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The study of hypermethylation in blood leukocytes of irradiated parents and their children

Research Article

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Abstract: Background: Accumulation of evidence about the epigenetic regulation of genome function suggests the necessity to explore new aspects of the genotoxic action of radiation on the human body. Methodology: A methylation-sensitive PCR assay was used to analyze promoter methylation of *p16/CDKN2A, p14/ARF, RASSF1A* and *GSTP1* genes in blood leukocytes from 103 unirradiated volunteers and 104 irradiated subjects (83 Chernobyl Nuclear Power Plant liquidators and 21 nuclear specialists). Additionally, 21 families whose fathers were nuclear specialists were examined. Results: A significantly elevated frequency of individuals with abnormal methylation of *p16/CDKN2A* and *GSTP1* genes was revealed in the exposed group compared to the control group ($p = 0.0097$ and $p = 0.005$, respectively). The occurrence of promoter methylation of *RASSF1A* gene significantly correlated with aging both in the control group ($r = 0, 213$; $p = 0.006$) and in the exposed individuals ($r = 0, 212$; $p = 0,031$). No methylated genes were found in the offspring of control families. Conclusion: Our study showed for the fist time that prolonged radiation exposure at low and medium doses is associated with hypermethylation of genes involved in the basic protective functions of cells; an effect that is persistent in blood leukocytes for significant periods after irradiation.

Keywords: *Human • DNA hypermethylation • CpG islands • p16/CDKN2A • p14/ARF • RASSF1A • GSTP1 • Leukocytes • Irradiation*

1. Introduction

Earlier, we and other investigators have demonstrated significantly elevated levels of chromosome aberrations and gene mutations in blood leukocytes of Chernobyl Nuclear Power Plant (ChNPP) accident liquidators (clean-up workers), either shortly or many years, following their irradiation [1-3]. In addition, examination of blood samples from the offspring of irradiated fathers-liquidators showed that these children had higher frequencies of analogous genetic disturbances in comparison with the children of nonirradiated parents despite the fact that they were not directly exposed to ionizing radiation. Data obtained point to the phenomenon of transgenerational radiation-induced genomic instability in children born to irradiated fathers [1,3].

However, accumulation of evidence about the epigenetic regulation of genome function suggests the necessity to explore new aspects of the genotoxic action of radiation on the human body. DNA methylation is a main epigenetic genomic modification, which not only plays an important role in gene regulation but is also crucial for maintaining stability of the genome.

This epigenetic event can be described as the attachment of a methyl group to a cytosine base without changing the original DNA sequence [4]. A variety of genotoxic agents are known to alter DNA methylation patterns [5-10].

Most methylated CpG dinucleotides are located in repetitive DNA elements which make up to 45% of the genome. Unmethylated CpG dinucleotides are clustered in regions known as CpG islands (CGIs) which are short genomic regions (500 bp to a few kb)

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characterized by relatively high CpG density and located in the proximal promoter region of approximately 75% of human genes [11]. Changes in DNA methylation patterns include global hypomethylation, gene-specific hypermethylation/ hypomethylation and loss of imprinting. The reality of these epigenetic modifications, induced by radiation, is shown in experiments using cell cultures and different animal models [5-9]. The bulk of the research is devoted to the assessment of DNA methylation in long interspersed nuclear element-1 *(LINE-1)* and *Alu* repetitive elements as a surrogate of genome-wide methylation. The results indicate that although the response of cells is predominantly in hypomethylation, hypermethylation occurs as well. One of the main mechanisms of gene inactivation is aberrant methylation of cytosines in CGIs associated with active promoters. In particular, hypermethylation CGIs of genes involved in carcinogenesis (tumor--suppressor genes and a number of other genes) leads to transcriptional repression and is revealed in cells of different malignant tumors [12-15]. At the same time, chronic X-ray irradiation (50 cGy, 10 days) of mice also induced hypermethylation of the *p16* tumor-supressor gene in the cells of the liver in a sex-specific manner [9]. However, there are no reports on the hypermethylation effects of radiation exposure in humans. It is believed that changes in methylation patterns are potentially reversible, although such epigenetic modifications can be stored for a long time and inherited [16].

