METHYLATION OF CELL CYCLE AND APOPTOSIS GENES' PROMOTERS IN EXPOSED INDIVIDUALS WITH SUBSEQUENT MALIGNANT NEOPLASMS

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DNA methylation plays an important role in carcinogenesis; there are many studies that investigate the degree of methylation of the entire genome, gene promoters, and non-coding elements in cancer cells, but much less information about changes of the methylation patterns in blood cells and links with the development of malignant neoplasms (MN). This study aimed to investigate the degree of methylation of promoter regions of cell cycle control and apoptosis genes (*BAX*, *MDM2*, *TP53*, *NFkB1*) in peripheral blood cells of persons chronically exposed to radiation with MN developing latently. The study included 200 persons chronically exposed to radiation from the Techa River, contaminated with nuclear wastes dumped into it. The level of methylation was assessed by real-time PCR. The participants were divided into exposed and control groups; comparing them, we found that in the former, the distribution of exposed individuals with latent MN by the degree of methylation of promoter regions of *BAX*, *MDM2* and *NFkB1* genes was significantly different from that in the latter ($p < 0.001$; $p < 0.001$; $p = 0.004$, respectively). It was established that, compared to the control group, the share of the test group participants with subsequent MN who had up to 10% of the *BAX* gene promoter regions methylated was significantly higher, and amounted to 98%, while in the control group this figure did not exceed 73% ($p < 0.00001$).

Keywords: chronic radiation exposure, gene methylation, CpG dinucleotides, carcinogenesis, the Techa River

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МЕТИЛИРОВАНИЕ ПРОМОТОРОВ ГЕНОВ КЛЕТОЧНОГО ЦИКЛА И АПОПТОЗА У ОБЛУЧЕННЫХ ЛИЦ, ВПОСЛЕДСТВИИ ЗАБОЛЕВШИХ ЗЛОКАЧЕСТВЕННЫМИ НОВООБРАЗОВАНИЯМИ

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Метилирование ДНК играет важную роль в канцерогенезе, в литературе встречается достаточно много исследований уровня метилирования всего генома, промоторов генов и некодирующих элементов в раковых клетках. При этом данных об изменении паттерна метилирования в клетках крови и связи с развитием злокачественных новообразований (ЗНО) существенно меньше. Цель работы — исследование уровня метилирования промоторных регионов генов контроля клеточного цикла и апоптоза (*BAX*, *MDM2*, *TP53*, *NFkB1*) в клетках периферической крови лиц, подвергшихся хроническому радиационному воздействию в латентном периоде развития злокачественных новообразований. Исследование проводили у 200 человек, подвергшихся аварийному хроническому радиационному воздействию в результате сбросов радиоактивных отходов в реку Течу. Уровень метилирования оценивали методом ПЦР в реальном времени. Было установлено, что распределение облученных лица с ЗНО в латентном периоде по уровню метилирования промоторных регионов генов *BAX*, *MDM2* и *NFkB1* статистически значимо отличалось от распределения в группы сравнения ($p < 0{,}001$; $p < 0{,}001$; $p = 0{,}004$ соответственно). Установлено, что в группе облученных лиц, которые впоследствии заболели ЗНО, доля лиц с уровнем метилирования до 10% промоторной области гена *BAX* была статистически значимо больше и составила 98% относительно группы сравнения, в которой доля таких людей не превышала 73% ($p < 0{,}00001$).

Ключевые слова: хроническое радиационное воздействие, метилирование генов, CpG-динуклеотиды, канцерогенез, река Теча

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To date, the potential usefulness of genetic factors in prediction of risks of malignant neoplasms (MN) has been investigated fairly well. For some MN, there were established highly reliable genetic markers, like *BRCA1* and *BRCA2* mutations for breast cancer and ovarian cancer [1], *TP53* mutations for breast, lung, stomach and intestinal cancers [2], *ATM* gene mutations for pancreatic and breast cancers [3]. However, polygenic nature of MN prevents determination of the role of such changes in radiation-induced carcinogenesis. Epigenetic indicators, including DNA methylation, which are modifiable by environmental factors like ionizing radiation, can underpin an alternative approach to the MN risk prediction.

