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EXPRESSION OF *REG1A* GENE IN RAT DUODENAL UPON LONG-TERM HYPOACIDITY AND WITH ADMINISTRTION OF MULTIPROBIOTIC "SYMBITER® ACIDOPHILIC" CONCENTRATED

The appearance of Reg1a gene's expression in rat's duodenal villus epithelial cells and the increasing of mRNA level of Reg1a in crypt epithelial cells upon hypoacidic conditions were shown. The level of Reg1α expression in villus epitheliocytes was decreased (in 1,6 times), while the level of Reg1α gene mRNA was similar to the control value in crypt epitheliocytes upon treatment of hypoacidic rats with multiprobiotic "Symbiter® acidophilic" concentrated.

Key words: hypoacidity, duodenal, gene expression, multiprobiotics.

Introduction. The *Reg1α* gene is an interleukin (IL)-6-inducible gene which encodes regenerative protein (regenerating islet-derived protein 1*a*) [11, 12]. REG Ia protein plays important roles in the process of wound healing in non-neoplastic gastric mucosa [11, 12]. Also it provides formation of endocrine islands and regeneration of pancreatic tissue upon pathological conditions and is also involved in the differentiation of pancreatic cells upon regeneration [10, 13].

In the small intestine REG I a protein is localized in Paneth cells and nonmature columnar cells of the human intestinal crypts, but never in functional villous epithelium [2]. Therefore, such topographical distribution is associated with growth and also with differentiation process in proliferative cells of intestinal epithelium [2].

The *Reg1α* gene does not express in healthy high differentiated tissues of many organs: liver, spleen, brain and so on [7, 15], while the appearance of this gene expression in above mentioned tissues (not in the active regeneration upon pathological conditions in some organs [15]) may be related with tumorigenesis [7, 11, 12, 16]. According to a scientific literature, besides these, increased expression of *Reg1α* gene encoding eponymous protein is associated with regeneration of pancreatic islet cells and diabetogenesis upon damage of the gland [10], with inflammation (ulcerative colitis, Crohn's disease and so on) and carcinogenesis in the gastrointestinal tract (GIT) [9, 11, 12, 16]. REG Ia overexpression is linked to STAT3 (signal transducer and activator of transcription 3 signaling) activation by activating the Akt/Bad/Bcl-xL pathway in human gastric cancer tissues. These data suggest that REG Ia protein plays a critical anti-apoptotic role in gastric tumorigenesis under STAT3 hyperactivation [11, 12].

Therefore, different alterations in *Reg1α* gene expression may lead to the development of various morbid conditions in GIT [2, 9, 11, 12].

In recent decades, proton-pump inhibitors (PPI) of gastric parietal cells, such as omeprazole, remain the most effective therapeutic agents against acid-related disorders [5, 13]. Development of dysbiosis is one of the key consequences of long-term hypoacidity. Colonization of GIT by opportunistic microbiota forms stable sources of endogenous infection and additionally promotes gastric carcinogenesis [13]. It is proved in clinical trials that probiotics are able not only to cure dysbiotic states, but also to immediately reduce damage ratio of GIT [4, 5].

Multiprobiotics of "Symbiter®" group (hereinafter referred to as Symbiter) are characterized by complexity, wide array of bioactivity and composition that is maximally close to nature microbial populations of human and animals [5].

Analysis of scientific literature showed lack of data on pattern of above mentioned gene expression in duodenal upon experimental or natural hypoacidity. Data on effect of probiotics on gene expression in duodenal upon these conditions are also absent.

Therefore, the aim of current investigation was to determine the expression of *Reg1a* gene in rat duodenal upon long-term injection of omeprazole and with administration of Symbiter.

Materials and methods. The international recommendations on performance of medical and biological investigations with the use of animals according to European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes were followed. Experiments were carried out on white non-strain male rats with initial weight around 180-200 g.

All animals were divided into four groups. The rats injected abdominally with 0,2 ml of physiological solution and 0,5 ml of water for injections orally were used as a control (first group). Animals of second group were treated with Symbiter (manufactured by LLC "O.D. Prolisok") orally (0,14 ml/kg) during 28 days. Hypoacidity (third group) was modeled by everyday intraperitoneal injection of omeprazole (14 mg/kg) during 28 days [14]. Fourth experimental group simultaneously with omeprazole obtained Symbiter in the same dose. Number of animals in each experimental group was 6.