In the present study we investigated the long-term epigenetic consequence of exposure to low and medium doses of radiation on liquidators of the ChNPP accident and nuclear specialists (exposed to tritium and tritium oxide), and also analogous disturbances in their offspring. The investigation was designed to evaluate hypermethylation promoter of genes associated with the cell cycle (*p16/CDKN2A, p14/ARF, RASSF1A*) and xenobiotic detoxification (*GSTP1*) using peripheral blood DNA from subjects who do not have tumor pathology.

2. Experimental Procedures

2.1 Subjects

In our work 103 unirradiated volunteers (control group) and 83 liquidators of the ChNPP were examined. The peripheral blood samples of 67 liquidators were collected at the Federal State Institution “Russian Scientific Center of Roentgeno-Radiology” (the Department of Radiation Medicine) where these patients underwent medical examination in 2004-2007. Moreover, 16 liquidators came to Federal Children’s Scientific and Practical Centre of Radiation Safety for examination of their children. The liquidators participated in ChNPP clean-up work within the 30-km zone in 1986–1987. The duration of their work varied from 2 to 6 months. The individual doses of liquiditators ranged from 50 to 460 mSv (the data of physical dosimetry). One-third of liquidators’ doses are unknown, but for those with documented doses the average was 221 mSv. Additionally, an average dose of 230 mSv was established by other investigators using chromosome FISH painting (retrospective biodosimetry) in a group of Chernobyl clean-up workers [2].

Twenty-one full families of fathers - nuclear specialists (All-Russian Research Institute of Experimental Physics, Sarov, Russia) who began to be occupationally exposed to tritium and tritium oxide 40 to 50 years ago were examined. Thirteen families had 1 child, 8 families had 2 children. Only one child was born before the father started working with tritium and tritium oxide. Other examined offspring had irradiated fathers. The summarized accumulated doses over a period of work with tritium ranged from 37 to 994 mSv. The duration of work of nuclear specialists with tritium and tritium oxide varied from 3 to 46 years. The time between irradiation of the fathers and conception of the children varied from a few months to 18 years.

Overall, the time between the end of clean-up work of the ChNPP liquidators and their examination varied from 17 to 21 years. The time between the end of work of the nuclear specialists with tritium and tritium oxide and their examination varied from 2 to 46 years, although for 48% of these irradiated subjects this parameter was more than 10 years.

All examined subjects were Russian residents of territories uncontaminated by radiation. At the time of examination none of the subjects had oncological diseases, and cases of acute radiation syndrome were not observed. Informed consent was obtained from all of the patients and the investigation was approved by the Ethical Committee of N.I. Vavilov Institute of General Genetics, Russian Academy of Sciences.

2.2 DNA methylation analysis

The methylation status of 4 genes was analyzed using a methylation-sensitive polymerase chain reaction (PCR) assay. These genes, including tumor suppressor genes, are associated with the cell cycle (*p16/CDKN2A, p14/ARF, RASSF1A*) and xenobiotic detoxification (*GSTP1*).

Genomic DNA was isolated from blood leukocytes using MagnetTM DNA MegaPrep1 kit (“Izogen“, Russia) according to the manufacturer`s protocol. Concentrations of isolated DNA were determined using Qubit® 2.0. Fluorometer и reagents Qubit™ dsDNA

HS Assay Kits (Invitrogen, USA) according to the manufacturer`s recommendation.

Restriction reactions were performed using the standard protocol ("Fermentas", Lithuania), which we modified and adapted to use small amounts of DNA. Genomic DNA samples (15 ng) were individually digested with methyl – sensitive AciI restriction enzyme (1 unit) in 10 x BufferO for 1 hour at 37°C. The total volume of the reaction mixture was 20 µl. Subsequently, an aliquot of this mixture (3.3 µl) containing 2.5 ng hydrolyzed DNA was used for amplification.

