The influence of the "bio-coat's" type of culture dishes on the primary cultures of lung cancer cells

JUSTYNA WOŚ[1], MONIKA PIECZYKOLAN[1], PAWEŁ RYBOJAD[2], ANDRZEJ JABŁONKA[2], KAROLINA OLSZEWSKA-BOŻEK[1], JACEK TABARKIEWICZ[1]

[1]Department of Clinical Immunology, Medical University of Lublin, Poland
[2]Department of Thoracic Surgery, Medical University of Lublin, Poland

Abstract

Lung cancer has the highest mortality rate among both women and men all over the world. A more complete understanding of the molecular pathogenesis and biology of human lung carcinomas may contribute to designing more effective therapies in the future. This paper focuses on primary cultures of human lung cancer cells and their application as models of malignant biological behavior.

The aim of the study was to evaluate the growth of lung cancer cells on various culture plates and to choose the most optimal and efficient surfaces.

Tissues from 6 lung cancer patients were included in the study. Cells were cultured on plates coated with different kinds of surfaces (polylysine, fibronectin, untreated glass and borosilicate glass). The number and viability of obtained cells were counted.

Lung cancer cells grew differently on polylysine, fibronectin and glass surfaces. The results of this experiment suggest that the best surface for the growth of lung cancer cells is polylysine surface (CC2). CC2 surfaces gave the greatest increase in cells count among four examined surfaces. The lung cancer cells cultured on the surfaces made of borosilicate glass did not proliferate, therefore these kinds of dishes are rather not suitable for the primary culture of lung cancer cells.

The kind of bio-coat of culture dishes has a significant impact on the growth and proliferation of cultured lung cancer cells.

Key words: *lung cancer, cell culture, growth factors, culture surfaces.*

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Introduction

Lung cancer (LC) is the leading cause of death from neoplasmatic diseases among men and women in Poland and all over the world [1-3].

For years, lung cancer has been one of the most important problems of modern civilization. Despite the development of medical sciences, doctors failed to achieve significant progress in the treatment of this terrifying disease. Dynamic development of certain branches disciplines from the borderland of medicine and biology such as immunology and genetics gives hope for the improvement of this situation.

Lung cancer is one of a few neoplasms, which has very well defined risk factors [2]. Long-term studies showed that there are approximately 60 different risk factors for the incidence of LC, 18 of which have a clear carcinogenic effect. The most important of them is active smoking, which is responsible for almost 85% of cases. Passive smoking is responsible for next 3-5% of LC. Other risk factors are occupation (exposure to asbestos, beryllium, vinyl chloride, lead, chromium, nickel, oil, coal, iron ore and radioactive elements), diet, genetic factors and others [1, 2].

Clinically, LC can be categorized as small cell carcinoma (SCLC) or non-small cell lung cancer (NSCLC).

Correspondence: Justyna Woś, Chodźki 4 A, 20-093 Lublin. Phone number: +48 81 75 64 840, e-mail: justyna.wos1@gmail.com

The latter includes squamous cell carcinoma, adenocarcinoma, large cell carcinoma [1, 4, 5]. These two main groups show striking differences in biochemical, morphological and molecular properties, as well as in development and response to therapeutic modalities [4]. In our study we focused on NSCLC tumors, which constitute the majority of lung cancers.

Cell cultures provide a good model for studying neoplasmatic diseases. The dynamic development of this method may provide a better understanding of this disease and give hope for discovery of effective treatment for cancer patients [4]. Two types of cultures are routinely performed. These can be divided into the primary culture or the culture of established cell lines [6].

The use of primary cultures is becoming more widespread, and they are used in many diverse studies. Recently, most authors have believed that the use of primary cell cultures of cells obtained from tumor biopsies is better and more exact than commercially prepared cell lines. The current authors believe that the use of primary cell cultures of tumor biopsies is more important than commercially prepared cell lines in understanding the mechanisms of neoplasmic diseases.

