent, followed by Europe.

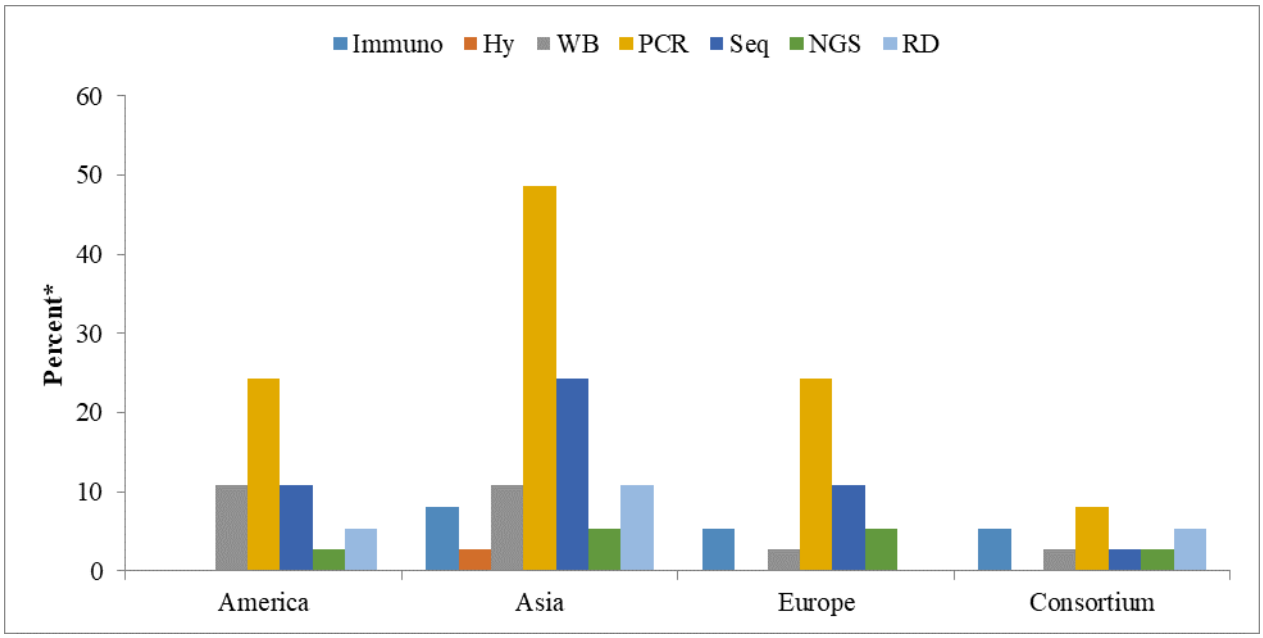

Figure 4. *Percent of methods in in vitro and clinical research through the regions for EGFR variants in NSCLC*

Legend: *counted in relation to total number of 37 full text articles for in vitro and clinical research. Abbreviations: Immuno-Immunostaining techniques; Hy-Hybridization techniques; WB-Western blot; PCR-Polymerase chain reaction; Seq-direct sequencing; NGS-Next generation sequencing; RD-Recently developed

DISCUSSION

Like other types of tumours, NSCLC is genetically and cellular heterogeneous, with complex signaling pathways, several different pathological features, and a specific tumor environment (25). In a small proportion of patients (8 - 15%), NSCLC is diagnosed at the initial stage, so the prognosis for most patients is poor (26). The key mutations for the development of NSCLC are numerous (ALK, ROS1, BRAF, V600E, NTRK1-3, RET, KRAS, and MET), and the most important among them are EGFR mutations (5, 27, 28). The most important EGFR mutations in lung tumors are deletions (del 19, del E746_A750), substitutions (L858R, T790M, D761Y) and duplications (29). In the treatment of NSCLC patients with activated EGFR mutations, the drugs from the group of tyrosine kinase inhibitors (TKIs) are mainly used