Digested and undigested DNA samples were used as templates for the amplification of the fragments of the promoters of the above genes (nucleotide sequences of gene CGIs were downloaded from http://genome.ucsc.edu). Table 1 shows the total number of AciI sites in the studied fragments of the *p16/CDKN2A, p14/ARF, RASSF1A* and *GSTP1* promoters. A 20 µl PCR was carried out in 10 µl Genepack kit ("Izogen", Russia), 10 pmol forward primer, 10 pmol reverse primer, 2.5 ng digested and undigested genomic DNA, and water. PCR was done using the GeneAmpR PCR System 9700. The amplification products were separated by electrophoresis in 2% agarose gel. Undigested DNA from the same subjects served as a positive control of amplification. An amplification mixture consisting of all PCR components, with the exclusion of DNA, was used as a negative control. We conducted amplification of *RAR-β2* as a control of DNA preservation after restriction (exception false-negative results due to star activity of enzyme), because this gene does not contain AciI endonuclease recognition and cleavage sites. PCR products digested DNA of second exon of *ING1* gene (CGIs is methylated) served as the positive control methylation (Figure 1).

If, on the amplification of studied fragments of gene promotors, relevant products were detected only for undigested DNA, the conclusion was drawn about the absence of analyzed CpG dinucleotides methylation. The presence of a faint, but clearly visible, band for digested DNA was considered as a positive result for methylation of a small amount of alleles in the sample DNA (Figure 1). In this case, the absence on an electrophoregram of a band for other genes, amplification of which was carried out using the same hydrolyzed DNA, indicated that a positive "signal of PCR", observed for one (or two) analyzed gene fragments, is the result of DNA methylation and there were no false-positive results due to incomplete digestion of DNA. Results were confirmed by threefold repeat methyl-sensitive PCR assays after performed digestation with restrictase AciI in three independent experiments

2.3 Statistical analysis

The statistical analysis was carried out by the standard methods with a WinSTAT 2003.1 package integrated into Excel, SPSS 20.0.0., WINPEPI (PEPI-for-Windows) [17]. The frequencies of methylation cases between the two groups were analyzed using Fisher`s exact test. We used multiple regression analysis to assess the association of exposure and age factor with hypermethylation of studied genes.

3. Results

The age of the examined liquidators covered a wide range from 38 to 76 years (on average, 57.6±1.05 years old). One hundred three unirradiated subjects, frequency matched by gender, age and smoking to the exposed groups, were used as referents. The age of unirradiated subjects at the time of examination was 36 to 77 years (on average, 54.0±1.1 years old).

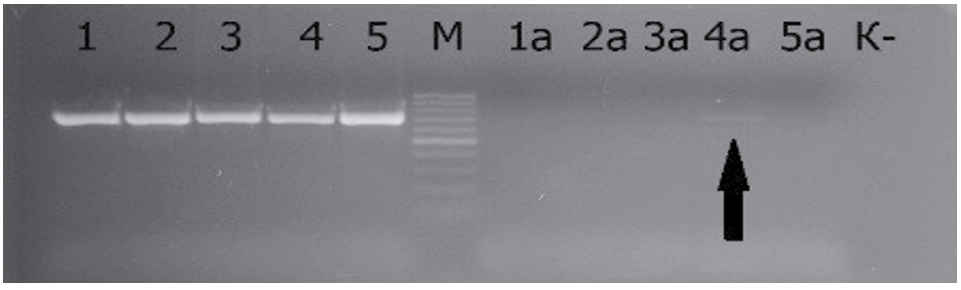
The average age of the fathers and mothers from the families of nuclear specialists at the time of examination was 53 to 75 years (on average 66.8 ± 1.26 years old) and 54 to 72 years (on average, 64.7 ± 1.14 years old), respectively. The age of offspring at the time of examination was 17 to 48 years. The control group consisted of children and unirradiated parents of similar ages from 22 families (16 families had 1 child, 3 families had 2 children). The age of the fathers, mothers and their children at the time of examination was matched with that of subjects of the control group.

The sensitivity of this methyl-sensitive PCR assay performed in our experimental conditions was