The analysis of tumor suppressor activity and differential gene expression in primary cultures reveals changes relevant to lung cancer progression [7].

Primary cell cultures have the advantage that their basic cells are removed from the *in vivo* environment and might therefore be expected to resemble the function of these cells *in vivo* [6] more closely. Their main disadvantage is that these cultures are reacting to continuous changes of *in vitro* environment, which can change cell composition of the culture. As a result, some cells in the mixed cultures die and others proliferate or differentiate [6].

Cell lines, derived from metastases, are not often representative of primary cancers [7]. Some cell lines may exhibit morphological changes after several passages. Moreover, the tendency of cultures to reduce the differential gene expression in lines suggests that heterogeneity of tumor cells biology may be unrepresented in cell lines culture system.

Furthermore, the question of how extensively long-term culture alters the biological properties of cell lines is unknown. These are the main reasons why primary cultures of malignant cells should be studied more thoroughly [7].

Cell culturing in glass or plastic dishes is one of the basic methods of research on normal and malignant cells. Disposable plastic is now most commonly used for the cell culture growth. However, since most plastics are hydrophobic, culture dishes are often specially prepared to create hydrophilic surfaces, which are preferred by many cell types. Biological coatings and chemical modifications may increase or reduce the proliferation of cultured cells [8]. To assure optimal proliferation and growth, some cells have to be provided with additional requirements such as an extracellular matrix substrate (for example fibronectin, polylysine, and collagen), cytokines or growth factors [4].

Over the past 20 years, many of the technical hurdles involved in growing primary cultures of human lung epithelial cells have been overcome, and a variety of more or less similar methods have been reported [7]. Thanks to continuous improvement in *in vitro* proliferation of cancer cells, it is possible to carry out more and more sophisticated experiments which may lead to a better understanding of pathogenesis of neoplasmatic diseases and development of new treatment modalities.

Materials and methods

Samples for the study were obtained from 6 patients diagnosed with non-small cell lung cancer who underwent surgical resection.

Four tumors were recognized as squamous cell carcinoma, one large cell carcinoma, and one mixed large and squamous cell carcinoma. In order to evaluate the best conditions for cell proliferation, four different types of culture plates, each coated with different substrate, were prepared:

1. Culture plates Lab-Tek®II Chamber Slide™ System 154917, 4 well Glass slide CC^2 treated (Nalge Nunc International; Germany) – chemically prepared growth surface on glass slide that mimics polylysine. The coat remains stable without refrigeration.
2. Culture plates BD BioCoat™ cell environments Human Fibronectin Cellware, 4-well CultureSlides 354559 (BD BioCoat™, Canada) – plates with a uniform application of human fibronectin. Fibronectin is involved in cell adhesion, growth, migration and differentiation.
3. Culture plates Lab-Tek®II Chambered Coverglasses System #1 Borosilicate Coverglass 136420 (Nalge Nunc International, USA) – The polystyrene chamber is mounted on a borosilicate slide with medical grade silicone adhesive and is not removable.
4. Culture plates CultureSlides, 4-well 354104 (BD Falcon™, USA) – These plates have polystyrene chambers of 4 well format on an untreated glass growth surface.

Establishment of cell culture from primary tumors

Tumor tissue was teased and minced with a scalpel into approximately 1-2-mm fragments [1]. Next, cells were extracted by enzymatic digestion. After that, $CD45^+$ cells were depleted with the use of Human CD45 Depletion Kit. Then $CD45^-$ were plated into four 4-well culture dishes in X-VIVO 15 (Lonza, Belgium) medium supplemented with 1% combination of antibiotics (penicillin-streptomycin-neomycin) and epidermal growth factor (1 µl/1 mL). The cultures were incubated at 37°C in a humidified 5% CO_2 atmosphere.