Crypts and villi of duodenal epithelial cells were extracted by means of low-temperature method [5]. RNA was isolated following *Chomczynski* [3]; cDNA was synthesized in 20 μl of reaction mix containing 2 μg of RNA, 1 mM dNTP, 50 U of reverse transcriptase "MultiScribe™ Reverse Transcriptase", corresponding buffer, 20 U of ribonuclease inhibitor "RNase Inhibitor" ("Applied Biosystems", США), 20 pmol of reverse primer. Synthesis was carried out in the following conditions: 37° C – 2 hour. Polymerase chain reaction was performed in 30 μl of reaction mix containing 10 μl of cDNA, PCR buffer, 200 μM of each dNTP, 30 pmol (1,0 μM) of each primer, 2,5 mM of $MgCl_2$ and 1,5 U of Taq DNA polymerase ("iTaq™", "Bio-Rad", США).

PCR amplifications consisted of an initial denaturing step of 94ºC for 4 min, followed by 35 (30 for *β-actin* – gene used as internal control of reaction due to its constitutive expression) cycles of 94ºC for 45 s, the annealing step (with optimal annealing temperature): *Reg1a* (608 b. p., 48º C – 45 s) and β-actin (521 b. p., 49º C – 40 s); the extending step at 72º C for 1 min 15 s (for *Reg1a*) or 1 min (for *β-actin*). Final extension step was performed upon 72º C for 5 min.

Such primer sequences were used in reactions: for *Reg1α* – forward – AGCCTGCAGAGATTGTTGAC and reverse – CCATAGGGCAGTGAGGCAAG; for *β-actin* – forward – TGGGACGATATGGAGAAGAT and reverse – ATTGCCGATAGTGATGAXCT. Separation of PCR products was performed electrophoretically in 1,6 % agarose gel with 0,5 x TBE buffer. For semi-quantitative analysis of amplicons expression based on densitometry the ImageJ 1.45s program was used. Indices of mRNA expression were calculated for each sample following *Konturek et al.* [8].

Mathematical and statistical processing of experimental data was performed using GraphPad Prism 4.03 ("GraphPad Software Inc.", USA). The normal Gaussian distribu-

tion of the data was verified by the Shapiro-Wilk normality test. Two-way analysis of variance (two-way ANOVA) and Bonferroni post tests were performed on obtained data. Statistical significance was set at $p \leq 0,05$. The data are expressed as means and standard deviations. For graphical data means and 95 % confidence intervals are used.

Results and discussion. PCR analysis of cDNA samples generated in the rat's duodenal crypt epithelial cells indicated the presence of a specific signal with the expected length (608 b. p.) for *Reg1a* gene both in the control and second groups of investigated animals. While mRNA of this gene wasn't detected in villous epithelium both of the control group and animals treated only with Symbiter (Fig. 1.).