Epigenetic modifications, including methylation, affect the expression of genes involved in carcinogenesis at different stages, from initiation to progression [4]. Hypermethylation of suppressor genes, mobile genetic elements, and oncogenes, is registered in tumor cells, the examples thereof including hypermethylation of tumor suppressor genes in non-small cell lung cancer, colorectal cancer, breast, prostate, and bladder cancer cases [5–7]. Hypomethylation of mobile genetic elements, such as Alu and *LINE-1,* as well as individual gene regions, was registered in breast, ovarian, hepatocellular, and stomach cancer cases [8, 9].

It should be noted that epigenetic marks reflect both the innate genetic background and the impact of environmental factors, which is important in the context of investigation of the effects exogenous factors have on carcinogenesis [10].

DNA methylation is tissue-specific, therefore, methylation patterns obtained from, for example, blood, cannot be easily extrapolated to tissues in which cancer grows [11]. However, this is possible, since the correspondence between DNA methylation in different tissues depends on the locus and the degree of inter-tissue correlation, and methyl marks can be inherited or form at early stages of development, as a consequence of which they will be detected in many tissues [12]. Changes of methylation patterns peculiar to the aging genes (epigenetic clock) may also be associated with the risk of development of various pathologies, including cancer [13–15].

There are published papers that report development of the MN risk prediction algorithms based on the analysis of blood cell DNA methylation. The phenotypic aging and mortality risk assessment algorithms based on the level of methylation of CpG-dinucleotides of DNA associated with age, plasma protein levels, smoking status, and key disease factors, were shown usable in the context of both overall and specific MN risk evaluation, including that for lung, prostate, breast, colorectal cancers [16-18]. A systematic review of studies investigating human blood DNA methylation established a stable relationship between breast cancer risk and global hypomethylation of blood cell DNA and epigenetic age [19].

However, despite the mentioned works, there is still no reliable evidence of the alleged link between DNA methylation patterns and MN development risks.

Cell cycle arrest and apoptosis are some of the mechanisms preventing cell's oncotransformation; with this in mind, we conducted this study seeking to assess the level of methylation of promoter regions of cell cycle control and apoptosis genes (*BAX*, *MDM2*, *TP53*, *NFkB1*) in blood sampled from individuals who were chronically exposed to radiation and subsequently had MN.

METHODS

Characteristics of the examined individuals

We determined the degree of methylation of CpG dinucleotides in promoter regions of peripheral blood *BAX*, *MDM2*, *TP53*, and *NFkB1* genes in people exposed to chronic low dose rate radiation emitted by the Techa River contaminated with liquid radioactive wastes dumped from the Mayak Production Association in 1950-1960. Individual doses accumulated by red bone marrow (RBM) were calculated for each participant using the Techa River Dosimetry System (TRDS) 2016 [20]. They were divided into two groups: a test group, which included 100 exposed persons who were subsequently diagnosed with MN (we collected blood samples prospectively, in the latent period, 5 years before MN developed), and a control group, which consisted of 100 exposed persons not diagnosed with cancer. In this study, the latent period was up to 5 years, because the level of methylation depends on various environmental factors and may change over time, consequently, a longer follow-up period would weaken the link with cancer risk. One of the previously published systematic reviews has shown that the DNA methylation patterns can change in different periods of observation [19].

The inclusion criteria were: residence in one of the 41 Techa riverside villages from 01.01.1950 to 31.12.1960; availability of the individual red bone marrow dose data calculated based on TRDS 2016 [20]. The exclusion criteria were: autoimmune diseases, hemoblastoses and malignant neoplasms at the time of blood sampling (including in 2023 for the control group).

The following MN were diagnosed in the test group 2002 to 2020: lip cancer (ICD 10 code C00 — 3 cases), cancer of digestive organs (esophagus, C15 — 1 case; stomach, C16 — 14 cases; transverse colon, C18.4 — 5 cases; rectosigmoid junction, C19 — 3 cases; pancreas, C25.9 — 8 cases), cancer of respiratory and thoracic organs (trachea, bronchus, lung, C34 — 19 cases), breast cancer (C50 — 16 cases), cancer of female genitalia (cervix, C53 — 7 cases; uterine body, C54 — 4 cases; ovary and uterine appendages, C56 — 3 cases), male genitalia (prostate gland, C61 — 8 cases); urinary tract (bladder, C67 — 6 cases; kidneys, C64 - 3 cases).

