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BASIC RESEARCH OF LUNG CANCER *IN-VITRO*: MEASUREMENT METHODS, NEW POSSIBILITIES AND PERSPECTIVE

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Abstract. *Epidemiological data indicate that in the last period there has been an increase in the number of malignancies and among them lung cancer is one of the most common forms. In vitro studies based on the usage of immortalized cell lines are an important source of scientific knowledge for understanding of the mechanism of cell growth, proliferation and cell death. In this paper, the most commonly used methods for in vitro research in NSCLC (non-small cell lung cancer) based on testing the effects of new compounds to determine the degree of apoptosis, necrosis, cell proliferation as well as their significance are discussed. So far, techniques of working with monolayer cultures have been mainly used. In the future, it is recommended to use a 3D system, knockout cell line and to conduct additional studies regarding the use of organoids or spheroids, as well as the application of new techniques to better understand the complex processes of carcinogenesis and the action of biologically active compounds.*

Keywords: *NSCLC, EGFR, cell line, mutation, in vitro method, MTT, Western blot, Flow cytometry, CRISP/cas*

1. Introduction

Epidemiological data indicate that recently there has been an increase in the number of malignancies. They are one of the predominant causes of death in our country and in world. The most common lung cancer type is non-small-cell lung carcinoma (NSCLC) (85%) highly associated with cigarette consumption [1, 2].

Figure 1. Lung cancer cell culture for in-vitro examination.

In vitro studies based on the use of immortalized cell lines are a valuable source of scientific knowledge for understanding of the mechanism of cell growth, proliferation and cell death. Also, *in-vitro* research can help us to apply new potential drugs, synthetic compounds, as well as to test their toxicity, which is not possible in humans.

Numerous cell lines isolated from patients with lung tumors used for research, which are described and characterized in great detail, include: H460, H1299, H1975, H23 and H358 (3). Cell lines are useful because they can be used not only to study the signaling processes associated with tumor formation, carcinogenesis, but also with the mechanism of action of newly synthesized drugs [4, 5].

2. Investigation of NSCLC

A review of the literature indicates that the most studied phenomena for NSCLC cell lines include the investigation of cell growth and cell death (apoptosis). Investigation of cell death receptors, cell signaling and mechanism of resistance to the newly synthesized drugs as potential therapeutic medicaments are investigated most often. Genomic and epigenomic phenomena involved in that process indicate the role of Rb (Retinoblastoma) genes, the Bcl-2 (B-cell lymphoma) family genes, the TNF (Tumour Necrosis Factor Family) genes, and perhaps the most studied EGFR (Epidermal Growth Factor Receptor) gene [6]. According to their function in the cell, they are dividing into pro-apoptotic ones that lead to apoptosis and anti-apoptotic ones that inhibit it. The most well-known mechanism of apoptosis cell regulation is through an increase in pro-apoptotic (Bax) and a decrease of anti-apoptotic proteins (Bcl-2), suppression of matrix metalloproteinases (MMP 2) [7, 8, 9].

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Recently, an enormous number of scientific publications indicate the significant role of epidermal growth factor receptor (EGFR) in NSCLC [10, 11, 12]. This trans-membrane receptor plays a key role in important cellular signaling pathways. EGFR is also a target for treatment, most often with tyrosine kinase inhibitors (TKIs) and monoclonal antibodies. For *in vitro* research, many potential drugs can be tested, and only those with the best effects can be included in further clinical phases of application. Apart from the best-known EGFR, the key molecules in the NSCLC cell signaling network are STAT3 (Signal transducer and activator of transcription 3), YAP1 (YES-Associated Protein 1), ALK (anaplastic lymphoma kinase), BRAF (v-Raf murine sarcoma viral oncogenic homolog B) [13, 14].

Looking for new and better treatment of patients with lung tumors, new compounds are constantly being tested as potential new drugs *in vitro* (Table 1).

