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ЭКСПРЕССИЯ ГЕНА *REG1A* В ЭПИТЕЛИОЦИТАХ ДВЕНАДЦАТИПЕРСТНОЙ КИШКИ КРЫС В УСЛОВИЯХ ДЛИТЕЛЬНОЙ ГИПОАЦИДНОСТИ И ПРИ ВВЕДЕНИИ МУЛЬТИПРОБИОТИКА "СИМБИТЕР® АЦИДОФИЛЬНЫЙ"

Выявлено экспрессию гена Reg1a в эпителиоцитах ворсинок и показано увеличение уровня мРНК этого гена в эпителиоцитах крипт двенадцатиперстной кишки в гипоацидных условиях. При введении мультипробиотика "Симбитер® ацидофильный" концентрированный в тех же условиях уровень экспресии Reg1a в эпителиоцитах ворсинок уменьшился в 1,6 раза, в то время как содержание мРНК Reg1a в криптах было на уровне контрольных значений.

Ключевые слова: гипоацидность, двенадцатиперстная кишка, экспрессия генов, мультипробиотики.

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PROMOTER DNA METHYLATION OF E-CADHERIN GENE IN MOLECULAR PATHOGENESIS OF ADHESIVE COMPLEX AND ACTIVATION OF EMT MARKERS *SNAIL* AND *TWIST1* IN MYELOPROLIFERATIVE LEUKEMIA

EMT – the epithelial-to-mesenchimal transduction is the basis platform of the tumor microenvironment. EMT causatively binding with the tumor progression, by which are modulated the migrational, invasive and metastatic potentials of the tumor cells. Therefore, EMT is the crucial microenvironment metastatic niche for the final cancer cell aggressiveness. Moreover, EMT underlie cancer stem cell induction and also controls tumor drug sensitivity modulating the response of cancer cells to chemotherapy. The earlier event of EMT metastatic cascade is the epigenetic deregulation of the E-cadherin-beta-catenin adhesive signaling. The promoter DNA hypermethylation of E-cadherine gene as the key adhesive molecules results in the switch the E-cadherin-beta-catenin adhesive signaling on the canonic beta-catenin-Wnt signaling associated with epithelial-mesenchimal transduction. Thus, the epigenetic regulation of EMT might be considered as the target for a new cancer epigenetic therapy strategies.

Key words: epithelial–mesenchymal transition, E – cadherin, aberrant DNA methylation.

Introduction. The epithelial-mesenchymal transition (EMT) is the key phenomenon in conception of tumor microenvironment [1]. EMT is the tissue-specific mechanism of morphogenesis and organogenesis, which takes place during embryonic development and associated with transformation of the epithelial cells into mesenchymal stem cells [2; 3]. In carcinogenesis EMT is chimerical organogenesis manifested as metastatic tumors. Thus, it is abnormal program leads to migrational, invasive and metastatic properties of tumor cells [4]. These processes implement at different stages of cell differentiation [5; 6]. The violation of epigenetic control in carcinogenesis is associated with aberrant promoter DNA methylation of E – cadherin gene – the key molecule of adhesive complex. This event leads to abnormal activation of EMT in tumor progression [7]. Thus, epigenetic deregulation of E-cadherin-beta-catenin adhesive signaling plays the key role in violation of epigenetic control in myeloproliferative leukemia.

The aim of our study was to investigate the epigenetic regulation of EMT as a result of aberrant DNA methylation of E – cadherin gene and its association with EMT modulating transcription factors *Snail* and *Twist1* in progression of myeloproliferative leukemia, such as chronic and acute leukemia (CML and AML).

Tumor microenvironment is the central factor of carcinogenesis, especially in tumor progression and metastasis [8]. The mesenchimal stem cells (MSCs) are progenitors of tumor stem cells (CSCs) in cancer progression. The morphological heterogeneity is crucial criterion in classification of tumors. Chromosomal aberrations find in different types of tumor cells [9]. Scientists suggested, that aneuploidy might explain genetic instability in tumors with genetic mutations (aneuploidy theory of carcinogenesis, Theodore Bovary, 1914). These changes in chromosomes may occur millions of nucleotides [10]. Even the most advanced program of sequencing of the human genome does not provide definitive answers about the mechanisms of carcinogenesis [11].

