The study of aberrant methylation in blood leukocytes of irradiated parents and their children

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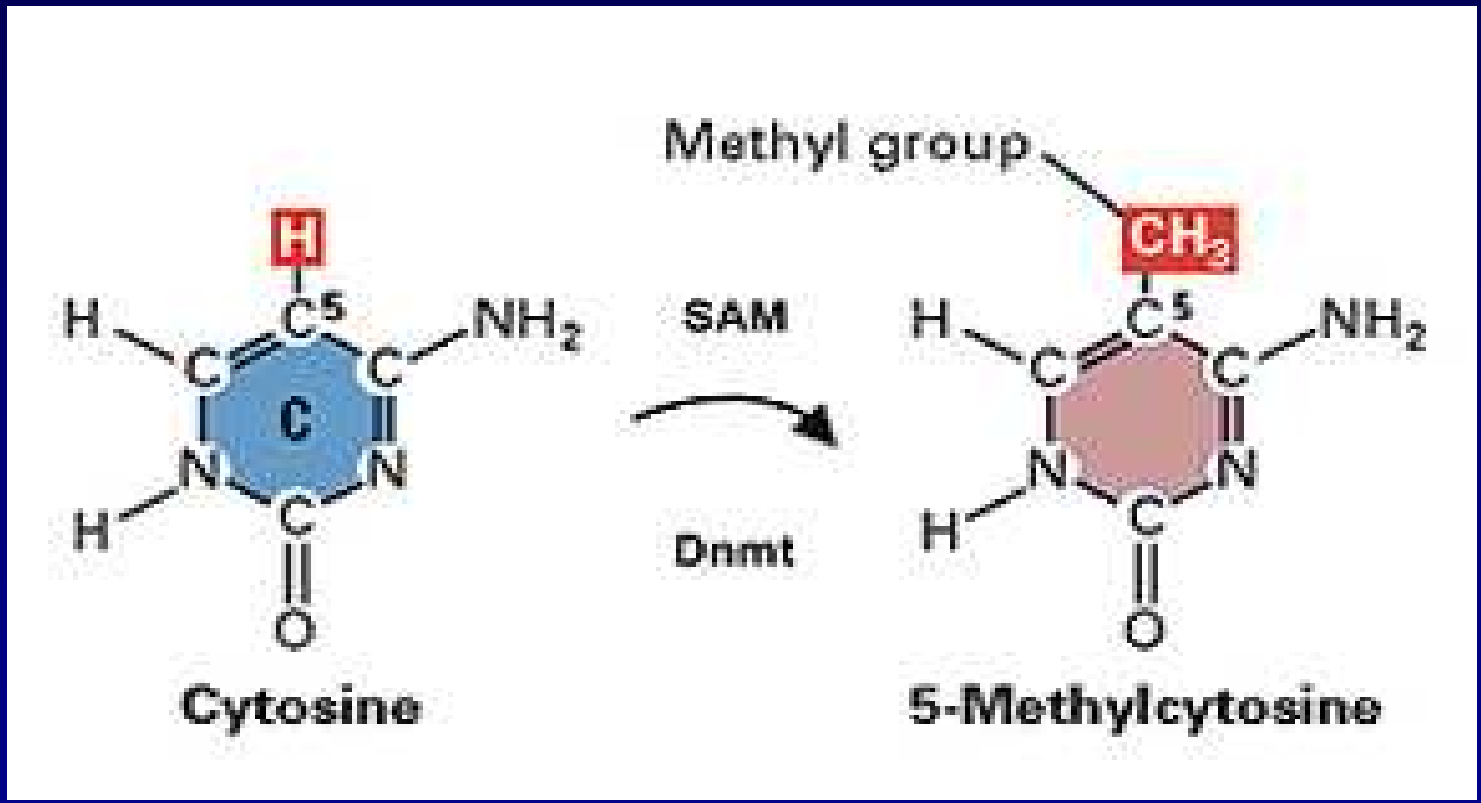
DNA methylation - a main epigenetic genomic modification



CpG islands (CGIs)

- short genomic regions (500 bp to a few kb)
- C+G $\geq$ 0.5, $\frac{\text{CpG obs}}{\text{CpG exp}} \geq 0.6$
- located in the proximal promoter region of approximately 75% of human genes
- Unmethylated CpG dinucleotides of CGIs are associated with active promoters
- hypermethylation of CGIs leads to transcriptional repression and gene inactivation

The aim of investigation: to evaluate long-term epigenetic consequence associated with hypermethylation promoter of genes of basic protective functions of cells in irradiated parents and their children blood leukocytes

Examined subjects

- **Liquidators (n=83) of the Chernobyl Nuclear Power Plant accident (ChNPP) in 1986-1987**

The individual doses ranged from 50 to 460 mSv (average dose 221 mSv).
The time between the end of clean-up work and examination varied from 17 to 2 years.