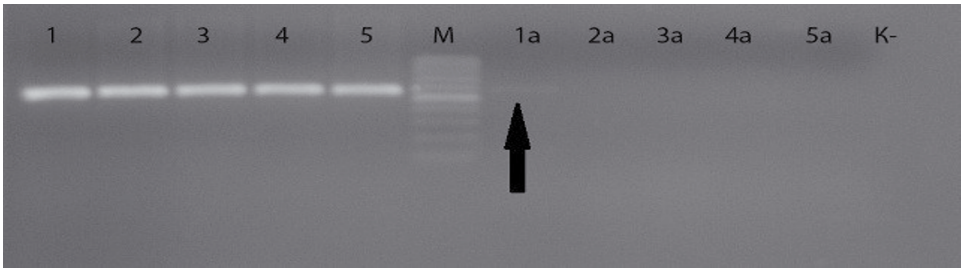
Gene	The total number of AciI sites in the studied fragments of promoters	The total number of CpG - dinucleotides in the analyzed restriction sites	The total number of CpG - dinucleotides in analyzed fragments of promoters	The analysed CpG - dinucleotides (%)
p16/CDKN2A	2	2	23	8,7%
p14/ARF	3	3	35	8,6%
RASSF1A	7	7	32	21,9%
GSTP1A	4	4	31	12,9%

Table 1. The analysed CpG - dinucleotides in gene promoters.

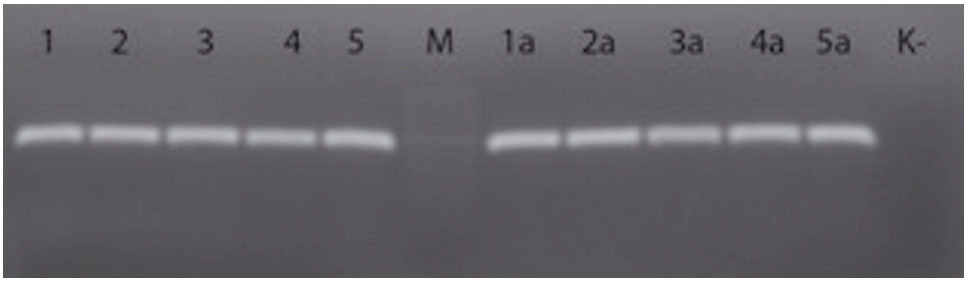
A.



B.



C.



D.

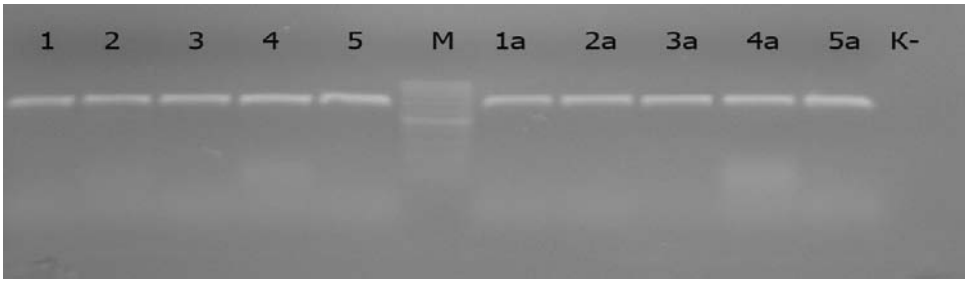


Figure 1. The analysis of promoter methylation of *p14/ARF* and *RASSF1A* genes in five liquidators (as an example) 1 - 5: undigested DNA samples; 1a – 5a: digested DNA samples. A - *RASSF1A* gene, B - *p14/ARF* gene, C - *RAR-β2* gene (control of DNA preservation after restriction), D - *ING1* gene (positive control methylation), M - molecular ladder (step - 50 bp), K – water (no DNA). The arrows indicate the detected cases of methylation.

established by using totally methylated control DNA (DNA treated with SssI methylase, "Fermentas", Lithuania) serially diluted with normal leukocyte DNA without methylation of the studied genes. PCR with 1:10, 1:100, 1:1000, 1:10000, 1:100000 diluted totally methylated control DNA produced detectable methylated bands (not shown). It is shown that the present system of methylation assay possesses the sensitivity to detect around 0.1-1.0% methylated alleles in DNA samples.