Table 1. Active substances and antibodies for *in vitro* research of NSCLC

Natural compounds	Chemical substances	Immunology Antibodies
Resveratrol [15] Cordycepin [16] Chelidonine [17] Osthole [18] Curcumin [19] Cypripedin [20]	Platinum-DNA adducts [7] Berberine-hydrochloride [8] Metformin [22] Quinacrine [23] Pimozide [24] Nelfinavir [25]	Imgatuzumab [25] Cetuximab, Necitumumab [26], Panitumumab, Matuzumab, Nimotuzumab

Berberine hydrochloride, platinum-DNA adducts, nitrogen heterocyclines are just some of the examples [7, 8, 21]. More and more scientific papers confirm that polychemio-therapy or their combinations in synergistic action of different compounds can be useful against cancer. Synergistic action enables the targeting of several targets of key signaling pathways that one drug alone fails. In cell line research, the synergy between the most commonly used TKIs was confirmed, as well as the combination of TKIs with other pharmacological substances [28, 29]. In addition to compounds that directly induce apoptosis, several chemicals have been described with the possibility to mediate and accelerate this process in combination with other compounds. Metformin and cypripedin as active chemicals, in combination with standard therapy (cisplatin), have higher activity and give better results in apoptosis induction [20, 22].

Compounds used in the treatment of various non-malignant diseases could be potentially applied in NSCLC. They include quinacrine (anti-malarial drug), pimozide (anti-dyskinesia agent) and nelfinavir (an antiretroviral drug) [23, 24, 25].

3. The most commonly used methods for *in-vitro* testing of NSCLC

Some of the methods used in last period for *in-vitro* investigation of NSCLC include MTT assay, Flow cytometry, Western blot and CRISPR/cas9.

3.1. MTT assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is the most used cell viability and proliferation assay since it was described in 1983 [30]. The essence of the reaction is that mitochondrial enzyme succinate-dehydrogenase reducts yellow tetrazolium salt in the blue colored formazan (Figure 2).

Figure 2. MTT test values; 96 microwell plates during the testing of the effects of chemical compounds

This test is an accurate indicator of cell viability because only enzymes from the active mitochondria of living cells lead to a reduction. The main disadvantage is that this conversion depends on metabolic rates and the number of mitochondria [31]. This method is suitable for high-throughput screening and miniaturization but requires numerous wash steps. The alternative to MTT assay might be the resazurin assay (RES), neutral red uptake (NRU), and sulforhodamine B (SRB) assays [32, 33]. Two more alternatives to the MTT test should be mentioned, and they are MTS (3- (4,5-dimethylthiazole-2-yl) -5- (3-carboxymethoxyphenyl) -2- (4-sulfophenyl) -2H-tetrazolium) and XTT (2,3-bis (2-methoxy-4-nitro-5-sulfophenyl))-5-[(phenylamino) carbonyl] -2H-tetrazolium hydroxide) assay [31, 19].

Using MTT assay, it is possible to investigate the effects of new synthesized chemicals or natural compounds with potential anti-proliferative effects on cancer cells as well as to assess the optimal concentration for application without toxicity [34]. Based on the IC50 value, it can be concluded which treatment and which concentration give the best results. Besides, the activity of the chemicals at different exposure times and whether the data have statistical significance are monitored. Anticancer activity in NSCLC cells has been confirmed using different natural origin compounds including Resveratrol, Cordycepin, Chelidonine [15, 16, 17].

3.2. Flow cytometry

Flow cytometry is a modern multi-parameter technique for the biomedical study of cell characterization that generates information from the interaction of individual cells labeled with multicolor antibodies in cell suspension and a laser light reflection for cell detection [35]. Cell classification is performed based on size and granularity. Using these methods, individual cell receptors, cell antigens, degree of apoptosis, necrosis, and cell proliferation can be

analyzed. Advances in technology have enabled the simultaneous measurement of as many as 40 markers on the cell surface [36]. Flow cytometry has advanced a lot in the last 30 years, mostly in the field of cell biology [35]. Until the discovery of monoclonal antibodies, flow cytometry was used only to measure the amount of DNA in cancer cells [37].

