COMPARATIVE CHARACTERISTIC OF LUNG CANCER STEM-LIKE CELLS GENERATED *IN VITRO* UNDER DIFFERENT CULTURE CONDITIONS

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Cancer stem cells (CSCs) play an important role in resistance to cancer treatment and recurrence developing. The aim of this study was to obtain cell culture of MOR line non-small cell lung cancer cells enriched in CSCs and to investigate its functional and molecular-genetic properties. Tumor spheroids (TS) of MOR cell line were generated in vitro under normal adhesive (0.2% carboxymethyl cellulose, CMC) or low-adhesive (2% agarose) culture conditions. Lateral population of TS was evaluated by flow cytometry with the use of R-123 fluorescent dye, the index of R-123 exclusion was also assessed. Expression of CD44, ALDHA1, CD133, SOX2 and NANOG mRNA was determined with RT-qPCR. It was found that regardless of the culture conditions tumor spheroids form a lateral population characterized by an increased dye exclusion index. Expression levels of CD44, ALDHA1, CD133, SOX2 and Nanog mRNA in TS cells obtained under low-adhesive (2% agarose) conditions were significantly higher than in monolayer cells and cells obtained using 0.2% CMC. Thus, the proposed method of culturing in low-adhesive conditions allowed to enrich significantly tumor spheroids of MOR line in cells with CSC properties.

K e y w o r d s: cancer stem cells, non-small cell lung cancer, spheroids, MOR cell line, CD44, ALDHA1, CD133, SOX2, NANOG.

According to WHO more than 2 million new lung cancer (LC) are detected in the world. Moreover, 61.1% of first time cancer identified patients did not live more than 1 year [1]. High lung cancer morbidity is observed in Ukraine, namely almost 12.54 thousand of new cases were detected in 2018 while at the same time 9.53 thousand patients were died according to the bulletin of National Chancer-Registry of Ukraine [2].

Combined approaches are considered to be the "standard" of treatment for patients with LC that include radical surgery, chemotherapy and radiation therapy. However, the recurrences and metastases may occur in 30-50% of patients including early stages [3]. The risk of recurrences and metastases after the combined treatment mainly determined

with population of cancer stem cells (CSCs) that are resistant to radiation and chemotherapy [4]. CSCs make up less than 1% of all tumor cells, but are able to significantly affect the disease course [5]. CSC biology has widely investigated in the most common malignancies past 20 years. The CSC exceptional plasticity and the complexity of the processes are promoting the development of new methods of their purification and approaches for "therapy" targeted to CSCs [6].

According to CSC theory only population of these cells has tumorogenic properties and is capable to support tumor growth and tumor metastasis [7]. Considering the processes associated with the properties of CSC, it becomes relevant the development of new therapeutic treatment strategies, name-

ly: stimulation of CSC differentiation, destruction of physiological niches, inhibition of migration ability and CSC chemoresistance, normalization of the immune response and the development of methods for personalized anti-CSC immunotherapy. The anti-CSC therapy is a new, promising strategy in oncology as it allows us to move away from old, established perceptions of the cancer pathogenesis. Investigation mRNA expression of the stemness genes and functional properties of CSCs in tumor cell lines may serve as a laboratory model for finding the effectiveness of new treatments.

Aim. To obtain MOR non-small cell lung cancer (NSCLC) cell culture enriched CSCs and to investigate its functional properties and expression of the stemness genes.

Materials and Methods

Cultural works. We used MOR non-small cell lung cancer cell line (ECACC, UK). Cells in the amount of 1.35×10^6 were placed in T25 culture flasks (TPP, Switzerland) in complete culture medium, which included DMEM (Gibco, USA), 10% ETC (Gibco, USA), 2 mM L-glutamine (Gibco, USA) and a mixture of 100 U penicillin and 0.1 mg/ml streptomycin (Gibco, USA). Cells were cultured in a CO_2 incubator at 37°C and 5% CO_2 atmosphere, cell culture was checked daily for growth patterns, confluence and color change of the culture medium. When the medium was changed to yellow, 75% culture medium was replaced.

