

Phenotypic heterogeneity in the NCI-H125 cell line affects biological activity using the epidermal growth factor receptor as target

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We evaluated the influence of some morphological changes of the NCI-H125 cell line in surface expression of the epidermal growth factor receptor (EGFR) and their impact on some biological activity assays using this molecule as target. Hematoxylin and eosin (H/E) staining, light microscopy immunocytochemistry, flow cytometric antibody-receptor binding test, cell viability determination and cell cycle analysis were performed. Phenotypic changes and inconsistency in EGFR expression were detected in NCI-H125 cell cultures. A significant reduction in the growth rate, mainly characterized by cell cycle arrest in the G0-G1 phase, was also evidenced. Differential distribution of cell viability in NCI-H125 subpopulations and its relationship with the EGFR surface expression were determined. Nuclear alterations observed in NCI-H125 were not apoptosis related. A lack of control of cell cultures affects the reliability and reproducibility of biomedical and biotechnological research using EGFR as target. Therefore, rigorous control of the above mentioned parameters in these experiments is recommended.

Keywords: cell viability, morphological changes, biological activity, epidermal growth factor receptor, nimotuzumab

Accepted September 8, 2012

The epidermal growth factor receptor (EGFR) is a 170 kDa membrane-bound protein (1) over-expressed in some human malignancies in comparison with normal tissues. The EGFR is considered an attractive therapeutic target for inhibiting tumor growth and metastatic progression (1–3). Based on these results, several molecules such as inhibitors of tyrosine kinase and monoclonal antibodies are in clinical trials or have records of treatment of epithelial tumors with EGFR over-expression.

Nimotuzumab (h-R3) is a humanized therapeutic anti-EGFR monoclonal antibody that has been granted approval for use in squamous cell carcinoma of head and neck,

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glioma and nasopharyngeal cancer in different countries (4). Since the introduction of nimotuzumab into medical practice, a flow cytometry-based measure has been used as quality control for potency and binding assays, taking an overexpressing EGFR cell line (NCI-H125) as target.

It is known that cell suspensions contain a heterogeneous cell population (viable and dead cells, cells in different phases of the cell cycle and cellular debris). However, using a flow cytometer it is possible to discriminate cells in a heterogeneous cell population based on the detection of their light scattering and fluorescence properties (5). Cell viability determination is usually necessary to discriminate viable and non-viable cells (6). Non-specific binding to dead cells introduces changes in the fluorescence signal (7).

For these reasons, experimental data from any flow cytometry cell-based binding assay might lead to unpredictable results and to misinterpretation if different cell types are included in subsequent analyses. In this work, we evaluated for the first time a variety of phenotypic and physiological changes in the NCI-H125 cell line using consecutive subcultures. Hence, the impact of these alterations in the results of some bioassays taking the EGFR as target was assessed.

EXPERIMENTAL

Monoclonal antibodies

We employed the h-R3 (anti-EGFR) (8) and T1h (anti-CD6) (9) monoclonal antibodies, produced at the Center of Molecular Immunology (Cuba) at a concentration of 5 mg mL⁻¹. The T1h irrelevant Mab was used as negative control.

Cell culture conditions

A human non-small cell lung cancer NCI-H125 (ATCC CRL-5801) cell line was kindly provided by the research direction (Center of Molecular Immunology, Cuba). A skin epidermoid carcinoma A431 (ATCC CRL-1555) cell line was obtained from the American Type Culture Collection (USA). Cells were grown in Dulbecco's modified Eagle's media (Gibco, USA) supplemented with 5 or 10 % heat-inactivated fetal bovine serum (Gibco), respectively. Cells were maintained at 37 °C in a humidified atmosphere of air containing 5 % CO₂ and were harvested using a trypsin-EDTA in dilution (Sigma-Aldrich, USA). Cells were sub-cultured 1:4.

Determination of cell viability by optical microscopy

Cell viability was detected by the trypan blue exclusion assay labeling of NCI-H125 and A431 cell line cultures with increasing passage. Briefly, cells were stained with 0.4 % trypan blue (Aldrich Chemical Company, USA) in phosphate buffered saline (PBS), followed by examination with a hemacytometer under an optical microscope. Cells which excluded the dye were considered viable and the data were expressed as percentage of viable cells by trypan blue exclusion.