The experiment included cultured cells from each of the 6 tumors in four different culture dishes. The cells were cultured in the above-mentioned conditions and then observed microscopically and counted after 0, 2, 4 and 6 days of incubation. Cells were counted under a reverse microscope at a magnification of 40× in 5 fields of vision in each of four wells on a plate. During observation of cells derived from one tumor 120 results for one type of plate were obtained.

After each counting, 1 µl of EGF was added to all wells filled with cultures. At the end of the culture procedures, the combined results of 6 experiments were used for statistical analysis.

Statistical analysis

All the data are shown as a mean ±SD. The differences between the groups were evaluated by two-way ANOVA followed by Bonferroni post-test (Graph Pad Prism software). The differences were considered significant at p-value < 0.05.

Results

The study assessed the influence of substance covering the surface of culture dish on the growth of non-small cell lung cancer cells in the primary culture. Cells were plated into four different surfaces: polylysine (CC2), fibronectin (BD BioCoat), borosilicate glass slide (Nalge Nunc), and untreated glass slides (BD 354140).

Lung cancer cells grew differently on glass and modified glass surfaces. In our experience the best surface for cell growth of lung cancer proved to be CC2 surface, similar to the polylysine substrate. On the first day of experiment (0) the average number of cells on the plate for 120 measurements was 24.67 ±33.25 and on the last day the number of cells increased to 40.58 ±40.96, n = 120. Culture on this surface gave the greatest increase in cells count among four examined coats. The plate is also characterized by a very good optical quality, which is very important in observation under the microscope.

Performed analysis of variance revealed, that variation among column means is highly significantly greater than expected by chance (p < 0.0001).

Bonferroni Multiple Comparison Test indicated statistically significant difference between the average cells number seeded on the plates CC2 at day 0 and the average cells number on these plates evaluated at sixth day. Such increase in cell number was observed only in cultures established on this type of surface. The worse result was obtained in cultures established on Nalge Nunc surface. In this case the average cell number on the sixth day was significantly lower than in other types of cultures.

Sample images of cultured cells on different surfaces are shown in Figure 1A-D. Numerical data are presented in the Table 1 and graphically in Figure 2A-B, as the average number of cells in visual field.

Discussion

Continuing studies of proliferated lung tumor cells has resulted in a better understanding of the pathology and development of lung cancer [9]. As part of our ongoing efforts to develop better models for the study of NSCLC, we evaluated the effect of different surfaces of culture containers on proliferation capabilities of lung cancer cells.

Preclinical cancer biology research and drug development traditionally rely on the use of cultured cells that are able to grow *in vitro* on artificial surfaces [10, 11]. A lot of debates have focused on the reliability of such culture cells as cancer models and their ability to predict the clinical outcome of novel therapies for patients [10, 12]. Recent attention has focused on the use of primary cultures of cancer cells as a better model for cancer *in vivo* [10].

Growth substrates may affect the morphology, differentiation and behavior of various cell types [8, 13]. Lung cancer cells showed significant differences in growth and proliferation on each of the tested surfaces. In our experiment cells were plated into four different surfaces (CC2, fibronectin, untreated glass, and borosilicate glass). Our study showed that the best surface for cell growth and proliferation of lung cancer cells is polylysine surface.

Many factors can influence the cell growth during ongoing culture, for example: the type of cell, the age and condition of the culture, nutrients, cell culture protocols, toxins, culture environment and selection of an appropriate surface. Slight changes in culturing conditions like fluctuations in CO_2 concentration or temperature may result in poor growth. Prolonged cultures may result in the depletion of nutrients or raising concentration of toxins [9, 14, 15]. Migration of cells in culture is very dependent on the composition of the culture medium. For example, some authors suggest that in medium containing EGF, epithelial cells are very migratory and form colonies consisting of widely spread cells [7]. Another thing is contamination by microbial agents e.g. bacteria. Antibiotics are used to minimize the risk of these contaminants.