Flow cytometry is based on the estimation of marker expression on the cell membrane using Annexin V (Annexin A5) and Propidium iodide for assessing the percentage of apoptosis [38]. In addition to these two, the terminal deoxynucleotidyl transferase (TdT) and acridine orange (AO) are used, and the results show whether there are breaks in the DNA and the type of cell death. Surface marker testing and cell cycle phase testing are equally important. From such research, conclusions can be drawn as to which substances lead to programmed cell death, as well as at what stage of the cell cycle they are most sensitive [18]. New principles of immunotherapy based on genetically engineered newly synthesized antibodies directed against anti-epidermal growth factor receptor (EGFR) have shown the ability to induce antibody-dependent cellular cytotoxicity (ADCC) and inhibit EGFR signaling. They also confirmed first *in vitro* conditions and estimated degree of apoptosis [26].

Flow cytometry immune profiling, immune cell-based biomarker signatures and immunotherapy, cell cycle distribution and apoptosis are the main possibilities of the flow cytometry method in NSCLC studies [9, 37]. This information is necessary to evaluate the effectiveness of therapy in the treatment of NSCLC. The introduction of specially designed antibodies confirms various signal pathways or parts of them. One example is the use of the anti-epidermal growth factor receptor – MB-encapsulated thiol-terminated silica nanoparticles (anti-EGFR/MB-SHSi) – for tumor detection [39]. That “Lab-on-a-chip platforms” are the future of molecular biology and medicine is also confirmed by new biotechnological discoveries by flow cytometry [40].

3.3. Western blot

In addition to the investigation of the genomic in NSCLC cell line, information about the protein state in the cells (proteomics) is very important. The most widely used technique over the last three decades to detect specific and the target protein has been a Western blot. Western blot is a method that examines the level of expression of a protein of interest and its phosphorylation status usually during the investigation of cell signaling.

This method consists of several steps, the first of which is the preparation of a cell lysate [41]. After appropriate homogenization or ultrasonic treatment of the cell lysate, denaturation of higher structures is necessary while preserving the negative charge. The negative charge is required for the proteins to separate in the process of electrophoresis, and then transferred to the membrane. Washing, blocking nonspecific antibody binding, and incubating the right antibody concentration are essential to minimize unbound antibodies in the background. We say that the Western blot method is semiquantitative because it does not show the absolute amount of protein of interest, but compares it with a predetermined control (Beta-actin) [38].

The main directions of research in NSCLS include an examination of changes in different cell markers and key signaling pathways during apoptosis (42, 43]. Advances in biotechnology are seen in the design of highly specific antibodies with high sensitivity. It is equally important that the test is cost-effective and that results in mutant EGFR are obtaining quickly. The Western blot method on the NSCLC model system is also used to investigate the effect of potential vaccines, substances of natural origin and drugs on the concentration and phosphorylation of EGFR protein [19, 42, 43]. Data about protein investigation indicate their regulatory role for EGFR signaling in NSCLC and give more information about several proteins like some of the fusion proteins that contribute to the oligomerization of the tyrosine kinase (EGFR) domain [44].

Western blotting has been used to investigate protein expression of circulating tumor cells (CTCs), cell migration, drug resistance as well as signaling pathways that are crucial for NSCLC cell survival and proliferation [45]. The results of protein testing help in the choice of therapy and also in understanding the benefits of vaccination in patients with NSCLC [42, 43, 46]. This method has advanced tremendously in the last decade and is becoming a more automated technique. The sensitivity and reliability of the method had been increased with the use of new automated western blotting machines, chemi-luminescent reagents, and imagers [47]. Changes in the material for separation, imaging and software data processing are responsible for this progress [48].

3.4. CRISP/cas9

Scientific research is not only based on availability and designed model systems. With the help of genetic engineering methods, they predict mutations of interest and direct the development of new model systems. CRISPR (clustered regularly interspaced palindromic repeats) technology allows us to examine *in vitro* how cell functions change with genome editing. An example is the insertion of 20 exons of the EGFR gene in NSCLC [49].