Evaluation of cell viability and EGFR surface expression by flow cytometry

Cells (0.25×10^6) of 3 consecutive cell passages or subcultures (SC) were incubated for 30 min with h-R3 and T1h antibodies at 4 °C. Stained cells were washed, pelleted and resuspended with an FACS Flow (Becton-Dickinson, USA). A secondary FITC-anti human antibody (Dako, Denmark) was added for 30 min and cells were washed as described before. Afterward, cells were resuspended with the FACSFlow and stained with propidium iodide (PI) at a final concentration of $4 \mu\text{g mL}^{-1}$. The percentage of positive cells and the mean of fluorescence intensity (MFI) on both viable and non-viable cells were measured using the FACScan flow cytometer (Becton-Dickinson). Cells that were stained red with PI were considered necrotic. Viable cells had a little or no red fluorescence.

Immunocytochemical detection of EGFR

Monolayers of NCI-H125 and A431 cell lines were fixed in cold acetone (Spectrum Chemical MFG, USA) and rehydrated in tris/saline buffer, pH 7.6 for 10 min at room temperature. The cells were then incubated for 1 hour with h-R3 or T1h antibodies. A secondary biotinylated-anti human antibody (Dako) was added followed by the ABC/HRP complex (Dako). The enzymatic activity was visualized with AEC (Dako) and cells were counterstained with methyl green (Dako).

Cell cycle analysis and apoptosis measurement

Cells (0.5×10^6) with different number of passages were incubated for 30 min with cold absolute ethanol (Spectrum Chemical MFG, USA) at 4 °C. Fixed cells were then stained with PI/RNase solution for at least 30 min at room temperature. Eight sub-samples of each subculture were prepared. The percentage of cells in sub-G0-G1, S and G2-M phase was analyzed using a FACScan flow cytometer equipped with a doublet discrimination module (DDM) and CellQuest software (version 2.0). The area under the curve was integrated and then the percentages of each population present were calculated using ModFit LT™ software (version 2.0, Verity Software House, USA). The sub-G0-G1 peak was measured to determine the apoptotic population.

For immunocytochemical detection of apoptosis, the ApopTag plus Peroxidase *in situ* detection kit (Oncor, USA) was used according to the manufacturer instructions.

Statistical analyses

GraphPad Prism 5 software (2007 GraphPad Software Inc., USA) was employed for data analysis. We compared changes in cell size, cell viability, percentage of specific recognition, MFI as well as the percentage of cells in G0-G1, S and G2-M phases of the cell cycle while increasing the number of cell passages. Mann-Whitney U test was used to compare two groups of variables. Kruskal-Wallis followed by Dunn's multiple comparison tests was used when three groups were compared.

RESULTS AND DISCUSSION

Cultured cells are generally homogeneous, with uniform morphology and chemical composition. The number of cells obtained in a culture is frequently not enough for their characterization, freezing and testing. In these cases, subcultures give the opportunity to expand the cell population (10).

NCI-H125 exhibited cell size heterogeneity measured by flow cytometry

In Fig. 1a, two cell subpopulations, identified as R1 and R2, were observed based on the forward scatter light (FSC-H) parameter analysis. Significant differences between R1 and R2 in all subcultures were detected ($p = 0.0079$, Fig. 1c). Also, the FSC-H increased significantly in R1 (from 220.5 to 267.6, $p = 0.0019$) as well as in R2 (from 358.1 to 426.5,