The use of growth factor supplements and more defined media has also highlighted the role of the substrate to which the cells are attached in regulating growth and differentiated function [6]. These attachment factors, such as fibronectin or collagen, are part of the complex *in vitro* environment in which a cell normally functions [6].

Primary cells are usually more fragile than established cell lines. This is due to the fact that they have to be extracted from the tissue by digestion. The choice of enzymes and the length of extraction process may have a significant impact on viability of the cancer cells [15-17]. Poorly executed cell culture procedures such as trypsynization can damage cell membrans.

The technique of primary cancer cultures has not often been mentioned in the literature. *In vitro* models based on primary cultures of human lung cancer cells are difficult to

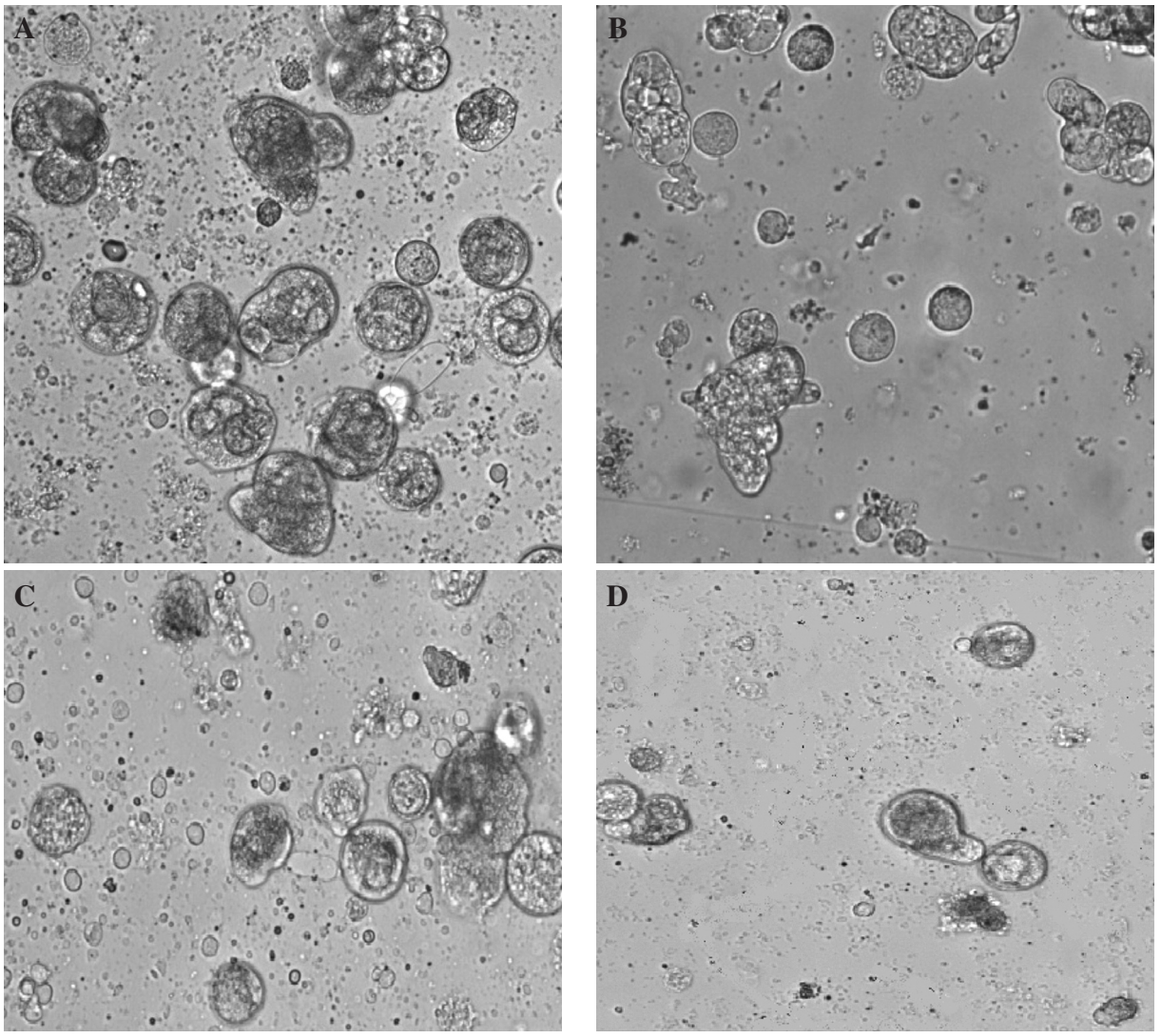

Fig. 1. Representative picture of culture cells from each surfaces tested. **A.** Cells cultured on CC2; **B.** Cells from BD BioCoat surfaces; **C.** Cells from BD 354140 plates; **D.** Cells cultured on Nalge Nunc.

develop [8]. In response to the difficulty of obtaining primary cell cultures, scientists developed the technique of obtaining cell lines. It turned out very quickly that these cell lines do not always provide the proper genotype needed for the disease under study [8].

Stevenson and Gazdar reported that it is easier to establish a cell line from the metastatic deposit than from the primary lung cancer [18]. Liu and Tsao found that establishment of cell lines from primary lung carcinoma is unpredictable and the success rate is low. In their experiment only in 8 out of 29 tumors it was possible to form cell lines [4]. Bepler *et al*. were able to derive only one line from 44 primary lung tumors [19].