Results of the CRISP/cas9 study on NSCLC cell lines show great potential in the treatment of patients with this type of tumor. The application of the CRISP/cas9 method in gefitinib TKI-resistant H1975 cells has shown the possibility of editing mutations of interest [50]. Elimination of mutant EGFR alleles leading to drug resistance (L858R) occurs to cell death and tumor reduction after treatment. The unique CRISP/cas9 method in NSCLC cell line studies cannot be omitted. CRISPR technology allows us to examine *in vitro* how cell function changes with genome editing.

4. New possibilities and perspectives in NSCLC research and treatment

In vitro research has great potential for discovering new target biomarkers and a better understanding of already discovered ones. For *in vitro* research to be valid, a reliable model must meet certain conditions. The cell line must maintain consistency and purity to be the right preclinical model. Infections (usually mycoplasma) and mixing of cell cultures are mentioned

as the most common problems in the study of cell lines [3]. Cell line research has a wide range of studies.

In vitro studies are suitable for testing such models of therapy that cannot be immediately applied in humans, such as determining the dose and type of radiotherapy, where they were previously a good model of testing. Recently, there have been very intensive studies of the combination of radio and immunotherapy depending on the expression and/or mutation of EGFR [9]. Data have shown that EGFR mutated cells are more sensitive to radiation and suffer cell death by apoptosis. It is an important guideline for further clinical trials.

One of the technology novelties in basic research with cell culture has given a new model of three-dimensional cell culture (3D cell culture) [51]. The advantages of new 3D model systems and high-throughput screening (HTS) tests are much more significant than "ordinary" preclinical drug trials. Cytotoxicity information is only part of the results. With the help of new methods, it is possible to identify a key signal pathway and what the response to e. g. transcription level is. The main conclusion is that the new 3D models better simulate the conditions prevailing in-vivo systems, mostly under the influence of extracellular matrix proteins [52].

Research into NSCLC cell lines for testing cell death, signaling pathways and bioactive substances are only a part of the possibility. The mixed methods research that will connect the active substance, signaling pathway and apoptotic death of cancer cells is the most important. Bufalin and PI3K / Akt Pathway, or Sesamin and Akt / p53 pathway in the induction of apoptosis are examples of multi-method works that give the complete picture of the subject of research [53, 54].

Resistance to TKI occurs in a large number of patients with NSCLC. The mechanisms of resistance in genes, such as HER2 and BRAF, have been insufficiently investigated, and resistant mutations in EGFR, c-MET, and ALK genes are best explained using classical methods based on Polymerase chain reaction (PCR) tests. [14]. Although the key genes involved in the development of resistance are known, patients still die from NSCLC.

This means that new target biomarkers must be set, and in this way targeted therapy should be improved. The first step is research involves in vitro studies on cell lines.

5. Conclusion

Mutation status, cell proliferation, expression and phosphorylation of proteins and cell cycle distribution provide complete information about cancer physiology and cancer growth. Literature data suggest that multi-method research and mixed investigation are important in the future for a better understanding of this complex process.

Besides, several techniques such as qPCR-HRM, MTT assay, Western blot and Flow cytometry need to be simultaneously applied [21, 42, 55].

Continuous investigation effects of a new potential drug are very important in oncology with aims to achieve better benefits to patients. Basic research is the one that provides guidelines for clinical research. It is equally important to examine whether the test compounds have toxic effects on healthy cells and whether their application would do more harm than good.

Future research should provide conclusions and guidelines on new therapeutic strategies in the treatment of NSCLC, as the emergence of drug resistance in patients is a major problem.

Cell lines are a good model on which the synergy of biotechnology and medicine can identify the key causes of tumor formation, angiogenesis, and metastasis. Advances in technology in the field of 3D cultures, organoids and knockout cell lines, make it possible to better understand the property of tumor heterogeneity *in vitro*.

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