Table 1 presents characteristics of the examined individuals.

The mean age of the examined persons with MN was 68.3 ± 0.7 years (from 51 through 86 years). More than half (54%) of members of this group were female. The average accumulated RBM dose there was 731.5 ± 68.3 mGy (dose range: 10.1–3,507 mGy).

By each of the studied genes, the number of people in test and control groups differed, but by age at the time of examination, sex and RBM dose, the groups were comparable (Table 2).

All participants of the study signed a voluntary informed consent form approved by the Ethics Committee of the Urals Research Center for Radiation Medicine.

Research methods

Genomic DNA isolated from frozen blood samples was denatured and converted with bisulfite using the EpiJET Bisulfite Conversion Kit (Thermo Scientific; USA), as per the manufacturer's protocol. After bisulfite treatment, we applied primers specific to the methylated DNA sites for amplification purposes. Methyl Primer Express Software V.1.0 (Applied Biosystems; USA) was used to construct the sequences of primers for PCR fragments of promoter regions of *BAX*, *MDM2*, *TP53*, *NFkB1*. We selected genes based on the results of earlier studies investigating their transcriptional activity and level of methylation of the gene's promoter regions in irradiated individuals [21, 22].

The oligonucleotides were synthesized by DNK Synthes (Russia). Table 2 shows sequences of oligonucleotides specific to the methylated DNA sequence.

Table 1. Characteristics of the studied groups

Group characteristics		Test group (patients with latent MN)	Control group			
			BAX	*MDM2*	*TP53*	*NFkB1*
Number of participants		*n* = 100	*n* = 73	*n* = 140	*n* = 69	*n* = 90
Age at the time of examination, years: M ± SE (min–max)		68.3 ± 0.7 (51–86)	71.7 ± 0.8 (59 – 87)	71.8 ± 0.5 (56–87)	70.4 ± 0.8 (58 – 84)	71.5 ± 0.7 (59–84)
Sex, person (%)	Male	46 (46)	26 (36)	51 (36)	17 (25)	29 (32)
	Female	54 (54)	47 (64)	89 (64)	52 (75)	61 (68)
Accumulated RBM dose, mGy, M ± SE (min-max)		722.5 ± 69.3 (10.1–3507.1)	542.4 ± 63.4 (10.1–2869.8)	617.6 ± 52.2 (10.1–3179.7)	507.6 ± 62.0 (10.0–2869.8)	765.8 ± 83.3 (10.1–3715.5)

Note: RBM — red bone marrow, M — mean; SE — standard error; *n* — number of people; (min–max) — range of values.

The status of methylation of the gene promoters was established with the help of real-time PCR and high resolution melt curve analysis (HRM analysis). The reaction was triggered in 20 µl of a 5x qPCRmix-HS (Eurogen; Russia) reaction mixture consisting of a highly recessive Taq-DNA polymerase with specific monoclonal antibodies, SYBR Green I dye, a mixture of dNTP, $Mg2^{+}$ and PCR buffer. For real-time PCR, we used a StepOnePlus Real-Time PCR System (Thermo Scientific; USA) amplifier. The temperature and time sequences for this procedure were as follows: first denaturation (95°, 5 minutes), denaturation (95°, 30 seconds), annealing (see Table 2 for annealing temperature for each gene, 30 seconds), and elongation (72°, 30 seconds) — 50 cycles; construction of the melting curve (95°, 10 seconds; 60°, 1 minute; 95°, 15 seconds; 60°, 15 seconds).

Bisulfite-converted samples of commercially available fully methylated DNA, CpG Methylated Human Genomic DNA (Thermo Fisher Scientific; USA), and unmethylated Human Genomic DNA Male (Promega; USA) were used as controls enabling assessment of methylation of the studied CpG islands of the gene promoter regions. The controls were mixed in the following ratios: 0/100, 5/95, 10/90, 25/75, 50/50, 75/25 and 100/0, respectively. The degrees of methylation for each control sample were 0%, 5%, 10%, 25%, 50%, 75% and 100%. For the analysis, we used HRM software (Applied Biosystems; USA); it was based on the comparison of the experimental DNA samples melt curve profiles with the standard samples, i.e., those with a known level of methylation. Based on the standard samples, the following degrees of methylation were distinguished: 0%; 0–5%; 5–10%; 10–25%; 25–50%; 50–75%; 75–100%. Experimental DNA samples were distributed accordingly.