The epithelial – mesenchymal transduction activates during carcinogenesis and leads to invasion and metastasis [12]. The EMT is activated by inhibition of the expression of E – cadherin protein [13, 14] and E – cadherin/β – katenin adhesive complex [15, 16]. Thus, mesenchymal phenotype is acquired by epithelial cells [17]. Epigenetic silencing of promoter DNA-methylation of E-cadherin is crucial event on primary stages in cancer [18]. This step results into the switch of the E – cadherine/β – catenin adhesive signaling [14] on the canonic β – catenin/Wnt signaling [19], which associates with epithelial – mesenchimal transduction.

The regulation of E-cadherin promoter occurs in two ways: transcription regulation through inhibition of factors *Snail, Slug, Twist1, Zeb1, Zeb2* in *E-box* region [20; 21] and epigenetic regulation through aberrant promoter methylation of E-cadherin gene [22; 23]. Unrestrained Wnt signaling and/or loss of the cell adhesion occur during progression of cancer [14, 22]. The key event of carcinogenesis is lack of the control over level of β – catenin, which may lead to the destabilization of adhesive complex [19, 24].

Materials and methods. The object of our study was samples of patient's peripheral blood from the National Institute of Cancer. In our work we used such methods as extraction of total DNA and genomic RNA, bisulfate modification of DNA using EpiTect Bisulfite Kit (QIAGEN), methyl – specific PCR of bisulfate modificated DNA and PCR with reverse transcription. In these methods primers to the promoter region of E–cadherine were used, where cytosine was replaced to thymine, which complementary to uracil (5'-TGGTTGTAGTTATGTATTTATTTTTAGTGGTGTT-3'-forward and 5'-ACACCAATACAACAAATC AAACCAAA-3'-reverse); primers to transcription factors *Snail* (560 bp) 5′-CAGACCCACTCAGATGTCAA-3′ (forward) та 5′-CATAGTTAGTCACACCTCGT-3′ (reverse), *Twist1* (130 bp) 5′-CGGGAGTCCGCAGTCTTA-3′ (forward) та 5′- TGAATCTTGCTCAGCTTGTC -3′ (reverse). We used program *TotalLab v. 2.01* for quantity estimation of amplification product.

Results and discussion:

In our work we used four samples of patent peripheral blood.

- Patient with CML (chronic myeloprolipherative lekemia).
- Patient with AML (acute myeloprolipherative lekemia).
- Patient with ET (essential trombocytosis).
- Negative control HD (healthy donor).

Total DNA was extracted from patient peripheral blood leucocytes with myeloproliferative leukemia. These DNA samples were exposed to bisulfate modification. Then methyl – specific PCR (MS-PCR) was conducted. Electrophoregramme of products of methyl – specific PCR for identification of promoter DNA – methylation of E – cadherin gene is demonstrating on Figure 1. The lack of amplification product was observed in patients with CML and AML, in compared to healthy donor. This indicates the presence of the aberrant promoter DNA – methylation of E – cadherin gene in patients with CML and AML.

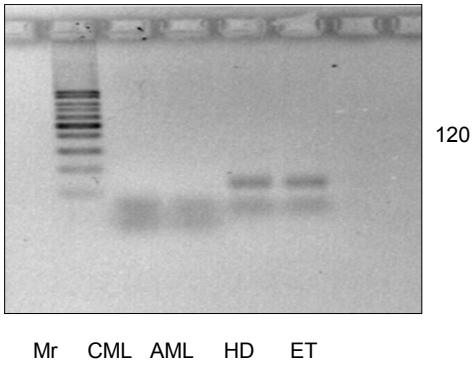

Fig. 1. Electrophoregram of products of methyl – specific PCR for identification of promoter DNA–methylation of E–cadherin gene

Electrophoregramms with amplification products of target genes *Snail* and *Twist1* (A) and the control gene *β – actin* (B) are demonstrated on Figures 2 and 3. The product of amplification was observed in patients with CML, AML and ET, which indicate expression of transcription factors *Snail* and *Twist1* in patients with various forms of leukemia.