Generation of tumor spheroids. TS were obtained in two ways. In the first way, 0.2% carboxymethylcellulose (CMC) was used. For this purpose, MOR tumor cell line in the amount of 1.35×10^6 was introduced into T25 culture vials in complete culture medium with the addition of 0.2% CMC (Sigma, USA) [8]. In the second way, a culture plate with a standard adhesive surface was pre-coated with a 2% agarose solution (AppliChem, Germany) prepared on distilled water [9]. Cells were cultured in a CO_2 incubator at 37°C and 5% CO_2 atmosphere cell culture was checked daily for growth patterns and formation of spheroids that were in the thickness of the culture medium. Cytometric and molecular-genetic analysis were performed on the 6th day of cultivation.

Flow cytometric studies. To investigate the side population (SP) of CSC, cells in the amount of 3×10^6 were added to 2 ml of Rhodamine 123 (R-123) (100 ng/ml) solution (Sigma, USA), incubated for 30 min at 37°C. 4 ml of cold DPBS was added to the

suspension of cells with R-123, the cells were centrifuged at 1000 rpm for 5 min. The cell pellet was resuspended in 0.5 ml of DPBS and left for 30 min at 37 °C. The residual fluorescence of cells with R-123 was determined by flow cytometry, the activation of the dye was performed by a laser with 488 nm wavelength, the fluorescence was determined using a 530/30 nm filter [10].

The level of cell line resistance was characterized using the R-123 exclusion index (REI). REI values were defined as R-123 mean fluorescence value in the tumor cells suspension to the fluorescence intensity of cells that excluded R-123. Samples were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, USA) equipped with two lasers (488 and 625 nm) using CellQuest-PRO for Mac computers.

Quantitative polymerase-chain reaction (qPCR). Total RNA from TS was isolated using "Ribo-prep" reagents (Amplisens, Russia) according to the manufacturer's protocol. Reverse transcription reaction was performed with "Reverta-L100" assay kit (Amplisens, Russia) according to the manufacturer's protocol. Gene mRNA expression was determined by qPCR with real-time detection on 7500 Real-Time PCR Systems (Applied Biosystems, USA) using specific primers for *CD44*, *ALDH1*, *CD133*, *SOX2* and *NANOG* genes and fluorochrome SYBRGreen (Termo Scientific, USA). The primer and probe sequences were selected using Primer Express® Software v3.0 and synthesized by Applied Biosystems (USA). Glyceraldehyde-3-phosphate dehydrogenase (GADPH) was used to normalize levels of mRNA for the relative quantification method of analysis. Forward and revers primers were used at 5 pM concentrations. Reaction mixture (25 µl) was contained 0.25 µl of each primer, 12.5 µl Maxima SYBR Green/ROX qPCR Master Mix (Applied Byosystems, USA), 7 µl deionized H_2O and 5 ng of cDNA. 45 cycles real-time PCR (94 °C – 15 sec, 60°C – 15 sec and 72°C – 30 sec) were run on 7300/7500 Real-Time PCR Systems, "Applied Biosystems", USA. Calculations were performed using the $2^{-\Delta Ct}$ relative quantification method. mRNA expression value was calculated by the formula:

$$x = 2^{-\Delta Ct},$$

where x – mRNA expression value, $\Delta Ct = Ct$ (GAPDH) − Ct (target gene).

Statistical analysis. Gaussian distribution of the group was checked with Shapiro-Wilk test. Statistical analysis included Mean ± SE for Gaussian dis-

tribution and Median ± Percentiles (Q_1 and Q_3) for non-parametric data. To compare the data in three groups, we used One-way ANOVA with Tukey post-hoc test for Gaussian distribution and Kruskal-Wallis test for nonparametric ones. Null-hypothesis of variables equality was rejected when $P < 0.05$. Statistical analysis was performed using Statistica 10.0 software package (Stasoft Inc., USA).

Results and Discussion

The small number of SCs in the tumors causes the problem of finding their specific markers. With the development of flow cytometric approaches, it has become possible to identify specific markers of CSCs, namely CD44, CD133, OCT4, SOX2, NANOG, ALDH1, CXCL12 [11], which allow isolating and characterizing these cells. Spheroid cell culture is used as a method of CSCs enrichment, which is based on their ability to grow without attachment to the substrate. Some researchers use the multicellular spheroid culture to enrich and maintain the CSC subpopulations for different cancer types [12]. Spheroid-forming cells feature stem-like properties and express CSC markers [13]. Cancer cell sources for the formation of TS may be different, but the general procedure for enriching CSC in vitro based on the unique ability of SC to survive and grow in spherical structures with limited nutrient intake conditions. So tumor cells do not receive enough nutrients and some of them will be dying, but stem-like cells feel adverse conditions and multiply by asymmetric division with production of growth factors. The main feature of such TS is the enrichment of tumor population with cells that have the characteristics of CSC [14].