Table 2-4 and Figure 2 summarize the methylation analysis of the irradiated (liquidators of the ChNPP accident and nuclear specialists) and unirradiated subjects. The cases of hypermethylation of studied gene promoters were revealed both in exposed individuals and in the control group. The frequency of unirradiated patients with the revealed anomalous epigenetic marking varied from 1.0% to 5.8% depending on the locus. Only one subject (0.97%) from the control group

Gene	Number of cases of hypermethylation of studied gene promoters (%)		OR (95% CI)*	p-value*
	Control subjects (n=103)	Exposed subjects (n=104)		
RASSF1A	6 (5,8)	9 (8,7)	1,53 (0.46 - 5.43)	0,593
p16/CDKN2A	1 (1,0)	10 (9,6)	10,85 (1,48 - 475,42)	0,010
p14/ARF	4 (3,9)	5 (4,8)	1,25 (0,26 - 6,49)	1,000
GSTP1	1 (1,0)	11 (10,6)	12,06 (1,68 - 524,26)	0,005

Table 2. Revealed cases of hypermethylation of studied gene promoters in examined subjects.

** - Fisher`s exact test (two-tailed)*

Gene	Correlation «age – gene methylation» (two-tailed p-value)	
	Control subjects (n = 162)	Exposed subjects (n = 104)
RASSF1A	0,213 (0,006)	0,212 (0,031)
p16/CDKN2A	0,043 (0,587)	0,130 (0,190)
p14/ARF	0,150 (0,056)	0,043 (0,667)
GSTP1	0,015 (0,854)	-0,161 (0,104)

Table 3. Correlation between age and gene methylation in examined subjects.

	B*	β**	p-value
	Methylation (*RASSF1A* + *p16/CDKN2A* + *p14/ARF* + *GSTP1*) ~status of subject + age		
Constant	-0,125±0,091		0,178
Exposure	0,187±0,060	0,203	0,002
Age	0,005±0,002	0,152	0,013
	Model as a whole		$2,0\times10^{-6}$
	Methylation (*p16/CDKN2A* + *p14/ARF* + *GSTP1*) ~ status of subject + age		
Constant	-0,045±0,089		0,615
Exposure	0,169±0,047	0,262	$7,1\times10^{-5}$
Age	0,001±0,001	0,059	0,365
	Model as a whole		$8,2\times10^{-6}$

Table 4. The multiple regression analysis of dependence of number of methylation genes on age and status of subject (control/exposed).

** - coefficient of linear regression; ** - standardized coefficient of linear regression (in units of standard deviations)*

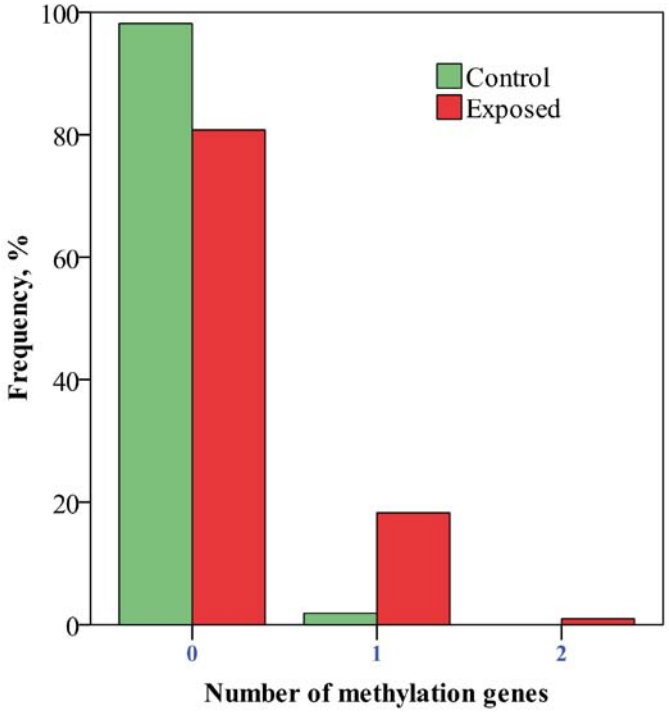


Figure 2. The distribution of the total number of hypermethylation cases of *p16/CDKN2A* and *GSTP1* gene promoters in examined groups.