Statistical analysis of the data

SPSS Statistics 17.0 software package was used for statistical processing of the results. Yates's chi-squared test enabled comparison of distribution of the participants by the level of methylation; the differences were considered significant at $p \leq 0.01$. To distinguish between methylation levels of 0 through 10% and over 10%, we used Fisher's exact test. The differences were considered significant at $p \leq 0.05$. Spearman's rank correlation coefficient (R) enabled correlation analysis designed to evaluate the effect of RBM dose and age on the degree of methylation; correlations were considered statistically significant at $p \leq 0.05$.

RESULTS

We found that by the degree of methylation of promoter regions of *BAX*, *MDM2* and *NFkB1*, test group (exposed individuals with latent MN) differed significantly from the control group (see Figure). It should be noted that in the vast majority of those who eventually developed MN, the level of methylation of the mentioned promoter regions did not exceed 10%, and the bulk of differences in distribution as compared to the control group were registered in this span. Thus, in the test group, the proportion of those who had *NFKB1* promoter region methylated by 0–10% was 100%, while in the control group this figure equaled 87%. At the same time, in the test group, there were 50% and 49% of those whose *NFKB1* promoter region was hypomethylated (0% methylation) and lightly methylated (methylation up to 5%), respectively, and in the control group these figures were 63% and 23%, respectively. As for the *MDM2* gene, the bulk of differences between test and control groups was also in the 0–5% span, with hypomethylation registered in 29% of the test group cases and light methylation (0–5%) in 62%, while in the control group promoter region of *MDM2* was hypomethylated in 55% of participants and lightly methylated in 41%. For *BAX*, the trend was similar: in 98% of test group participants, the level of methylation was below 10%, and 2% exhibited hypermethylation (50 through 75%) of this gene promoter region. It is worth noting that in the control group, we registered all the designated spans of level of methylation of BAX's promoter region.

Given the relative uniformity of distribution by the levels of methylation, we divided the sample into two groups: group 1, methylation up to 10%; group 2, methylation over 10% (Table 3).

Table 2. Characteristics of the used oligonucleotides

Gene	Primer sequences (5'-3')	Number of CpG sites	Amplicon length	Primer length	Ta
BAX	F: GAGGGGTAGAAATTTTCGGAT R: ATAATACGAACGACAAACCCG	10	181	21 21	59
MDM2	F: TTTGTCGGGTTATTAGTGTGAAC R: CCTTTTACTACAATTTCGAAACGTA	6	130	23 25	60
TP53	F: GTAGTTTGAACGTTTTTATTTTGGC R: CCTACTACGCCCTCTACAAACG	11	135	25 22	61
NFkB1	F: GTAGGAAGAGGAGGTTTCGTTATC R: ACCGATAACTACGTACAAACCGA	14	122	24 23	60

Note: F — forward primer; R — reverse primer; Ta — annealing temperature.