- **Full families (n=21) of fathers - nuclear specialists (All-Russian Research Institute of Experimental Physics, Sarov, Russia)**

The summarized accumulated doses over a period of work with tritium and tritium oxide ranged from 37 to 994 mSv

The time between the end of work and examination varied from 2 to 46 years (for 48 % of irradiated subjects – more than 10 years)

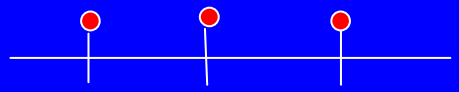
The time between irradiation of the fathers and conception of the children varied from few months to 18 years

- **Unirradiated subjects (n=103) and unirradiated full families (n=22) of similar ages**

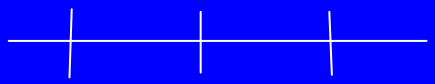
DNA methylation analysis

Genomic DNA was isolated from blood leukocytes

Restriction

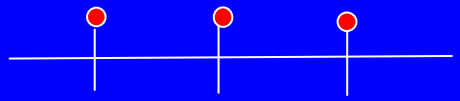


Methylated DNA



Unmethylated DNA

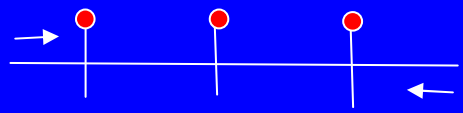
AciI (5'…C↓C GC…3')

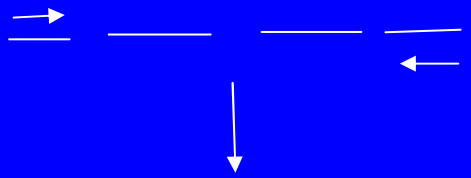


Restriction sites are not cleaved

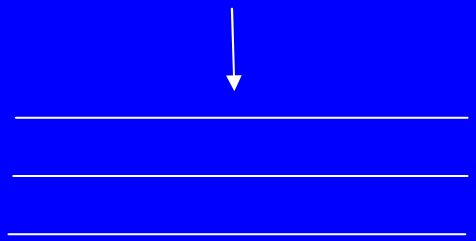
Restriction sites are cleaved

PCR





no product of amplification



Amplification of DNA fragment

electrophoresis in 2% agarose gel

Nucleotide sequence of analysed fragment of promoters of *p16/CDKN2A* gene (as example)