had methylation of the studied CpG – dinucleotides of two genes (*RASSF1A* and *p14/ARF*). The frequency of irradiated patients with the revealed hypermethylation was 8.7%, 9.6%, 4.8% and 10.6% for *RASSF1A, p16/CDKN2A, p14/ARF, GSTP1* genes, respectively. Six irradiated individuals had methylation of the studied CpG – dinucleotides of two genes. As shown in Table 2, frequency of patients with the revealed hypermethylation of *RASSF1A* and *p14/ARF* genes did not differ significantly between control and exposed groups. Promoter methylation of at least one of the analyzed genes was observed in 28.92% exposed individuals and significantly exceeded (p = 0.001) such rate in a one-age control group (10.68%). A significantly elevated frequency of individuals with abnormal methylation of *p16/CDKN2A* and *GSTP1* genes was revealed in the exposed group compared to the control group (p = 0.0097 and p = 0.005, respectively). The distribution of the total number of hypermethylation cases of *p16/CDKN2A* and *GSTP1* gene promoters is presented in Figure 2. It is shown, that the frequency of patients with the revealed hypermethylation of these genes (one and/or two genes) in the exposed group is higher compared to the control group (effect size: OR = 12.02, 95% CI = 2.76 - 108.05, p-value = 5.3×10^{-5}).

To evaluate the significance of the age factor in the induction of abnormal methylation, healthy young subjects (age ≤ 35 years, 48 subjects) were added to our control group (results were received previously). The occurrence of promoter methylation of *RASSF1A* gene significantly correlated with aging both in the control group (r = 0.213; p = 0.006) and in the exposed individuals (r = 0.212; p = 0.031). No similar trend was found for other genes (Table 3). Along with this, no dependence of the promoter methylation of *RASSF1A* gene on exposure factor was revealed. On this account, we did the multiple regression analysis of dependence methylation on "age + exposure" in two variants: 1) for all studied genes; 2) for all studied genes excepting *RASSF1A* gene. As seen in Table 4, the growth in the number of methylated loci of a total set of studied genes is due to either the age or the exposure factor. However, it has been shown that the growth in the number of methylated loci of a set of genes *p16, p14* and *GSTP1* (*i.e.* exception of *RASSF1A* gene) is exclusively due to the fact of exposure.

Table 5 summarizes the methylation analysis of the children and parents from families of fathers-nuclear specialists and from control families. The cases of hypermethylation of studied gene promoters were revealed in DNA samples from parents (father and mother) both in nuclear families and in the control families. No significant differences were found in the frequency of irradiated and unirradiated fathers with

The studed genes	Number of cases of hypermethylation of studied gene promoters (%)					
	Families of nuclear specialists			Control		
	Father (n = 21)	Offsprings (n = 28)	Mother (n = 21)	Father (n = 22)	Offsprings (n = 25)	Mother (n =22)
RASSF1A	3 (14.29)	0	2 (9.5)	2 (9.1)	0	2 (9.1)
p16/CDKN2A	2 (9.5)	0	0	1 (4.5)	0	0
p14/ARF	0	0	1 (4.76)	1 (4.5)	1 (4)	1 (4.5)
GSTP1	1 (4.76)	1 (3.57)	0	1 (4.5)	0	0

Table 5. The methylation analysis of the children and parents from families of fathers-nuclear specialists and from control families.

Note: The frequencies of methylation cases in children, fathers, mothers from the families of nuclear specialists were compared to those of analogous controls.

the revealed promoter hypermethylation of studied genes that probably was due to the small number of examined individuals. However, a trend towards a higher frequency of cases of promoter methylation of *p16/CDKN2A* gene in exposed fathers (9.5%) as compared to the unirradiated fathers (4.5%) was discovered. The frequencies of mothers with revealed hypermethylation was almost the same in control families and in nuclear families. No methylation genes were found in the offspring of control families. Only one offspring (3,6%) from the control group had methylation of the studied CpG – dinucleotides of *GSTP1* gene. It should be noted that no association between the number of methylation genes in nuclear specialists and summarized accumulated doses over a period of work with tritium was revealed (r = 0.217, p = 0.172).

4. Discussion

In small amounts of templates in DNA samples (around 0.1 - 1.0%) from irradiated and unirradiated subjects we revealed the methylation of CpG – dinucleotides of gene promoters using the above-described methodology, which is characterized by the optimization and standardization of conditions of restriction and amplification. The presence of a faint but clearly visible band considered as a positive result for methylation of small amounts of alleles in the DNA sample was noted by other researchers [12-14], who used methylation-sensitive PCR or methylation-specific PCR assay. The obtained data point to a high sensitivity of the method used. We detected that the sensitivity of the methyl-sensitive PCR assay performed in our experimental conditions is around 0.1-1.0% methylated alleles in DNA samples, in agreement with results obtained by other researchers [13,14]. Recent results confirm cases of the promoter hypermethylation of several genes (*APC, CCND2, GSTP1, p15, p16, RARbeta2, HCAD et al.*) in blood plasma DNA samples or blood leukocytes DNA sample from healthy subjects [14,18].