We conducted a series of *in vitro* experiments using MOR line of NSCLC (ECACC, UK). MOR is adhesive tumor cells and able to form PS on the 6 culture day in the 0.2% CMC or 2% agarose conditions. The cells formed aggregates of irregular shape which varied greatly in size after culture in 2 % agarose. The diameter of the largest aggregates was 30-100 μm in 0.2% CMC culture and 60-140 μm in 2% agarose culture on the 6 culture day. One of the CSC detection methods is a functional test that allows evaluating the exclusion efficiency of fluorescent dyes from cells, such as R-123 and Hoechst 33342 [15]. CSCs have ability to exclude these dyes due to the high expression of the cell membrane transporters, including various proteins of the ABC family. The CSC population with excluded

R-123 is called SP. For a number of malignant tumors, CSCs in SP is characterized by high tumorigenic activity in immunodeficient mice transplantation, the ability to self-renew, differentiate and reproduce morpho-functional heterogeneity of the tumor. Therefore, one of the study tasks was to identify SP of the CSCs and determine their content using a functional test that characterizes the activity of transporter proteins.

Our studies have shown that TS generation method statistically significantly affected the SP cell amount (F = 4.74, $P = 0.022$; Fig. 1 and Fig. 2). According to Tukey's HSD test, the using of 0.2% CMC led to a statistically significant increasing in 2.18 times the number of SP cells compared to cells in monolayer ($P = 0.03$; Fig. 2). Number of SP cells obtained in 2% agarose conditions also increased 1.64 times compared to adhesive cells.

The average fluorescence level (GeoMean) in SP cells did not change significantly in all groups whatever from the cell culturing conditions. Analysis of REI showed that different culture conditions (adhesive cells, 0.2% CMC or 2% agarose) statistically significantly influence on REI in MOR cells (F = 5.27, $P = 0.017$). Moreover, after applying of Tukey's HSD test we found a statistical difference of REI between SP obtained with 0.2% CMC and 2% agarose ($P = 0.007$). Therefore, MOR cells in SP characterized by an increasing of REI on the 6 culture day regardless of the culture conditions (by adding 0.2% CMC or 2% agarose).

The next task was to determine the effect of different *in vitro* culture conditions on mRNA expression of the stemness genes of MOR cells. Namely, the expression of *CD44* and *CD133* surface markers, *NANOG* and *SOX2* transcription factors and *ALDH1* enzyme were studied in MOR cell TS (Fig. 3 and Fig. 4).