The aim of the Gazdar and Oie study was the development a representative panel of lung cancer cell lines. They used these lines to identify antigens, growth factor and responses to cytotoxic therapies. They found out that differentiated tumor cells would have nutritional requirements similar to the surrounding tissue and that differentiated tumor cells had its own unique growth requirements [9]. This seems to prove that many cancer experiments cannot be based on line cells models because they are often obtained from metastatic localisations which are located in tissues requiring completely different conditions for growth and proliferation.

Results similar to our experience, were obtained by Scholz and coworkers [8]. They examined the growth of several cell types on different surfaces (e.g. CC2, polystyrene, permanox or glass). Scholz suggested that the CC2 substrate may induce changes in cell behavior, such

Table 1. The average value of the number of cells from each day for all types of surfaces calculated total for all patient

	CC2	BD BioCoat	Nalge Nunc	BD 354 104
Day		**mean value ±SD**		
0	24.67 ±32.24 *n* = 120	26.63 ±32.59 *n* = 120	20.92 ±30.31 *n* = 120	26.71 ±31.57 *n* = 120
2	19.58 ±30.31 *n* = 120	21.71 ±30.51 *n* = 120	15.04 ±21.71 *n* = 120	19.63 ±26.20 *n* = 120
4	32.60 ±40.81 *n* = 120	24.85 ±32.96 *n* = 120	16.35 ±20.14 *n* = 120	23.80 ±31.60 *n* = 120
6	40.58 ±46.22 *n* = 120	30.92 ±36.17 *n* = 120	16.58 ±25.25 *n* = 120	28.29 ±36.36 *n* = 120