In the present work we investigated for the first time whether hypermethylation of CGIs in promoter of genes involved in the basic protective functions of cells are induced in healthy subjects by prolonged exposure to radiation at low and medium doses. Obtained results point to the significance of radiation as a factor in the induction of revealed epigenetic modifications in human blood leukocytes for prolonged periods after exposure.

To date, it was established that DNA methylation is a labile enough epigenetic characteristic, although the molecular mechanisms of this “lability” remain unclear. For example, it was revealed that there are numerous epigenetic differences in MZ twins and that such epigenetic variation can serve as a new source of putative molecular substrates for discordance of MZ twins [19]. A number of environmental factors, both chemical and radiation have been found to alter the epigenome, including DNA methylation [5-10]. It should be emphasized that most of the investigations devoted to evaluation of the epigenetic effects of radiation (X - rays and γ – rays, range of doses of 0.1 to 2.5 Gy) was performed using cell cloning techniques. Most of the research is devoted to the assessment of DNA methylation in long interspersed nuclear element-1 (*LINE-1*) and *Alu* repetitive elements as a surrogate of genome-wide methylation. The results indicate that although the response of cells is predominantly in hypomethylation, hypermethylation occurs as well [5,6]. For example, observed epigenetic changes were mostly in DNA hypermethylation at repetitive elements *SAT2* and *MLT1A* in progeny of irradiated HPV-G cells after 20 passages, but hypomethylation, which is not identified with respect to the sequence context, was also

detected [6]. Reduction of various epigenetic indicators (the levels of global genomic hypomethylation, trimethylation of histone H4-Lys20, methyltransferases Dnmt1 and Dnmt3a/b, methyl-binding proteins MeCP2 and MBD2) has been observed in experiments using rodents exposed to acute (2.5 Gy) or chronic (0.5 Gy) irradiation [7,8]. Hypermethylation of the *p16* tumor-supressor gene in mouse exposed to chronic low-dose radiation (X-ray, 0.5 Gy, 10 days) was revealed. Sex and tissue-specific differences in *p16* promoter methylation was demonstrated. In male liver tissue, *p16* promoter methylation was more pronounced than in female tissue. In contrast, no significant epigenetic changes were noted in the muscle tissue of exposed males and females. Radiation also did not affect significantly methylation status of *MGMT* promoter. It was proved that chronic low-dose radiation exposure is a more potent inducer of epigenetic effects than the acute exposure [9].

The molecular mechanism by which radiation induces aberrant methylation remains unclear. Although, it cannot be excluded that reactive oxygen species induced by radiation, possibly cause an increase in the activity of DNA methyltransferases. Further, DNA strand breaks induced by irradiation exposure may cause DNA methyltransferases to bind with higher affinity at specific sites.