(30). All drugs used in the treatment of NSCLC were tested on cell lines first (12). In preclinical, *in vitro* research, it is possible to examine how new, potential drugs act at the molecular, subcellular, and cellular levels, but also to test a large number of substances in a short time (31, 32). The three most commonly used NSCLC cell lines for *in vitro* EGFR testing are A549 (HIF1A -/-/-, lung carcinoma, human epithelial cells with ZFN knockout modification), PC9 (an undifferentiated cell line derived from human adenocarcinoma tissue), and H1299 (also known as NCI-H1299 or CRL-5803, a human lymph node-derived cell line) (20). The T790M mutation is the cause of acquired resistance to TKI, so the genetic basis of acquired resistance is examined on the NCI - H1975/GR cell line with the T790M mutation (12). Advances in cell culture design provide preclinical models that simulate the growth and evolution of malignant cells, reveal the importance of the tumor microenvironment, and predict the patient response (20, 33, 34). By examining a precisely designed cell line with a mutation of interest, it is possible to conclude the key signaling pathways that take place in malignant cells (12).

This research aimed to examine the methods most commonly used in the analysis of EGFR in NSCLC cell lines and clinical studies in the examined decade (2010 - 2020), with a focus on geographic areas as well. Data was obtained from the analyses of internationals papers reported in several most important literature data bases. The obtained data originate from the analysis of scientific papers that used only cell lines for NSCLC, as well as research studies about EGFR variants in cell lines and clinical application.

When testing EGFR varinats *in vitro*, in the time span of 2010 - 2020, the most used method was the western blot. Proteome research can provide information on signaling molecules of the cell cycle, proliferation, and apoptosis (35 - 38). After the western blot method, MTT cytotoxicity test and PCR were in the second and third place. With the help of sequencing and NGS techniques, it was possible to analyze a large number of gene variants and mutations from a wide range of tumor types (18, 39). On the contrary, the use of the immunostaining techniques lessens than in previous decades indicating that new techniques are increasingly used not only in research but also in application in modern diagnostics and clinical practice (38, 40 - 42). Most of the basic papers examining the relationship between NSCLC and EGFR have been published in Asian countries, followed by countries on the European continent. In both geographic regions, western blot is the most commonly used assay, followed by colorimetric MTT assay, and PCR in the third place.

Even a large number of tests were observed in this study, not all of them used to analyze EGFR variants on the NSCLC cell line are shown in Figure 1 and Figure 3. The other methods, not frequently used but no less important were the following: Genome Alteration Print (GAP), Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein 9 (CRISPR/Cas9), Water-soluble tetrazolium-1 assay (WST-1 assay), Receptor Tyrosine Kinase Protein Array assay (RTK assay), Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS), Restriction Fragment Length Polymorphism (RFLP). Due to detailed characterization of the genome and transcriptome of the malignant cells, these methods help to examine drug resistance, methylation status, etc. (15, 23, 40 - 44).

The multidisciplinary approach and modern technologies advanced basic research and improved biology, medicine, and oncology. One example is three-dimensional (3D) printing platforms for establishing new 3D cell cultures enriched with cancer stem cells (45). Organoids of lung tumors are another example that shows how important basic research is and that they are crucial in the process of tumor individualization (46). The genomic changes and tissue structure of the primary tumor are maintained in the organoids. After the treatment of the organoids, a specific response to the drugs was obtained, based on which a therapeutically personalized approach to the patient is possible (47).

Certain studies that matched the inclusion criteria for this review examined at the same scientific question both EGFR variants on NSCLC cell lines and NSCLC patients' samples. Thus, they could not be separated to either term "clinical or basic". The PCR method sequencing and western blot are used as the most common methods in the analysis of EGFR variants in combined research. The advantage of PCR is that it is performed in a semi-automated manner and it was improved over time, being more valid and more sensitive (12). There are several different types of PCR methods used in EGFR variants research in NSCLC, e.g. allele-specific real-time PCR, PCR-Invader, peptide nucleic acid-locked nucleic acid (PNA-LNA) PCR clamps, ddPCR (Droplet Di-

gital PCR) (48 - 50). The largest number of papers combining EGFR variants and NSCLC research in cell lines and clinical studies have been publish on the Asian continent, similar to described research on NSCLC cell cultures research.