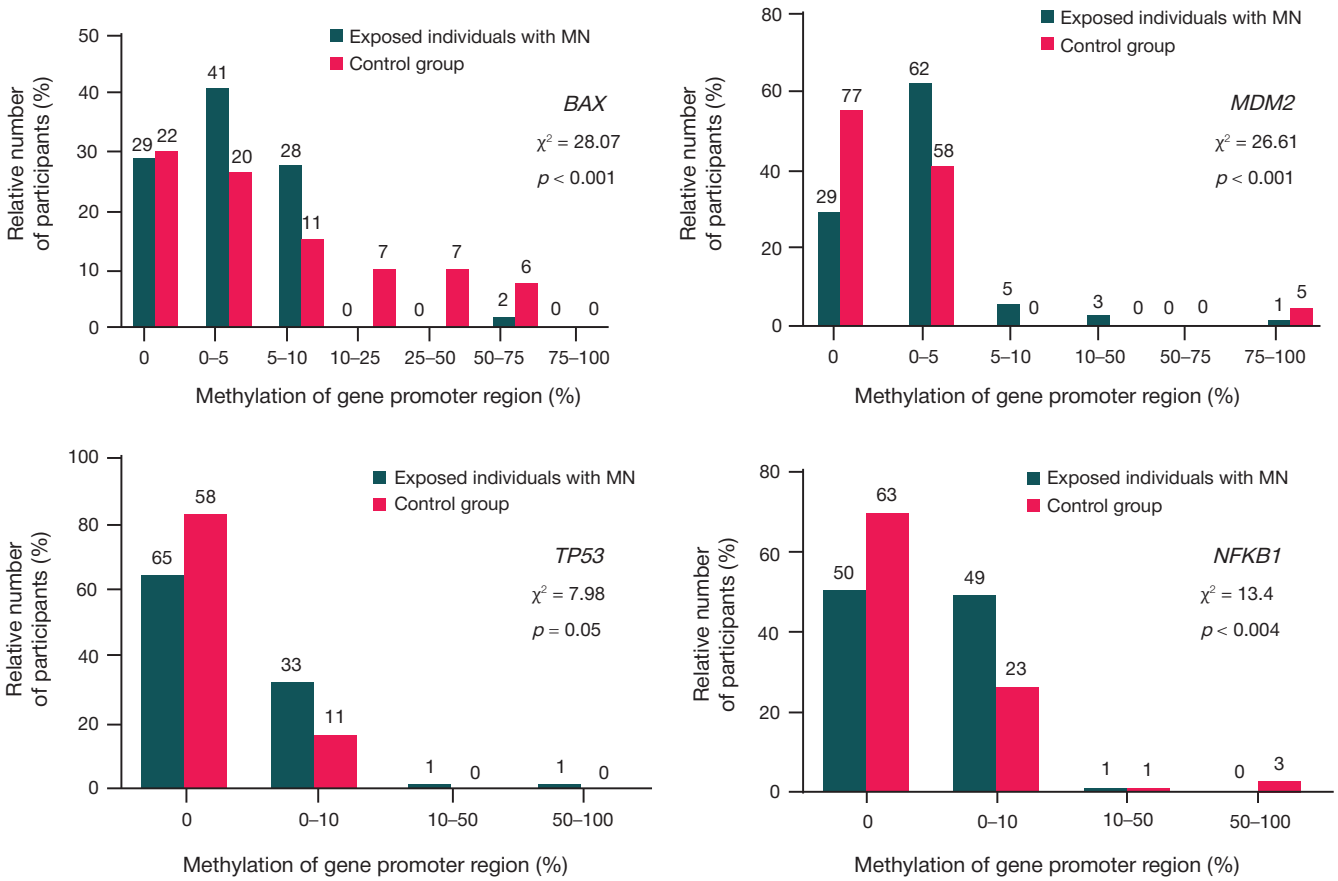

Fig. Distribution of the participants by level of methylation of CpG-dinucleotides of the studied gene promoter regions. Chi-squared test value incorporates Yates correction

We revealed significant differences only for *BAX*, the pro-apoptotic gene. Compared to the control group, the share of test group participants who had its promoter regions methylated for up to 10% was significantly higher ($p < 0.00001$).

Methylation is a dynamic process that may depend on a number of factors, including age and radiation dose. With this in mind, we correlated the levels of methylation with RBM dose and age at the time of examination. This analysis revealed no dependence of the methylation pattern on RBM dose and age in the test group, and in the control group, we registered a weak negative correlation between *BAX* and TP53's promoter regions methylation and age of the participants ($R = –0.35$; $p = 0.002$ and $R = –0.28$; $p = 0.02$, respectively) (Table 4).

DISCUSSION

The subjects of this study were the cell cycle (*MDM2*, *TP53*) and apoptosis (*BAX*, *NFkB1*) genes. By distribution of the levels of methylation of *BAX*, *MDM2* and *NFkB1* promoter regions, test group (exposed individuals, subsequent MN) differed significantly from the control group (exposed individuals, no subsequent MN). However, having divided the sample into two groups by the degree of methylation (up to 10% and above 10%), we discovered statistically significant differences only for *BAX*. In the test group, 98% of the participants had the levels of methylation between 0 and 10%, while in the control group, this figure did not exceed 73%.