CD44 is an adhesive protein that participates in cell-cell and cell-intercellular matrix interactions through to the hyaluronic acid. CD44 molecule is also involved in lymphocyte recirculation, lymphocyte activation, myelopoiesis, lymphopoiesis, and angiogenesis [16]. CD44 is considered a CSC marker in some solid tumors, including breast, pancreas, head and neck, hepatocellular liver cancer, non-small cell lung cancer and colon cancer [17]. CD44+ colon CSCs show greater tumorigenicity and the ability to form colonies *in vitro* compared to CD44− colon CSCs. CD133+ adenocarcinoma cells have been shown to be resistant to cisplatin and
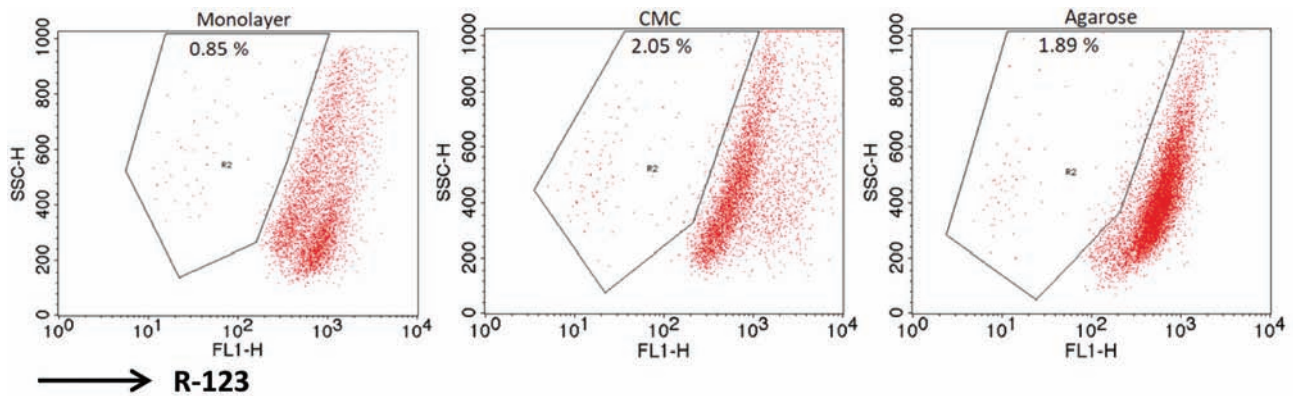
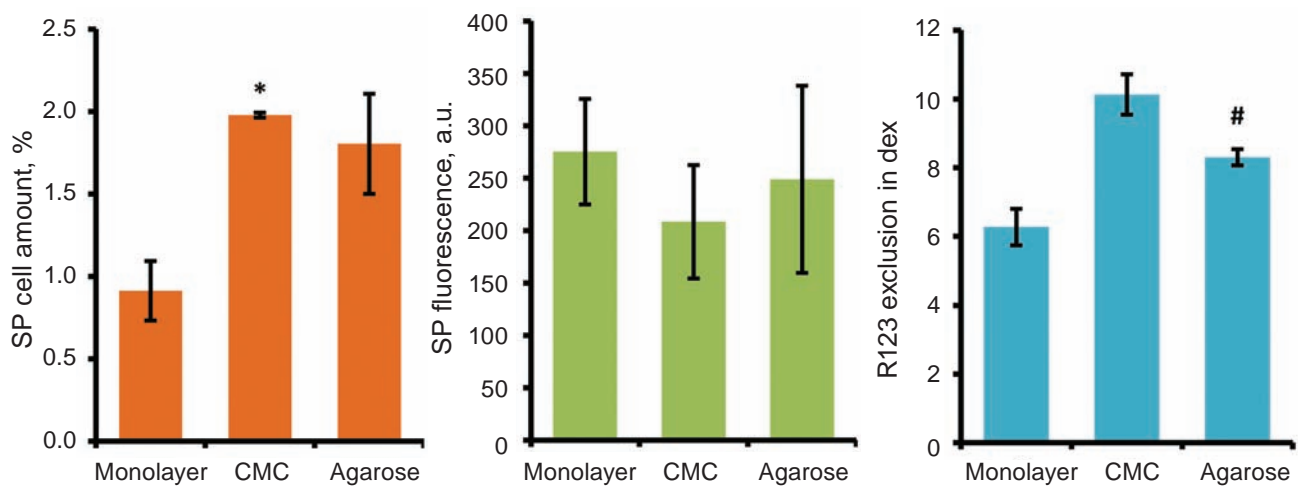*Fig. 1. The SP cells after R-123 exclusion by MOR cells obtained under different conditions*

*Fig. 2. The exclusion effect of the fluorescent dye R-123 by SP cells of MOR cell line (n = 10); *P < 0.05 compared to the value in the monolayer group; #P < 0.05 – compared to the value in cells under 0.2 % CMC*