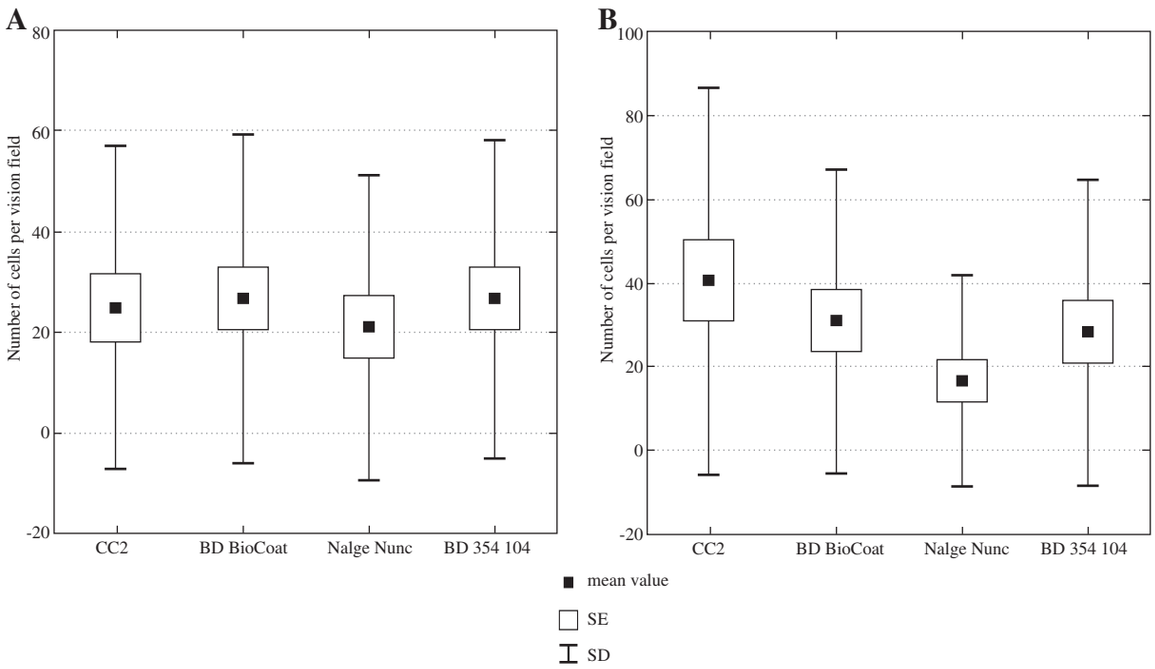

Fig. 2. A. Comparison of the number of cells observed in the test vessels on assumptions culture; **B.** Comparison of the number of cells observed in the test vessels on the last day of culture.

as differentiation. He also stated that many cells prefer culture plates with high surface energies [8].

The ability of CAM-coated to support the growth of cells from human tumour biopsies was evaluated by Price *et al*. [20]. CAM "Cell-Adhesive-Matrix" is a matrix of protein plus 50-70% fibronectin and fibrinogen. Successful growth was obtained in 41%. The data of this study showed that CAM was not better for establishing tumor cells growth.

Kleinman [21] analyzed the role of collagenous matrices in the adhesion and growth of cells. He reported that collagen gels improved cell growth in many cases. Collagen substrates enhance the growth as well as the differentiation of many cells in culture observed with other substrates such as plastic and glass [21].

The ability of tumor cells to proliferate continuously *in vitro* is also determined by their ability to respond to a specific set of autocrine and paracrine growth factors or growth inhibitors [3, 4]. Siegfried demonstrated that many kinds of tumor cells in primary culture require specific growth factors. The results suggest that EGF and TNF-α are important in NSCLC cell growth [17, 22, 23].

Continuing studies on cell cultures may allow to understand cancer biology and lead to development of new therapeutic approaches.

Conclusions

Based on our experience and the available literature on this subject we can draw the following conclusions:

1. The kind of culture plates has a significant impact on the growth and proliferation of cultured lung cancer cells.
2. Different cells require a suitable surface for attachment.
3. Cell growth in culture is a complex mechanism which depends on many factors.
4. CC2 modified soda lime glass provides a good surface for primary lung tumor cells growth and proliferation.
5. The borosilicate coated surface does not support lung tumor cells growth.