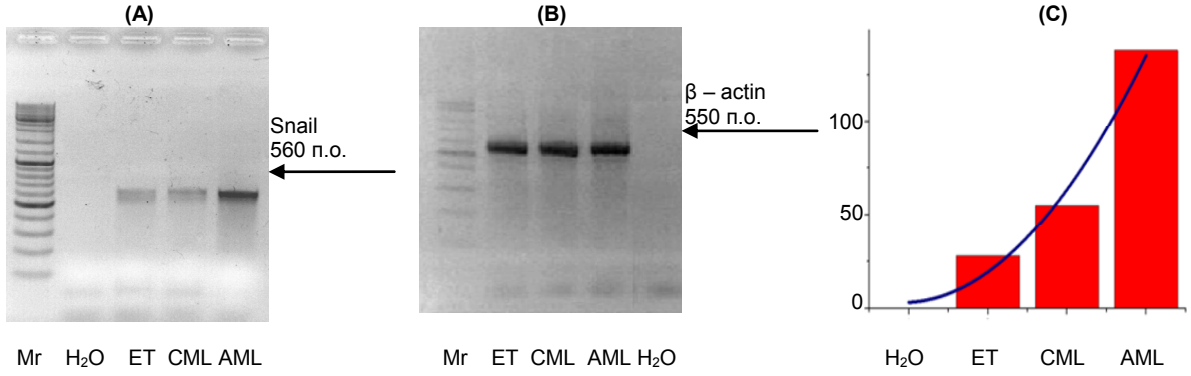

Fig. 2. Electrophoregramms with amplification products of target genes *Snail* (A) and the control gene *β – actin* (B) and graphical interpretation of the results (C)

Using densitometry analysis we revealed, that amount of amplified products of *Snail* gene increased at 2, 54 times, respectively, in patients with AML, in compared to patients with CML. The amount of referent gene *β – actin* was not significantly changed in all experimental samples.

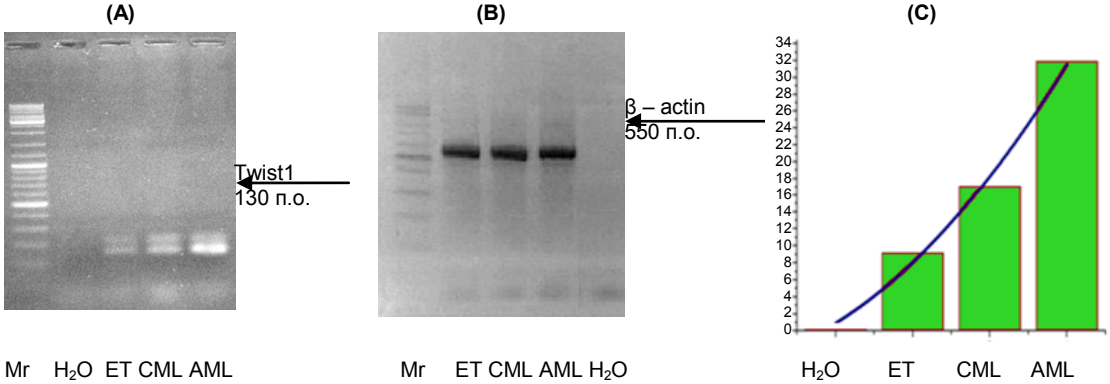

Fig. 3. Electrophoregramms with amplification products of target genes *Twist1* (A) and the control gene β – actin (B) and graphical interpretation of the results (C)

Using densitometry analysis we revealed, that amount of amplified products of *Twist1* gene increased at 1, 88 times, respectively, in patients with AML, in compared to patients with CML. The amount of referent gene *β – actin* was not significantly changed in all experimental samples.

Thus, using such methods, as bisulphate modification and methyl–specific PCR we indicate aberrant *CpG*-promoter methylation of E-cadherin gene in patients with CML and AML, whereas myeloproliferative disease essential trombocytosis (ET) has not this epigenetic modification. These results are similarly to the existing literature about the progression of metastatic tumors in various types of cancer [25-28], including the progression of leukemia [29, 30].

The mRNA expression of *Snail* and *Twist1* observed in progression of CML and AML. Chronic and acute forms of leukemia associated with reciprocal translocation t (9; 22) (q34; q11) between long arms of 9 and 22 chromosomes. This translocation results into fusion gene *bcr-abl* [31; 32]. Therefore, chimerical gene *bcr-abl* can be genetic marker of CML and AML, whereas ET has not this Ph-translocation.