It should be emphasized that our investigation was performed using white blood mature cells, which regularly renew and arise as a result of active proliferation and differentiation of their precursors - stem hemopoietic cells. Firstly, methylation revealed in leukocytes of exposed people can be explained by some proportion of cells with these epigenetic modifications derived from irradiated stem precursors as a result of renewal of circulating blood cells. At present there is sufficient evidence in favour of transmission of DNA methylation status from dividing cells to its mitotic descendants [16]. Thus, biallelic hypermethylation of *MGMT* gene promoter was revealed in myeloid precursor cell line, derived from the bone marrow of a patient with therapy-related myelodysplastic syndrome who had no overt post - myelodysplastic syndrome leukemia [20]. Secondly, genotoxic effects in irradiated subjects are the cause of both external and internal exposure as a result of accumulation of long-lived radionuclides in the organism. Thirdly, taking into account a large array of experimental evidence, there is a relationship between epigenetic disturbances, genomic instability, and bystander effect. It has been shown that bystander factors persist in Chernobyl liquidator blood serum for more than 20 years after irradiation [21]. On the one hand, hypermethylation itself in the regulatory regions of the studied genes, leads to a decrease in expression of genes which are responsible for the maintenance of genomic stability (the control of the cell cycle and apoptosis, detoxification of xenobiotics etc). On the other hand, we should not exclude genomic instability itself as a cause of the coordination disruption of the methylation processes, including induction of the aberrant epigenetic marking. Moreover, as it is known, the enzyme activity in the process of copying the methylation pattern during replication is attributed to the DNMT1 methyltransferase [16]. DNA methylation errors with age were found to be associated with increased methylation within active proliferative mitotic cells [22]. For example, in a highly mitotic tissue such as epidermis, DNMT1 was expressed in epidermal progenitors and lost during differentiation. It can be assumed that DNMT1 is required for sustained repression of differentiation. It has been shown that *CDKN2A* and *CDKN2B* are two major sites of age-dependent methylation that is observed in multiple tissues. Probably, DNMT1 also was required to repress *CDKN2A* and *CDKN2B* genes, two cyclin-dependent kinase inhibitor genes that may inhibit adult stem cell self-renewal [23]. It is possible that there is the above-described “aberrant” compensatory mechanism, as increased levels of predictors of apoptosis (cells with the CD95+ immunophenotype expressing the FAS marker of apoptosis) have also been observed in liquidators of the ChNPP accident [1].

The age of the examined liquidators covered a wide range from 38 to 76 years. The mean age of volunteers from control group was matched with that of liquidators to eliminate the possibility of DNA methylation by aging. As a whole, dependence of the aberrant hypermethylation of gene promoters on the age was shown. In this regard, our results are consistent with findings of other researchers. In recent years, age-related disorders in genomic methylation (total hypomethylation and gene-specific hypermethylation/hypomethylation) have been revealed [24-26]. These epigenetic changes are identified either in white blood cells or other tissues of healthy individuals who do not have tumor pathology. In the wide-genome investigations it was established that DNA methylation sites which are located within CGIs more often become hypermethylated with age as compared to sites outside an island. As it is shown age-related hypermethylation was revealed for thousands of sites, and hypermethylation is more site-specific than hypomethylation. Tissue-specific epigenetic changes are frequently located outside CGIs and associated with decreased methylation, and common methylation changes show the opposite trend [24]. Hypermethylated sites are overrepresented among genes that are involved in DNA binding, transcription regulation, processes of anatomical

structure and developmental process, cortex neuron differentiation [26]. As it was demonstrated age-related promoter hypermethylation was revealed only for *RASSF1A* gene. In other investigations the significant dependence between promoter hypermethylation of *RASSF1A* and *GSTP1* genes and age of healthy patients was revealed for cells of prostate ($r=0.27$; $p = 0.01$ and $r = 0.59$; $p < 0.0001$, respectively) [25]. However, in the above-mentioned work no age-related hypermethylation in the samples of blood plasma DNA was revealed, neither for *GSTP1* gene nor for *p16* gene [18].

No CpG methylation of promoter regions was found in non-exposed offspring born from irradiated fathers. One the one hand, this has to be expected, because methylation, if present, was lost by reprogramming during embryogenesis [27]. One the other hand, the age of examined patients, as a rule, did not exceed 40 years. Therefore, acceleration of 'epigenetic rates' (as a result of genomic instability), that may be expected, was not observed in terms of studied age period. Besides, our examined group can not be considered as representative at present.

The recent data show, that aberrant hypermethylation of some genes observed in malignant cells in patients with oncological diseases, was revealed in leukocytes of peripheral blood of these subjects also [28,29]. In the present work on blood leukocytes of healthy subjects we observed methylation changes which reproduce gene hypermethylation found in malignant cells. Additionally, hypermethylation of some genes in leukocytis blood DNA samples was revealed in patients with nononcological age-related disease, above all in those with cardiovascular conditions [30].

In conclusion, our study showed for the fist time that prolonged radiation exposure at low and medium doses is associated in healthy subjects with hypermethylation of genes involved in the basic protective functions of cells, that is revealed in blood leukocytes in remote periods after irradiation of human body. Additional studies are required to define the role of hypermethylation of these and other genes in induction of age – related oncological and nononcological disease. Further research of different epigenetic changes in the offsprings of irradiated parents is also necessary.

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