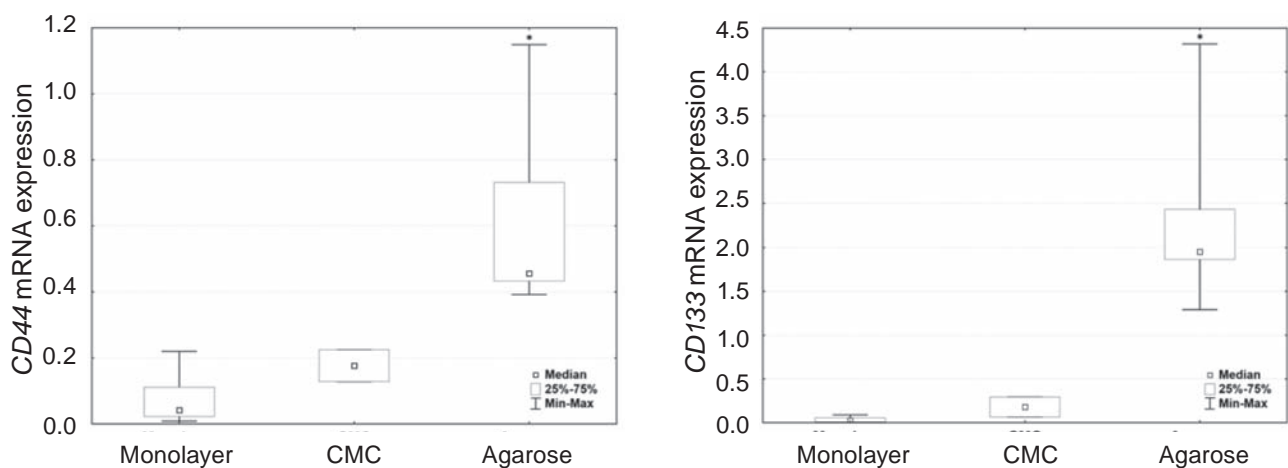*Fig. 3. The mRNA expression of CD44 and CD133 surface markers in MOR cells cultured in different conditions (n = 10); *P < 0.05 – compared to monolayer MOR cells*
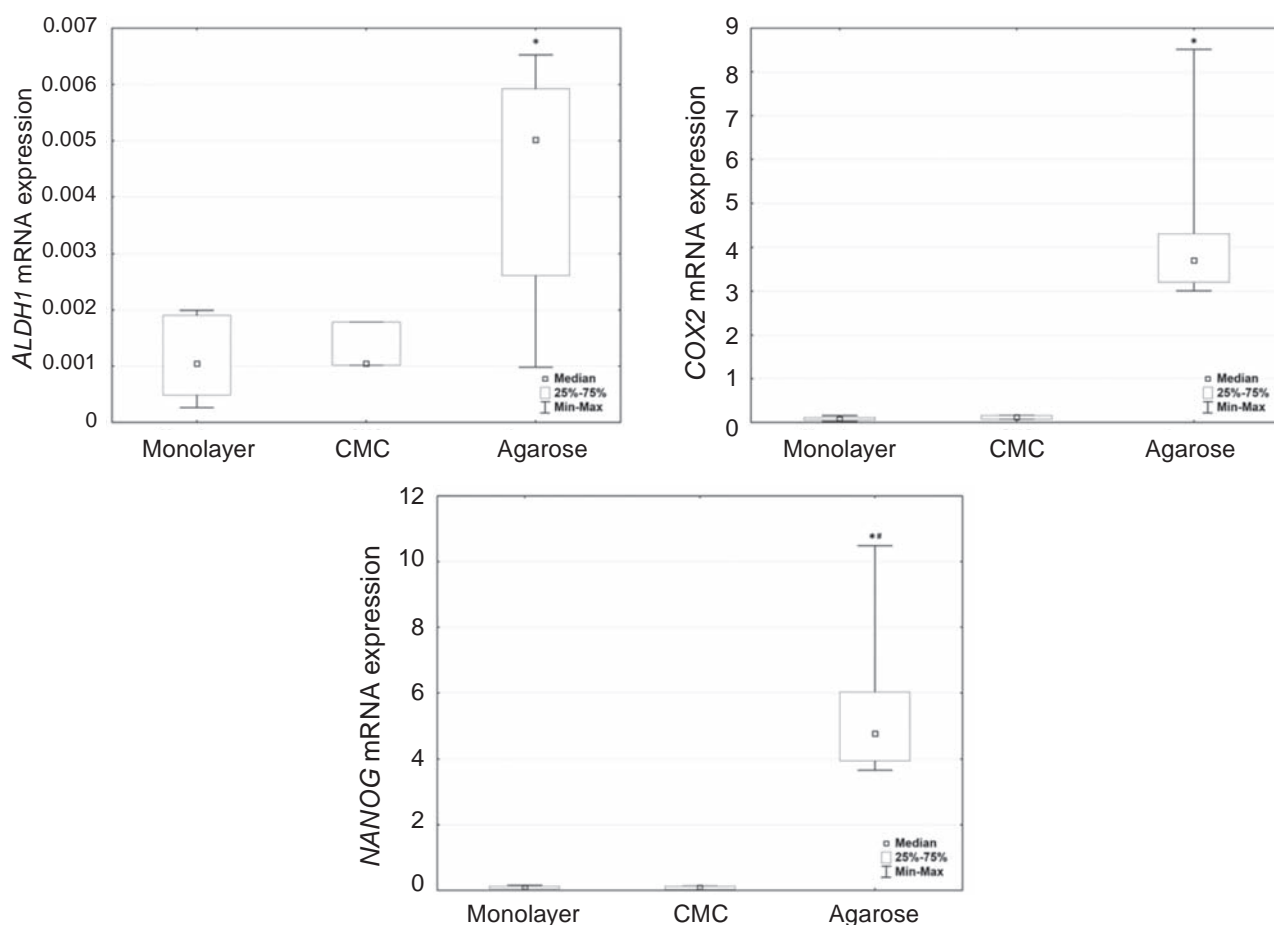
Fig. 4. ALDH1 and transcription factors mRNA expression in MOR cells cultured under different conditions (n = 10); * P < 0.05 – compared to mRNA expression value in monolayer cells; #P < 0.05 – compared to mRNA expression value in cells under 0.2% CMC