BAX is a member of BCL-2 family; it induces apoptosis and is considered a potential tumor suppressor [23]. Normally, in response to genotoxic damage, the p53 protein alters the level of expression of genes involved in mitochondrial-mediated apoptosis, and activates *BAX*, inter alia [24]. At the same time, tumor cells suppress pro-apoptotic genes, seeking to survive and metastasize. It is important to note that decreased concentration of the BAX protein is associated with mutations in the *Tp53* gene [25]. According to our studies, exposed individuals with latent MN have *BAX* promoter in blood cells hypomethylated, which may affect the transcriptional activity of this gene. It is interesting

Table 3. Cases of methylation of CpG islands of the promoter regions of *BAX*, *MDM2*, *TP53*, *NFkB1* in the study sample

Gene	Level of methylation	Control group N (%)	Exposed individuals with latent MN N (%)	*p*-value
BAX	0–10%	53 (72.6)	98 (98)	$p < 0.00001$
	Over 10 %	20 (27.4)	2 (2)	
TP53	0–10%	69 (100)	98 (98)	$p = 0.51$
	Over 10 %	0 (0)	2 (2)	
MDM2	0–10%	135 (96.4)	96 (96)	$p = 0.99$
	Over 10 %	5 (3.6)	4 (4)	
NFkB1	0–10%	87 (96.6)	100 (100)	$p = 0.10$
	Over 10 %	3 (3.6)	0 (0)	

Note: *p* — is the level of statistical significance of differences between groups, as given by Fisher's exact test.

Table 4. Spearman's rank correlation coefficients (R), dependence of the degree of methylation of the studied gene promoter regions on RBM dose and patient's age at the time of the study. The p-value for Spearman's correlation coefficients is given in parentheses

Gene	Control group		Exposed individuals with latent MN	
	RBM dose	Age at the time of examination	RBM dose	Age at the time of examination
MDM2	–0.03 (0.69)	0.09 (0.31)	–0.06 (0.53)	–0.05 (0.64)
BAX	–0.55 (0.64)	–0.35 (0.002)	0.08 (0.44)	0.08 (0.45)
TP53	–0.08 (0.53)	–0.28 (0.02)	–0.01 (0.99)	0.07 (0.48)
NFkB1	0.14 (0.19)	0.10 (0.34)	0.04 (0.70)	–0.02 (0.86)

to note that findings of a previous study that involved residents of the Techa riverside villages and investigated expression of mRNA of apoptotic genes: in those whose RBM dose exceeded 522 mGr, transcriptional activity of *BAX* was increased significantly [21]. Another study looked into death of peripheral blood lymphocytes in the same cohort, and found that in the exposed with obligate precancers, the level of respective apoptosis was higher than in those who were also exposed but had no precancer [26].

There is a sufficient number of published works investigating profiles of methylation of DNA in cancer cells, with colon, breast and lung cancers being the most common neoplasms considered [27]. At the same time, there are significantly fewer retrospective studies that look into methylation of DNA in normal tissues (for example, blood) before the onset of the disease, studies that seek cancer risk predictors; moreover, most of them consider genes associated with changes in the chronological age (epigenetic clock) [13, 15, 17]. There are, however, isolated studies of proto-oncogenes and tumor suppressor genes. One of them analyzed patterns of methylation of 17 genes potentially indicating predisposition to breast cancer, including cell cycle regulation genes, and found that, compared to the control group (no breast cancer), patients suffering the disease had the intragenic repeating element of the ATM gene hypermethilated [28].

Thus, the results of this study demonstrate that epigenetic modifications (degree of methylation) in the peripheral blood DNA can potentially be used as markers of radiation-induced carcinogenesis. In addition, identification of epigenetic changes in tissues and cells not involved in the pathological process allows clarifying the causes of pathological conditions. However, definitive determination of epigenetic markers of carcinogenic effects of radiation requires additional studies involving expanded samples and factoring in the analysis of the level of methylation registered in tumor tissues.

CONCLUSIONS

By distribution of the levels of methylation of *BAX*, *MDM2* and *NFkB1* promoter regions, test group (individuals exposed to chronic low dose rate radiation, with RBM doses from 10.1 to 3,507 mGy, latent MN) differed significantly from the control group. The share of the test group participants who had up to 10% of the *BAX* gene promoter regions methylated was significantly higher, and amounted to 98%, while in the control group this figure did not exceed 73%. There was revealed no dependence of the level of methylation of the studied gene promoter regions on RBM dose.

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