Previous results show that the most commonly used methods in testing EGFR variants in clinical studies differ from those used in the field of basic research for the same period (2010 - 2020) (24). These two studies have in common that the largest number of papers in which *in vitro* and *in vivo* EGFR variants were tested was published on the Asian continent. The three countries with the large number of published papers on EGFR gene and NSCLC are China, Japan, and Korea. The results of the global screening worldwide highlighted Asian countries as high-risk groups that have a high frequency of oncogenic EGFR alterations and a high prevalence of lung cancer in non-smokers (51, 52). After making conclusions about the initiation, promotion and progression of tumors, it is necessary to examine hypotheses related to the clinical picture, drug resistance, and patient survival (53, 54).

Both types of research, *in vitro* and clinical studies, have their disadvantages and advantages and the examination of any type of tumor cannot be fully examined without immortalized cell lines and samples obtained from patients. In order to improve the overall survival and quality of life of patients, it is important to examine the impact of newly synthesized substances, substances of natural origin and which key genes, their variants and frequencies have an impact on the outcome of treatment (31, 54 - 57).

CONCLUSION

Western blot, MTT, and PCR are the commonly used methods in testing EGFR variants on NSCLC cell lines. Sequencing, PCR, and western blot are the three most commonly used methods in combined (*in vitro* and clinical) studies of EGFR variants in NSCLC. Common characteristics of both types of research are that the majority of the published papers on EGFR gene variation in NSCLC originate from the Asian continent in the last decade.

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Najčešće korišćene metode u *in vitro* istraživanjima nesitnoćelijskog karcinoma pluća tokom prošle decenije

Jovana Todosijević Jovanović[1], Jasmina Obradović[2], Vladimir B. Jurišić[3]

[1]*Univerzitet u Kragujevcu, Fakultet za nauku, Institut za biologiju i ekologiju, Kragujevac, Srbija*
[2]*Univerzitet u Kragujevcu, Institut za informacione tehnologije, Departman za nauku, Kragujevac, Srbija*
[3]*Univerzitet u Kragujevcu, Fakultet medicinskih nauka, Kragujevac, Srbija*

S A Ž E T A K

Uvod/Cilj. Receptor epidermalnog faktora rasta (engl. *epidermal growth factor receptor* – EGFR) jedan je od ključnih tumorskih markera nesitnoćelijskog karcinoma pluća (engl. *non-small cell lung cancer* – NSCLC) i jedna od smernica za izbor terapijskih procedura. Cilj rada je da proceni zastupljenost raznih metoda koje se koriste u istraživanju EGFR varijanti na ćelijskim linijama NSCLC-a i u kombinovanim istraživanjima (NSCLC ćelijske linije i uzorci dobijeni od bolesnika) u poslednjoj deceniji, u različitim geografskim oblastima.

Materijal i metode. Studija je obuhvatila 185 članaka u punom tekstu u kojima su mutacije EGFR–a ispitivane na NSCLC ćelijskim linijama i 37 članaka u punom tekstu u kojima su analizirane EGFR varijante u kombinovanim istraživanjima, objavljenim od 1. januara 2010. do aprila 2020. godine. Deskriptivna statistika urađena je korišćenjem pivot tabele u originalnom softveru Microsoft Excel 2007.

Rezultati. Dobijeni rezultati pokazali su da su *western blot*, MTT test citotoksičnosti i lančana reakcija polimeraze (engl. *polymerase chrain reaction* – PCR) najčešće korišćene metode u proučavanju EGFR varijanti na NSCLC ćelijskim linijama. U kombinovanim istraživanjima EGFR varijanti, na ćelijskim linijama i uzorcima dobijenim od bolesnika, najčešće se koriste sekvenciranje, PCR i *western blot*. Najveći broj objavljenih radova obeju grupa istraživanja objavljen je u Aziji.

Zaključak. Postoji razlika u pregledu najčešće korišćenih metoda u testiranju varijanti EGFR-a u istraživanjima na NSCLC ćelijskim linijama i u kombinovanim studijama. Zajednička karakteristika obeju vrsta istraživanja je ta što većina objavljenih članaka u punom tekstu o varijanti EGFR gena kod NSCLC-a potiče sa azijskog kontinenta u poslednjoj deceniji.

Ključne reči: **nesitnoćelijski karcinom pluća, ćelijske linije raka pluća, receptor epidermalnog faktora rasta, polimorfizmi, metode**