References

1. Collins LG, Haines C, Perkel R, Enck RE (2007): Lung cancer: diagnosis and management. Am Fam Physician 75: 56-63.
2. Radzikowska E, Głaz P, Roszkowski K (2002): Lung cancer in women: age, smoking, histology, performance status, stage, initial treatment and survival. Population based study of 20 561 cases. Ann Oncol 13: 1087-1093.
3. Moody TW (1996): Peptides and growth factors in non-small cell lung cancer. Elsevier Science Inc 17: 545-555.
4. Liu C, Tsao MS (1993): Proto-oncogene and growth factor/receptor expression in the establishment of primary human non-small cell lung carcinoma cell lines. Am J Pathol 142: 413-423.
5. Wroblewski JM, Bixby DL, Borowski C, Yannelli JR (2001): Characterization of human non-small cell lung cancer (NSCLC) cell lines expression of MHC, co-stimulatory molecules and tumor-associated antigens. Lung Cancer 33: 181-194.
6. Mather JP, Roberts PE (1998): Introduction. In: Introduction to cell and tissue culture. Theory and Technique. Plenum Press, New York; 1-8.
7. Peehl DM (2005): Primary cell cultures as models of prostate cancer development. Endocr Relat Cancer 12: 19-47.
8. Scholz WK (2008): Bulletin. Cell Adhesion and Growth on Coated Or Modified Glass Or Plastic Surfaces. Nalge Nunc International No. 13.
9. Gazdar AF, Oie HK (1986): Cell culture methods for human lung cancer. Cancer Genet Cytogenet 19: 5-10.
10. Daniel VC, Marchionni L, Hierman JS et al. (2009): A primary xenograft model of small-cell lung cancer reveals irreversible changes in gene expression imposed by culture in vitro. Cancer Res 69: 3364-3373.
11. Baker FL, Spitzer G, Ajani JA et al. (1986): Drug and radiation sensitivity measurements of successful primary monolayer culturing of human tumor cell using cell-adhesive matrix and supplemented medium. Cancer Res 46: 1263-1274.
12. Hynes RO (1999): The dynamic dialogue between cells and matrices: implications of fibronectin's elasticity. Proc Natl Acad Sci USA 96: 2588-2590.
13. Siegfried JM, Owens SE (1988): Response of primary human lung carcinomas to autocrine growth factors produced by a lung carcinoma cell line. Cancer Res 48: 4976-4981.
14. Margarit G, Belda J, Casan P, Sanchis J (2005): Expansion of primary bronchial epithelial cell culture. Arch Bronconeumol 41: 524-527.
15. Duchesne GM, Eady JJ, Peacock JH, Pera MF (1987): A panel of human lung carcinoma lines: establishment, properties and common characteristics. Br J Cancer 56: 287-293.
16. Loh PM, Clamon GH, Robinson RA et al. (1984): Establishment and characterization of four new human non-small cell lung cancer cell lines. Cancer Res 44: 3561-3569.
17. Siegfried JM (1987): Detection of human lung epithelial cell growth factors produced by a lung carcinoma cell line: use in culture of primary solid lung tumors. Cancer Res 47: 2903-2910.
18. Stevenson H, Gazdar AF, Phelps R et al. (1990): Tumor cell lines established in vitro: an independent prognostic factor for survival in non-small cell lung cancer. Ann Intern Med 113: 764-770.
19. Bepler G, Koehler A, Kiefer P et al. (1988): Characterization of the state of differentiation of six newly established human non-small-cell lung cancer cell lines. Differentiation 37: 158-171.
20. Price P, Bush C, Parkins CS et al. (1991): Evaluation of cell attachment matrix (CAM) coated plates for primary culture of human tumor biopsies. Radiother Oncol 21: 282-285.
21. Kleinman HK, Klebe RJ, Martin GR (1981): Role of collagenous matrices in the adhesion and growth of cells. J Cell Biol 88: 473-485.
22. Piyathilake CJ, Frost AR, Weiss H, et al. (2000): The Expression of Ep-CAM (17-1A) in squamous cell cancers of the lung. Hum Pathol 31: 482-487.
23. Fisher ER, Paulson JD (1978): A new in vitro cell line established from human large cell variant of oat cell lung cancer. Cancer Res 38: 3830-3835.