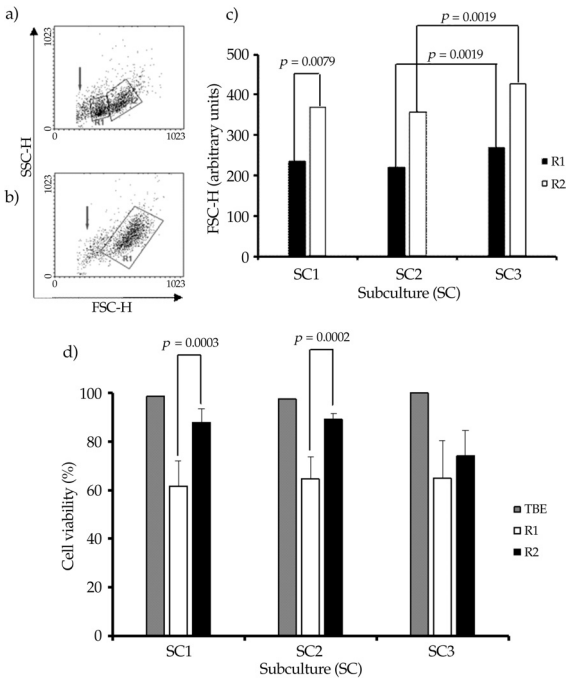


Fig. 1. Size heterogeneity and cell viability distribution in NCI-H125 cell line as a function of cell passage number. a) and b) Flow cytometric dot plots with gates encompassing populations present in NCI-H125 and A431 cell lines, respectively. Cellular debris is represented by arrows. Correlated measurements of FSC (cell-surface area or size) and SSC (cell granularity or internal complexity) permit differentiation of R1 and R2 cell subpopulations in NCI-H125 and their gating to define the characteristics (size) included in further analysis. c) Distribution of R1 and R2 cell subpopulations of NCI-H125 cell line increasing the subculture (SC) number. Statistically significant differences in R1 and R2 were detected. d) Cell viability determinations prior to flow cytometry assay (TBE) and PI staining. Statistically significant differences were observed when R1 and R2 subpopulations were compared in SC1 and SC2. Mean $\pm$ SD, $n \geq 3$.

$p = 0.0019$) in consecutive subcultures. A431 cell line used as control exhibited a homogeneous cell population (Fig. 1b).

NCI-H125 cell line subpopulations show differences in cell viability

No differences in cell viability were observed by optical microscopy using the trypan blue exclusion (TBE) method. Unexpectedly, cells in R1 (cells with apparently no morphological changes) showed low viability using the PI exclusion method. Statistically significant differences for R1 from 61.6 to 64.9 % ($p = 0.0003$) and for R2 from 74.1 to 89.1 % ($p = 0.0002$) in SC1 and SC2 were detected. In R1, it is not easy to distinguish cells from debris using a bivariate plot of FSC-H vs SSC-H (side scatter light) (11). Non-viable cells usually show low FSC-H signals, probably owing to the fact that the refractive index of their cytoplasm is similar to the surrounding medium (7). Therefore, cells in R1 remain merged with debris and non-viable cells from R2. No significant difference in cell viability between R1 and R2 in SC3 was noticed (Fig. 1d). Also, a not statistically significant shift in cell viability was observed in R1 and R2 concerning the cell passage number. A431 cell line, used as control, exhibited no changes in cell viability and a homogeneous cell population (data not shown).

Differences in cell viability affect the percentage of h-R3 Mab positive cells

When the binding of h-R3 Mab in all cells (including both viable and dead cells) in R1 and R2 were compared, as a function of cell passage, statistically significant differences were detected in SC1 and SC2 ($p = 0.0079$ for both). In contrast, when dead cells were excluded from the analysis, the binding of h-R3 was higher than 98.0 % independently of cell subpopulations and cell passage. However, a statistically significant difference between R1 and R2 was observed in SC3 ($p = 0.0317$).

EGFR surface expression decreases as a function of cell passages

The levels of EGFR surface expression were investigated by flow cytometry. In NCI-H125, the MFI diminished significantly with increasing cell passage. When only viable cells were considered in the analysis, statistically significant differences were detected between subcultures for both R1 and R2 ($p = 0.0081$ and $p = 0.0090$, respectively). Similar results were found when all cells were analyzed ($p = 0.0019$ for both R1 and R2). The amount of fluorescent signal detected is proportional to the number of fluorochrome molecules on the cell and, therefore, proportional to the number of EGFR molecules expressed per cell as recognized by h-R3 Mab (11). The decrease in the MFI of EGFR positive cells could result from the drastic declination of EGFR on cell surface. It is known that the number of specific sites per cell varies widely depending on differential ageing of cell lines or growth conditions (12).

On the other hand, no significant differences in MFI were obtained when R1 and R2 were compared in each subculture on both viable and all cells. A431 cell line exhibited no significant difference in both percentages of positive EGFR cells and MFI range in all subcultures (data not shown).