Conclusion. Epigenetic transcriptional deregulation of E – cadherin gene at the expense of aberrant methylation of *CpG*-promoter in CML and AML, associated with expression's analysis of transcriptional factors *Snail* and *Twist1,* that modulated EMT, was shown.

Also we indicate that expression of *Snail* and *Twist1* significantly increase in progression of myeloproliferative diseases in such direction: essential thrombocythemia (ET); chronic myeloid leukemia (CML); acute myeloid leukemia (AML).

Aberrant hypermethylation of E – cadherine gene and expression of transcription factors *Snail* and *Twist1* play key role in progression of metastatic phenotype and might be considered as the target for a new cancer epigenetic therapy, include myeloproliferative leukemia.

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ПРОМОТОРНЕ ДНК-МЕТИЛЮВАННЯ ГЕНУ Е-КАДГЕРИНУ В МОЛЕКУЛЯРНОМУ ПАТОГЕНЕЗІ АДГЕЗИВНОГО КОМПЛЕКСУ ТА АКТИВАЦІЇ ЕМТ МАРКЕРІВ – *SNAIL* І *TWIST1* ПРИ МІЄЛОПРОЛІФЕРАТИВНИХ ЛЕЙКЕМІЯХ

Епітеліально-мезенхімальна трансдукція (ЕМТ) – це основна платформа мікрооточення пухлини. ЕМТ причинно пов'язана з раковою прогресією, модулюючи міграційний, інвазивний і метастатичний потенціали ракових клітин. Тому, ЕМТ є важливою метастазуючою нішою мікрооточення пухлини в остаточній агресивності ракових клітин. Більш того, ЕМТ лежить в основі індукції стовбурових ракових клітин та модуляції відповіді ракових клітин на хіміотерапію. Ранньою стадією в індукції ЕМТ є епігенетична дерегуляція Е-кадгерин-бета-катенін адгезивного сигналінгу. Аберантне ДНК-метилювання гену Е-кадгерина, як ключової адгезивної молекули, призводить до молекулярного "переключення" Е-кадгерин-бета-катенін адгезивного сигналінгу на канонічний бета-катенін-Wnt сигналінг, що пов'язується з трансдукцією або дедиференціацією епітеліальних клітин в мезенхімальні. Таким чином, епігенетична регуляція ЕМТ може розглядатись як таргетна мішень для нової стратегії у терапії раку.

Ключові слова: епітеліально–мезенхімальна трансдукція, Е–кадгерин, аберантне ДНК–метилування.

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ПРОМОТОРНОЕ ДНК-МЕТИЛИРОВАНИЕ ГЕНА Е-КАДГЕРИНА В МОЛЕКУЛЯРНОМ ПАТОГЕНЕЗЕ АДГЕЗИВНОГО КОМПЛЕКСА И АКТИВАЦИИ ЭМТ МАРКЕРО – *SNAIL* И *TWIST1* ПРИ МИЕЛОПРОЛИФЕРАТИВНЫХ ЛЕЙКЕМИЯХ

Эпителиально–мезенхимальная трансдукция – это основная платформа микроокружения опухоли. ЭМТ связана с опухолевой прогрессией, модулируя миграционный, инвазивный и метастатический потенциал раковых клеток. Поэтому, ЭМТ – это важная метастазирующая ниша микроокружения опухоли в окончательной агрессивности раковых клеток. Более этого, ЭМТ лежит в основе индукции стволовых раковых клеток и модуляции ответа раковых клеток на химиотерапию. Ранней стадией в индукции ЭМТ есть эпигенетическая дерегуляция Е-кадгерин-бета-катенин адгезивного пути. Аберрантное ДНК-метилирование гена Е-кадгерина, как ключевой адгезивной молекулы, приводит к молекулярному "переключению" Е-кадгерин-бета-катенин адгезивного пути на канонический Wnt-бета-катенин сигнальный путь. Именно он связан с трансдукцией или дедифференциацией эпителиальных клеток в мезенхимальные. Таким образом, эпигенетическая регуляция ЭМТ может быть мишенью для новой стратегии в терапии рака.

Ключевые слова: эпителиально–мезенхимальная трансдукция, Е – кадгерин, аберрантное ДНК-метилирование.