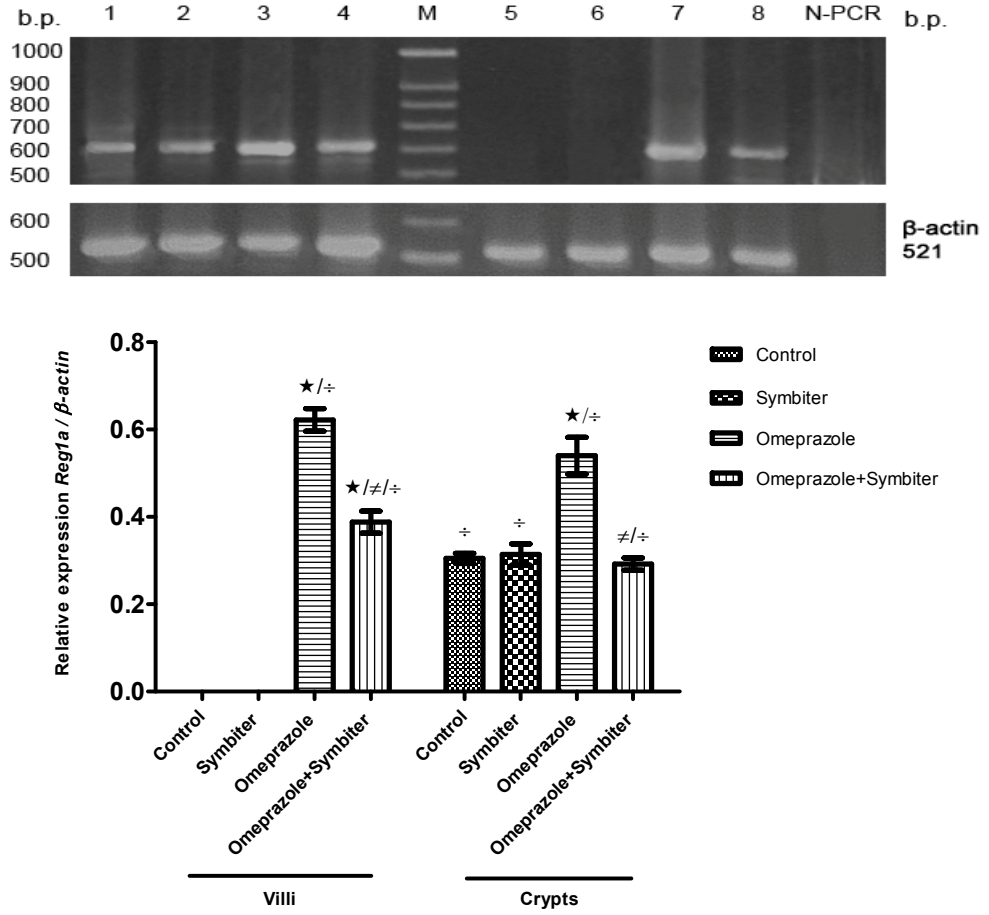

Fig. 1. Level of *Reg1a* gene mRNA in rat duodenal upon long-term hypoacidity and with administration of multiprobiotic "Symbiter® acidophilic" concentrated.

M – molecular mass marker; villus epithelial cells: 1 – control; 2 – Symbiter; 3 – omeprazole; 4 – omeprazole + Symbiter; crypts epithelial cells: 5 – control; 6 – Symbiter; 7 – omeprazole; 8 – omeprazole + Symbiter; N-PCR – negative PCR control; * – $p \leq 0,0001$ in relation to control; # – $p \leq 0,0001$ in comparison with animals treated with omeprazole; ÷ – $p \leq 0,0001$ villi in comparison with crypts. The levels of above mention gene's expression did not significantly differ *in the* crypt epithelial cells of the control and second groups from one another. On the other hand, the levels of *Reg1α* mRNA in duodenal samples of animals treated with omeprazole during 28 days and rats upon simultaneous administration of omeprazole with multiprobiotic Symbiter as significantly differentiate both in villous epithelium and crypts as between analyzed groups of animals ($F = 348,1$, $DFn = 3$, $DFd = 40$, $p \leq 0,0001$) (Fig. 1., Table. 1.).

Table 1. **Level of *Reg1a* gene mRNA in rat duodenal upon long-term hypoacidity and with administration of multiprobiotic "Symbiter® acidophilic" concentrated, (m ± SD, n = 6)**

Groups of animals	Epithelial cells	Relative expression *Reg1a / β-actin*
Control	villi	0
	crypts	$0,305 \pm 0,0110^{\div}$
Symbiter	villi	0
	crypts	$0,314 \pm 0,0231^{\div}$
Omeprazole	villi	$0,622 \pm 0,0247^{*\div}$
	crypts	$0,540 \pm 0,0403^{*\div}$
Omeprazole + Symbiter	villi	$0,388 \pm 0,0242^{\neq\div}$
	crypts	$0,292 \pm 0,0135^{\neq\div}$

Notes: SD – standard deviation;
** – $p \leq 0,0001$ in relation to control;*
≠ – $p \leq 0,0001$ in comparison with animals treated with omeprazole;
÷ – $p \leq 0,0001$ villi in comparison with crypts.

In functional villous epithelium of duodenal we found mRNA of *Reg1α* gene upon long-term hypoacidity. At the same time in fourth group of rats the level of this gene was in 1,6 times lower than in animals injected with omeprazole ($p \leq 0,0001$).