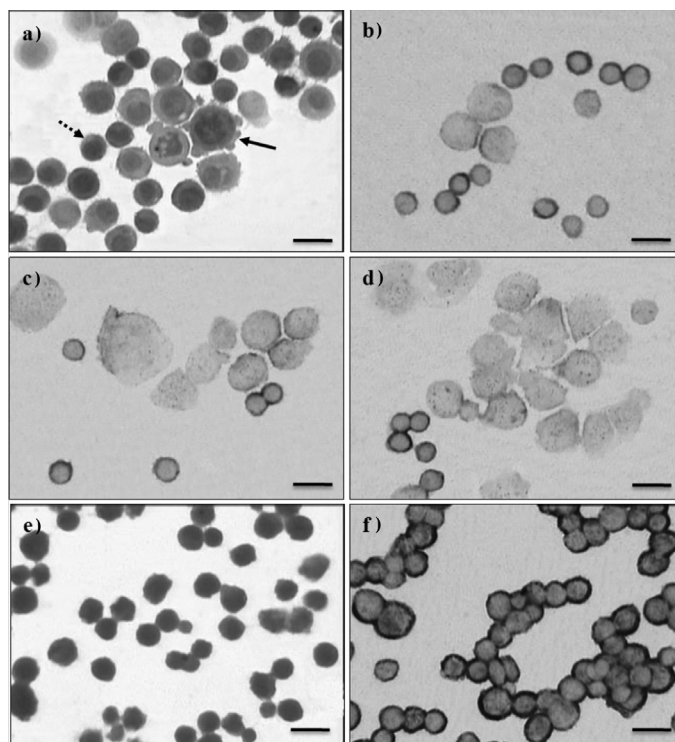


Fig. 2. Hematoxylin and eosin (H/E) staining and light microscopy immunocytochemistry assays as a function of cell passages. a) and e) H/E staining. a) R2 cell subpopulation (continuous arrow) exhibited some morphological changes, such as larger cell size, irregularities on the plasmatic membrane and nuclear alterations. No detectable morphological changes were observed in R1 subpopulation (discontinuous arrow) and in A431 cell line (e) h-R3 immunorecognition in NCI-H125 and A431 cell lines. b)-d) Differences in reaction intensity in R1 and R2 subpopulations were detected. R1: intense, homogeneous and finely granular recognition are located in the entire plasmatic membrane. R2: moderate to weak and negative reactivity of h-R3, located only in zones of the plasmatic membrane. Also, an increase in the R2 cell number in the function of cell passages was evidenced. f) Intense, homogeneous and finely granular immunostaining of h-R3 Mab in A431. Black bar – 20 μ m.

Morphological changes in NCI-H125 were supported by light microscopy analysis

In order to corroborate the heterogeneity detected in NCI-H125 by flow cytometry, both hematoxylin and eosin (H/E) staining and immunocytochemical assays using h-R3 Mab were performed. Figs. 2a and 2e show the H/E staining of NCI-H125 and A431 cell lines, respectively. Similar to flow cytometry measures, two different cell subpopulations in NCI-H125 were detected. The cells with increased FSC-H parameter detected by flow cytometry exhibited some morphological changes, such as larger cell size, irregularities on the plasmatic membrane, nuclear damages and intracytoplasmatic vacuoles

(Fig. 2a). However, no detectable morphological alterations were observed in either the smaller cell subpopulation of NCI-H125 or in the A431 cell line (Fig. 2e).

Expression of EGFR on the cell surface in function of cell passages is shown in Figs. 2b-d. Differences in the reaction intensity in cell subpopulations were evidenced. Two different patterns of immunostaining were observed. A weak, heterogeneous and incomplete finely granular pattern located mainly in the plasmatic membrane and also in the cytoplasm of larger cells was detected. In the small cell fraction as well as in the A431 cell line, intense, homogeneous and complete staining, mainly located in the plasmatic membrane, was obtained by h-R3 Mab (Figs. 2b-d, f, respectively).

The total number of EGFR molecules has been reported to be increased in larger A431 cells (5). Our results in NCI-H125 are in agreement with those obtained in A431. However, we observed some changes in the expression, localization and distribution of EGFR molecules in R2 detected by immunocytochemistry. Cells in R2 displayed larger cell size and MFI probably due to a larger number of EGFR molecules expressed on the cell membrane. The apparently contradictory results obtained by flow cytometry and immunocytochemistry suggest differences in method sensibility. In general, the flow cytometry-based technique is considered more sensitive than conventional immunocytochemical analysis for the detection of small numbers of molecules on cell surface. However, the receptor surface density in each subpopulation and the surface area have not been established (5).

NCI-H125 cell line exhibited growth rate reduction

In normal cultures, high density of cells limits nutrient perfusion and creates local exhaustion of peptide growth factors, leading to cell cycle arrests and decreasing cell proliferation (10). Our cells were harvested in the exponential growth phase and then stained with PI and analyzed by flow cytometry.