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PERMEABILIZED SECRETORY CELLS OF RATS EXORBITAL LACRIMAL GLANDS AS A MODEL FOR CA^{2+}-TRANSPORT SYSTEMS STUDY

The conditions of isolation and permeabilization of the rat exorbital lacrimal gland secretory cells were optimized. Optimal amount of digitonin required to exorbital lacrimal gland cells permeabilization was 50 μg / 0.5 million cells, and optimal incubation time – 10 min. Thus obtained permeabilized cells were stained with rhodamine 123 and chlortetracycline, indicating the functional integrity of mitochondria and endoplasmic reticulum. Permeabilized exorbital lacrimal gland cells is convenient model for study of Ca2+-transport systems functioning.

Keywords: lacrimal gland, secretory cells, isolation, permeabilization, digitonin, Ca2+-transport system.

Introduction. Research of lacrimal gland function in general, and their Ca^{2+}-transport systems particularly, are important and promising area of modern physiology. In particular, such common diseases as dry eye syndrome and Sjogren's syndrome are accompanied by lacrimation decrease [2, 26, 33], and it is known that lacrimal secretion is a calcium-dependent process [29, 33].

Comparatively with other exocrine glands, little is known about the Ca^{2+}-transport systems functioning of secretory cells of the lacrimal gland. Perhaps this is partly due to methodical difficulties in obtaining a sufficient number of functionally intact isolated secretory cells of lacrimal glands.

Permeabilized cells are a convenient system for study of many intracellular processes, including mechanisms of signal transduction in conditions that are close to the native ones, because in this case the correlation between Ca^{2+}-transport systems of different organelles are preserved. Cells membrane permeabilization makes intracellular Ca^{2+}-transport systems accessible for specific activators and inhibitors, that in normal conditions do not permeate through plasma membrane.

The aim of this work was to optimize the conditions of isolation and permeabilization of the rat exorbital lacrimal gland secretory cells and assess the possibility of their use for the intracellular Ca^{2+}-transport systems study.

Materials and methods. Experiments were performed on nonlinear white rats with 160-250 g weight, that were kept in a stationary conditions of vivarium. All manipulations with animals were carried out according to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 18.III.1986) and the Law of Ukraine "On protection of animals from cruelty" (2006).

After chloroform anaesthesia and decapitation the exorbital lacrimal glands *(glandula orbitalis externa)* were quickly removed from the body and dissociated from the connective tissue. For lacrimal cells isolation modified method described by Herzog et al. was used [9].

The amount of cells was counted using camera Horyaeva. For this study the drop of investigated suspension was placed under the cover glass, put under a microscope and, if cells accommodated evenly, cells were counted in 225 large squares of the camera. The number of cells in 1 ml of suspension was calculated using the formula:

$$x = \frac{n}{225} \cdot 0.25 \cdot 2 ,$$

where x – number of million cells in 1 ml of suspension; 0.25 – conversion factor for volume; 2 – dilution by solution containing trypan blue; n – amount of cells in 225 large squares of the Horyaeva camera.

The integrity of the plasma membrane was controlled visually under a light microscope using dye trypan blue. For this purpose 0.2 % trypan blue solution, that was on the basis of nominal noncalcium extracellular medium, was mixed with a suspension of cells in the same volume and after 2–3 min examined under a microscope. Lack of cellular colour indicated the integrity of the plasma membrane.

Cells membrane permeabilization was accomplished by digitonin at 37 °C in medium that was close to intracellular. The concentration of free Ca^{2+} in the medium was assigned by Ca^{2+}/EGTA buffer and calculated using the program Maxchelator (http://maxchelator.stanford.edu).

After permeabilization cells were washed twice by intracellular solution without digitonin. Permeabilization grade was estimated visually using trypan blue.

The functioning of the Ca^{2+}-transport systems was estimated by changes of Ca^{2+} content in cells, after incubation with agonists or antagonists. Ca^{2+} content was determined using metalochromic staining agent arsenazo III. Ca^{2+}-ATPase activity was evaluated based on changes in the content of inorganic phosphate in the medium, which was determined by UV detection. Protein concentration was determined by Lowry method [18]. The lacrimal gland secretory cells respiration was recorded by polarographic method [4] using a propeller stirrer [19].

Mathematical-statistical data processing was performed using the software package Microsoft Excel. Statistical difference between groups was determined by t-Student's test, for statistically significant were taking changes with $P<0.05$.