In crypts epithelium of animals treated only with omeprazole for 28 days the level of *Reg1α* mRNA was in 1,8 times higher than control values ($p \leq 0,0001$). While upon simultaneous administration of multiprobiotic Symbiter this parameter was approximately in 1,9 times lower than in animals of third group ($p \leq 0,0001$). In animals treated only with Symbiter this parameter was similar to the control value (Fig. 1., Table. 1.).

It is well known, that the main function of crypts is reproduction of cell, which are able to regenerate intestinal epithelial tissue. It is well known, that the crypt base columnar cells are only proliferative cells and that the mid-crypt columnar cells proliferate and also differentiate, whereas the crypt top columnar cells never divide, but continue to differentiate and then move along the lines of villi [2, 13].

As it was demonstrated, *Reg1a* gene mRNA expression was observed in Paneth cells, in the glands of Lieberkühn and nonmature columnar cells of the human intestinal crypts, but never in the mature functional villous epithelium [2]. Therefore, REG protein may be associated with growth and early differentiating of intestinal epithelium, as has been proposed for pancreas [2].

In our experiment we demonstrated the elevation of *Reg1a* mRNA level in crypt epithelial cells upon hypoacidic conditions (Fig. 1., Table. 1.). It can be assumed about intensification of inflammatory processes in duodenal [2, 9].

The appearance of *Reg1a* gene's expression in duodenal villus epithelial cells in rats treated only with omeprazole (Fig. 1., Table. 1.) may be associated with possible neoplasia in epithelial cells on later stages of pathology process development [2, 9, 11, 12, 16].

Thus, the obtained changes in expression of *Reg1a* gene in rat duodenal villus and crypts epithelial cells upon hypoacidic conditions should point out the development of teratoid displacements in duodenal tissue. Multidirectionality in alterations of *Reg1a* gene's expression in villus and crypts epithelial cells is determined by their structural and functional features.

Among probable mechanisms of Symbiter's action on gene expression in rat pancreas, firstly, it should be pointed out its ability to liquidate dysbiosis and bacterial colonization of GIT – it was observed in a number of investigations [1]. As a consequence, the burden of pathogenic microbiota is removed from GIT and associated organs [4, 5]. Furthermore, multicomponent probiotics such as Symbiter are able to increase *de novo* synthesis of the main low-molecular cellular antioxidant – reduced glutathione and, thus, to raise its content both in GIT and duodenal [1]. So, preliminary treatment with probiotics can ameliorate the rate of oxidative stress, inflammatory processes and damage of the duodenal [1].

Conclusion. In summary, we have shown, that long-term experimental hypoacidity is accompanied by changes in expression of *Reg1a* gene in rat duodenal. While upon simultaneous administration of multiprobiotic "Symbiter® acidophilic" the expression pattern of this gene is similar to control. The level of *Reg1α* expression in villus epitheliocytes was decreased, while the level of *Reg1α* gene mRNA was similar to the control value in crypt epitheliocytes upon treatment of hypoacidic rats with multiprobiotic "Symbiter®" acidophilic" concentrated. Based on the obtained data, it can be assumed, that there is some potential risk of duodenal carcinogenesis upon long-term use of omeprazole (and probably other PPIs). The final explanation of molecular mechanisms underlying the changes in expression of *Reg1a* gene in rat duodenal upon long-term hypoacidity and with administration of multiprobiotic Symbiter requires further experiments.

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ЕКСПРЕСІЯ ГЕНА *REG1A* В ЕПІТЕЛІОЦИТАХ ДВАНАДЦЯТИПАЛОЇ КИШКИ ЩУРІВ В УМОВАХ ДОВГОТРИВАЛОЇ ГІПОАЦИДНОСТІ ТА ПРИ ВВЕДЕННІ МУЛЬТИПРОБІОТИКА "СИМБІТЕР® КОНЦЕНТРОВАНИЙ АЦИДОФІЛЬНИЙ"

Виявлено експресію гена Reg1a в епітеліоцитах ворсинок та зростання рівня мРНК цього гена в епітеліоцитах крипт дванадцятипалої кишки за гіпоацидних умов. При введенні мультипробіотика "Симбітер® ацидофільний" концентрований за тих же умов рівень експресії Reg1a в епітеліоцитах ворсинок зменшувався в 1,6 рази, у той час як вміст мРНК Reg1a в криптах був на рівні контрольних значень.

Ключові слова: гіпоацидність, дванадцятипала кишка, експресія генів, мультипробіотики.

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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.