Figs. 3a-c show the evaluation of cell cycle phases based on DNA content in the NCI-H125 cell line as a function of cell passage. The histogram showed that DNA distribution increased significantly in the G0-G1 phase. The flow cytometry analysis of the NCI-H125 cell cycle distribution revealed a statistically significant shift ($p < 0.0001$) into the G0-G1 phase in the three subcultures studied (Fig. 3d).

The subculture with the major fraction of cells exhibiting morphological alterations (SC3) had an increase in the G0-G1 fraction from 60.2 to 90.4 % compared to SC1. A statistically significant reduction in the S and G2-M fractions from 23.0 to 4.5 % ($p < 0.0001$) and from 15.8 to 4.0 % ($p = 0.0003$), respectively, was also detected. There were no changes in cell cycle distribution in the A431 cell line subcultures (Fig. 3e).

In a similar study performed previously, no change in the percentage of positive A431 cells in the cell cycle phases depending on the selected subpopulation was evidenced. Furthermore, no substantial variations in the affinity of EGFR for EGF (epidermal growth factor) during the cell cycle have been suggested either (5). In our study, a significant reduction in the number of sites per cell in the function of cell passage number was detected. Reduction in the growth rate could be caused in part by conformational changes on the EGFR surface that lead to a disruption in the mechanism of ligand-induced signal generation, affecting cell proliferation (1).

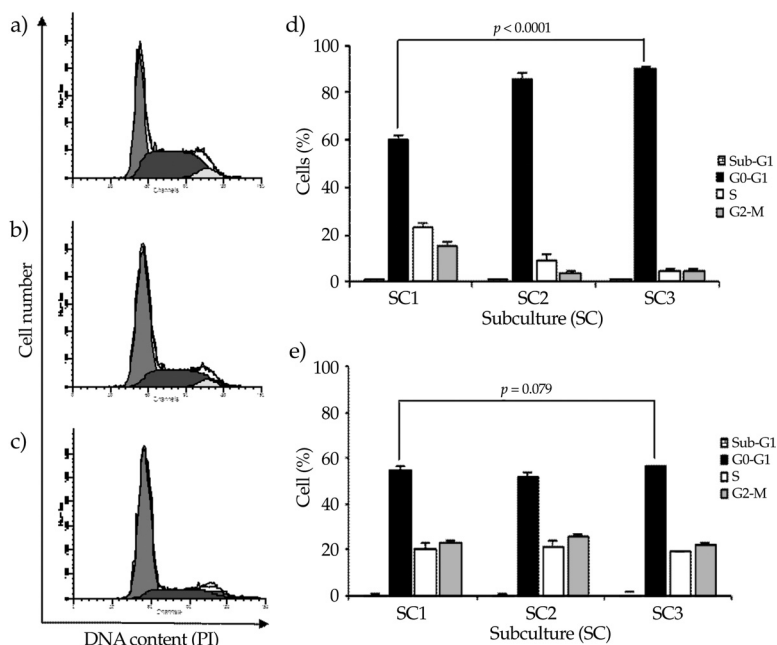


Fig. 3. Flow cytometry analysis of cell cycle phases based on DNA content as a function of the cell passage number. a)-c) Typical DNA graphs (sub-G0-G1, G0-G1, S and G2-M) of NCI-H125 cell line. Reduction of the growth rate mainly characterized by cell cycle arrest in the G0-G1 phase, c) as compared to a) and b) to d) NCI-H125 cell cycle phases of different passage number. Statistically significant reduction in the S and G2-M fractions in NCI-125 cell line was observed. No detectable changes in cell cycle distribution in A431 subcultures were observed. e) No significant sub-G0-G1 phase of cell cycle (apoptotic cells) in NCI-H125 and A431 cell lines was evidenced. Mean $\pm$ SD, $n \geq 3$.

Nuclear alterations of NCI-H125 cell line were not apoptosis related

To further evaluate the number of apoptotic cells in cultures of continuous cell passage, we investigated the DNA cell cycle in consecutive subcultures. Fragmented DNA was observed in a sub-G0-G1 peak in DNA histograms. No significant sub-G0-G1 phase of cell cycle (apoptotic cells) in both NCI-H125 and A431 cell lines in the three subcultures was evidenced (Figs. 3d and e).

We also investigated DNA fragmentation by optical microscopy using the Apoptag system (TUNEL enzymatic modification). No labeled-DNA (apoptotic cells) was detected in the two cell lines studied as a function of cell passage number (data not shown).