ALDH1+ cells to paclitaxel and taxol. The results of our studies showed that MOR cell TS obtained in low-adhesion conditions (2% agarose) the level of *CD44* mRNA expression was in 8.3 times and 3.3 times higher compared to adhesion tumor cells and cells obtained when using 0.2% CMC respectively (H =15.08, *P* = 0.0005; Fig. 3). It was also found that *CD133* mRNA expression level was 69.9 times and 12.7 times higher in MOR cell TS obtained under low-adhesion culturing conditions compared to adhesive tumor cells, and TS cells obtained using 0.2% CMC (H = 15.75, *P* = 0.0004). Thus, *CD44* and *CD133* markers mRNA expression levels in the MOR line TS obtained under low-adhesion culture conditions were significantly higher compared to the values in the cells obtained by other culture methods. The obtained results coincide with the data of the authors who studied the lung cancer CSCs and their characteristics. Same data was obtained Leung

E.L. et al., the subpopulation of CD44+ NSCLC cells were capable of spheroid body formation and *in vivo* tumor initiation [18]. Hardavella G. et al. showed that co-expression of CD90 further narrowed down the stem cell population as spheroid-forming cells were mainly found within the CD44+ CD90+ subpopulation with expression of mesenchymal markers N-Cadherin and Vimentin, increased mRNA levels of the embryonic stem cell related genes *NANOG* and *OCT4* and increased resistance to irradiation, therefore suggesting the CD44+ CD90+ population as a good candidate for the lung CSCs [19].

The next studied markers were SOX2 and NANOG transcription factors and ALDH1 intracellular enzyme. ALDH1 is a detoxification enzyme that oxidizes intracellular aldehydes and converts retinol into a retinoic acid. Hyperexpression of A*LDH1* leads to increased proliferation provides resistance to alkylating agents and protection of stem cells from

oxidative stress and promotes their existence [20]. ALDH1A1 is one of ALDH 19 isoforms expressed in humans and is considered a specific marker for the identification, isolation, and monitoring of colon CSCs, however, according to some data, ALDH1 is also a marker of common SCs in various tissues [21]. The association of ALDH1 high levels with poor prognosis and metastases in breast cancer patients was noted [22].

Our data showed that mRNA expression level of ALDH1 enzyme in MOR cell TS obtained under low-adhesion culture conditions (2% agarose) increased in 3.95 times and 3.34 times compared to monolayer and TS obtained under 0.2% CMC culture conditions respectively (H = 6.62, *P* = 0.04; Fig. 4).

In 1994, *SOX2* gene as a member of *SOX* family was also found in humans. It is located on 3q26.3-q27 chromosome and encodes a protein consisting of 317 amino acids. *SOX2* plays a key role in supporting stem cell populations, determining their death and the necessary factor for reprogramming of somatic cell pluripotency [23].