In addition, mycoplasma infection of NCI-H125 cell cultures was also detected at the end of experiments (data not shown). The maintenance and use of cell cultures of several cell passages make them susceptible to accidental mycoplasma infection. These microorganisms can virtually affect any measurable cell parameter, without causing obvious phenotypic changes in the infected cells (13). In this way, experiments aimed to

evaluate the possible impact of mycoplasma infection in NCI-H125 cell cultures are in progress.

Cell lines will usually undergo a limited number of subcultures or cell passage numbers. This limit is determined by the number of duplications before the cells stop growing because of senescence. After that, cells accumulate numerous abnormalities and deprivation of specialized functions, leading to the death of cultures (10). The stress of *in vitro* culture conditions has been also considered responsible for the increase in the number of non-viable cells (14). On the other hand, variability of the conventional binding techniques measuring both the number of classes of sites and the number of sites per cell has been previously reported. Such variation has been suggested to be associated with ageing of the cell line as well as with differences in growth conditions (5, 12). Also, impact of the cell passage number on the variability of cell-based biological assays has been recently reported (15).

CONCLUSIONS

In summary, this work offers evidences of the morphological changes in the NCI-H125 culture cell line, characterized by cell size heterogeneity, irregularities on the plasmatic membrane, nuclear damages and intracytoplasmatic vacuoles, as a function of cell passage number. We have also reported a reduction in EGFR surface expression levels and culture growth rate as well as the differential level of EGFR detection, depending on cell viability. These results underline the importance of suitable control of cell culture parameters as well as adequate selection of the region of interest in flow cytometry assays using the EGFR as target.

Acknowledgements. – The authors thank Claudia Calvo for inter-institutional coordination as well as Aymara Nieto Ph. D. and Mr. Antonio Vallin for their critical revision of the manuscript. The authors are also grateful to the Direction of Microbiology of the National Center for Animal and Plant Health (Havana, Cuba) for excellent support to the mycoplasma testing program. This work was supported by the Center of Molecular Immunology, Havana, Cuba.

Acronyms. – ABC/HRP – avidin-biotin-peroxidase complex; AEC – 3-amino-9-ethyl-carbazol; ATCC – American type culture collection; DDM – doublet discrimination module; DNA – deoxyribonucleic acid; EDTA – ethylenediaminetetraacetic acid; EGF – epidermal growth factor; EGFR – epidermal growth factor receptor; FITC – fluorescein isothiocyanate; FSC-H – forward scatter light; H/E – hematoxylin and eosin; Mab – monoclonal antibody; MFI – mean of fluorescence intensity; PBS – phosphate buffered saline; RNase – ribonuclease; PI – propidium iodide; R – region; SC – subculture; SSC-H – side scatter light; TBE – trypan blue exclusion; TUNEL – terminal transferase dUTP nick end labeling.

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S A Ž E T A K

Fenotipska heterogenost u staničnoj liniji NCI-H125 utječe na biološku aktivnost na receptore za epidermalni faktor rasta

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U članku je opisan utjecaj morfoloških promjena na staničnoj liniji NCI-H125 koje su povezane s površinskom ekspresijom receptora za epidermalni faktor rasta (EGFR) i njihov utjecaj na biološko djelovanje. U radu je korišteno bojanje s hematoksilinom i eozinom (H/E), imunocitokemijska mikroskopija, test vezanja protutijela na receptor pomoću protočne citometrije, testovi vijabilnosti stanica i analiza staničnog ciklusa. U staničnoj kulturi NCI-H125 detektirane su promjene fenotipa i nedosljednost u ekspresiji EGFRa. Primijećena je značajna redukcija rasta, uglavnom zbog zadržavanja staničnog ciklusa u G0-G1 fazi, te različitost u vijabilnosti NCI-H125 subpopulacije i povezanost s ekspresijom EGFRa na površini. Primijećena različitost u jezgri NCI-H125 stanica nije povezana s apoptozom. Nemogućnost kontroliranja stanične kulture utječe na pouzdanost i ponovljivost biomedicinskih i biotehnoloških istraživanja koja su usmjerena na EGFR. Stoga se preporuča stroga kontrola svih opisanih parametara.

Ključne riječi: vijabilnost stanice, morfološke promjene, biološko djelovanje, receptori za epidermalni faktor rasta, nimotuzumab

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