SOX2 is involved in the development of a large number of malignant neoplasms types and refers to the marker of CSC. It has been established that the presence of *SOX2* serves as a prognostic marker in esophageal and lung cancers [24]. The *SOX2* high expression is associated with a poor prognosis, short progression free period, low-degree of differentiation, lymph node involvement, T3-T4 stages and distant metastases in colon cancer patients [25]. Therefore, the results of our studies showed that *SOX2* mRNA expression level in TS obtained in low-adhesion conditions (2% agarose) much higher compared to adhesive tumor cells and TS obtained by culture with 0.2% CMC (H = 13.47, *P* = 0.0012; Fig. 4).

It is known that NANOG transcription factor controls the maintenance of the cell pluripotent state and their ability to self-reproduce. NANOG high expression level stimulates tumor growth and metastasis of breast cancer cells [26]. Our studies showed that the using of low-adhesion culture conditions (2% agarose) statistically significant increased *NANOG* mRNA expression level in TS compared to monolayer and 0.2% CMC culture conditions (H = 11.45, *P* = 0.0033; Fig. 3).

According to the Wefers C. et al., the use of DC vaccines loaded with NANOG-specific anti-gens, which were obtained from TS of tumor lines is a very promising area of immunotherapy for cancer patients [27].

Therefore, the results of our studies showed that *CD44, ALDH1, CD133, SOX2* and *NANOG* mRNA expression levels in TS of MOR line obtained in low-adhesion conditions (2% agarose) significantly exceed the following values in monolayer cells and cells obtained using 0.2% CMC which are typical for CSC. This approach allows maximally enriching the cell lines with CSC and then using them for antitumor immunotherapy.

It should also be noted that obtaining spheroids from tumor cell lines in vitro is a complex technological process that requires the use of high-cost reagents. As a result of our research, it was found that the use of 2% agarose allows to partially replace special vials with low-adhesion properties, which are quite expensive, which can significantly reduce the cost of cultural work.

In summary, it can be noted that an important aspect in the study of the biology of CSCs is their special metabolic properties. Cultivation method which allows to enrich the cell population with stem-like cells, will further allow to isolate this CSCs and more detail describe them. Molecular genetic markers not only identify CSCs, but are also important for the manifestation of their properties, as they are associated with the activation of genes and signaling pathways that regulate their stemness, and therefore they are often considered as potential targets for targeted therapy. In turn, a deeper understanding of the molecular and biological characteristics of these cells will make a significant contribution to solving the problem of cancer therapy.

ПОРІВНЯЛЬНА ХАРАКТЕРИСТИКА СТОВБУРОПОДІБНИХ КЛІТИН РАКУ ЛЕГЕНЬ ЗА РІЗНИХ УМОВ КУЛЬТИВУВАННЯ *IN VITRO*

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Ракові стовбурові клітини (CSCs) відіграють важливу роль у стійкості за лікування раку та розвитку рецидивів. Мета роботи – отримати збагачену на CSCs культуру клітин лінії MOR недрібноклітинного раку легені та дослідити її функціональні та молекулярно-генетичні властивості. Пухлинні сфероїди (ПС) лінії MOR генерували *in vitro* в стандартних (з додаванням 0,2% карбоксиметилцелюлози, СМС) або низькоадгезивних (з 2-им% розчином агарози) умовах культивування. Бокові популяції ПС досліджували методом протокової цитофлуориметрії з використанням флуоресцентного барвника R-123, також оцінювали індекс виключення R-123 із клітин. Рівень експресії мРНК генів *CD44, ALDHA1, CD133, SOX2* та *Nanog* визначали методом кількісної ПЛР у режимі реального часу. Встановлено, що незалежно від умов культивування ПС утворювали бокову популяцію зі збільшеним індексом виключення R-123. Виявлено, що рівень експресії мРНК *CD44, ALDHA1, CD133, SOX*2 та *NANOG* у ПС лінії MOR, отриманих за низькоадгезивних умов (2% агарози) вірогідно перевищує відповідні показники в клітинах моношару та клітинах, отриманих за використання 0,2% СМС. Отже, запропонований метод культивування ПС недрібноклітинного раку легені MOR у низькоадгезивному середовищі дозволяє досягти максимального збагачення на клітини, що мають властивості CSC.

К л ю ч о в і   с л о в а: стовбурові пухлинні клітини, пухлинні сфероїди, лінія MOR, *CD44, ALDHA1, CD133, SOX2, NANOG.*

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