F ⟶

GGATTTCTTTTTAACAGAGTGAACGCACTCAAACACGCCTTTGCTGGCAGGCGG

GGGAGCGCGGCTGGGAGCAGGGAGGCCGGAGGGCGGTGTGGGGGGCAGGTGG

GGAGGAGCCCAGTCCTCCTTCCTTGCCAACGCTGGCTCTGGCGAGGGCTGCTTC

CGGCTGGTGCCCCCGGGGGAGACCCAACCTGGGGCGACTTCAGGGGTGCCACA

TTCGCTAAGTGCTCGGAGTTAATAGCACCTCCTCCGAGCACTCGCTCACGGCGT

CCCCTTGCCTGGAAAGATACCGCGGTCCCTCCAGAGGATTTGAGGGACAGGGTC

GGAGGGGGCTCTTCCGCCAGCACCGGAGGAAGAAAGAGGAGGGGCTGGCTGGT

⟵ R

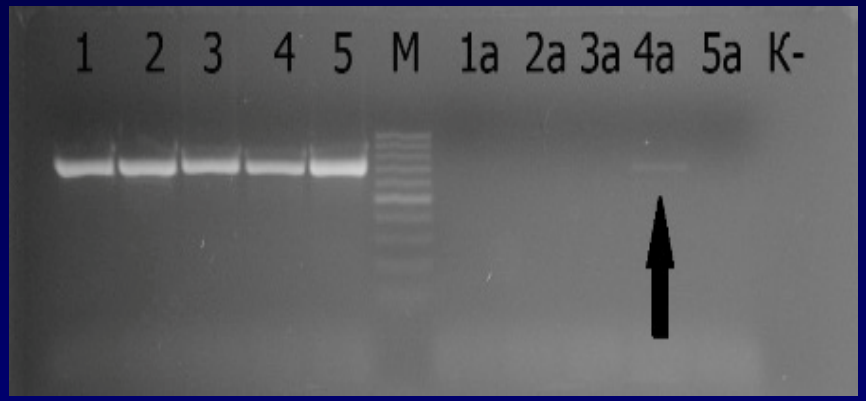
CACCAGAGGGTGGGGCGGACCGCGTGCGCTCGGCGGCTGCGGAGAGGGGGAG

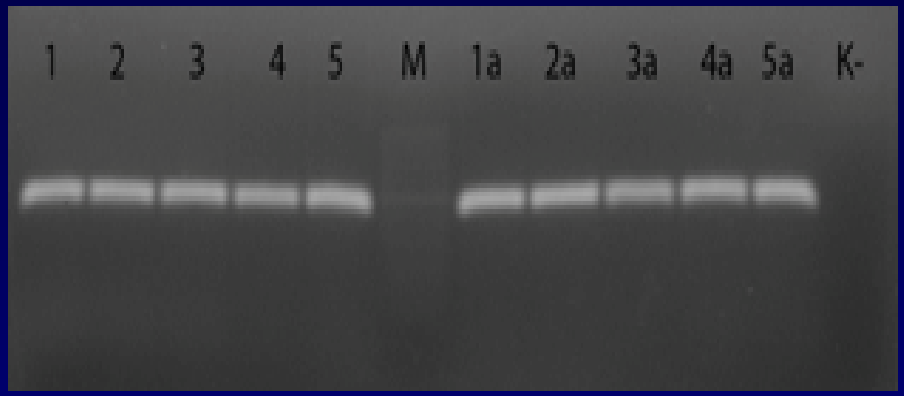
AGCAGGCAGCGGGCGGCGGGGAGCAGCATG ⟶

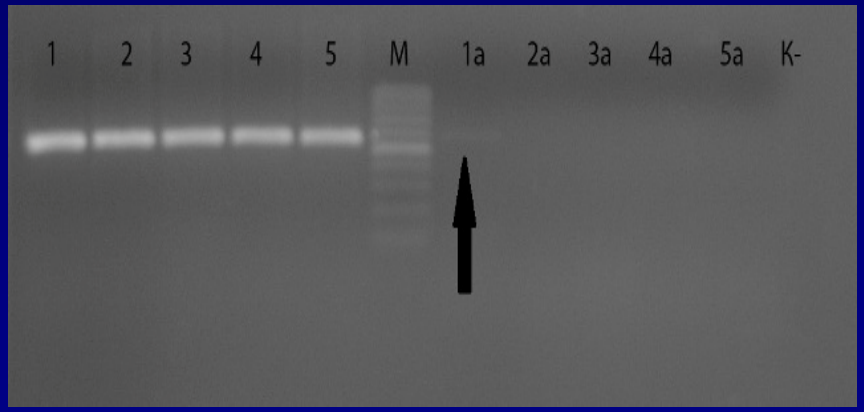
The analysed CpG - dinucleotides in gene promoters

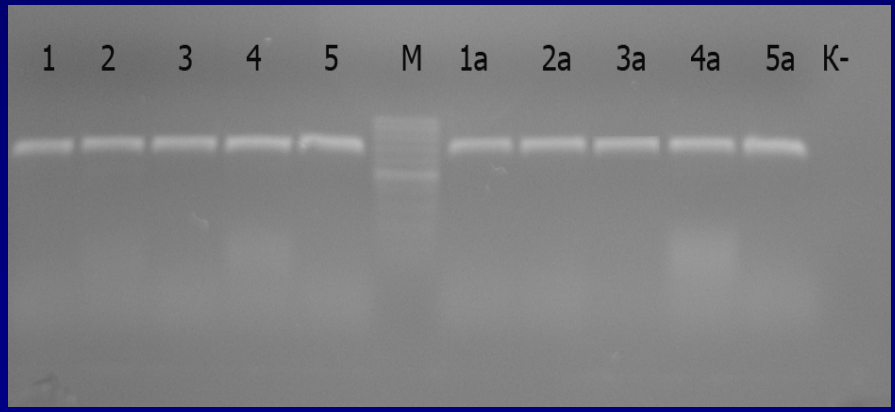
Gene	Function	The total number of AciI sites in the studied fragments of promoters	The total number of CpG - dinucleotides in analyzed fragments of promoters	The analysed CpG - dinucleotides (%)
p16/CDKN2A	Cell cycle	2	23	8,7 %
p14/ARF	Cell cycle	3	35	8,6 %
RASSF1A	Cell cycle	7	32	21,9 %
GSTP1	Xenobiotic detoxification	4	31	12,9%

The analysis of promoter methylation of *p14/ARF* and *RASSF1A* genes in five liquidators (as an example)









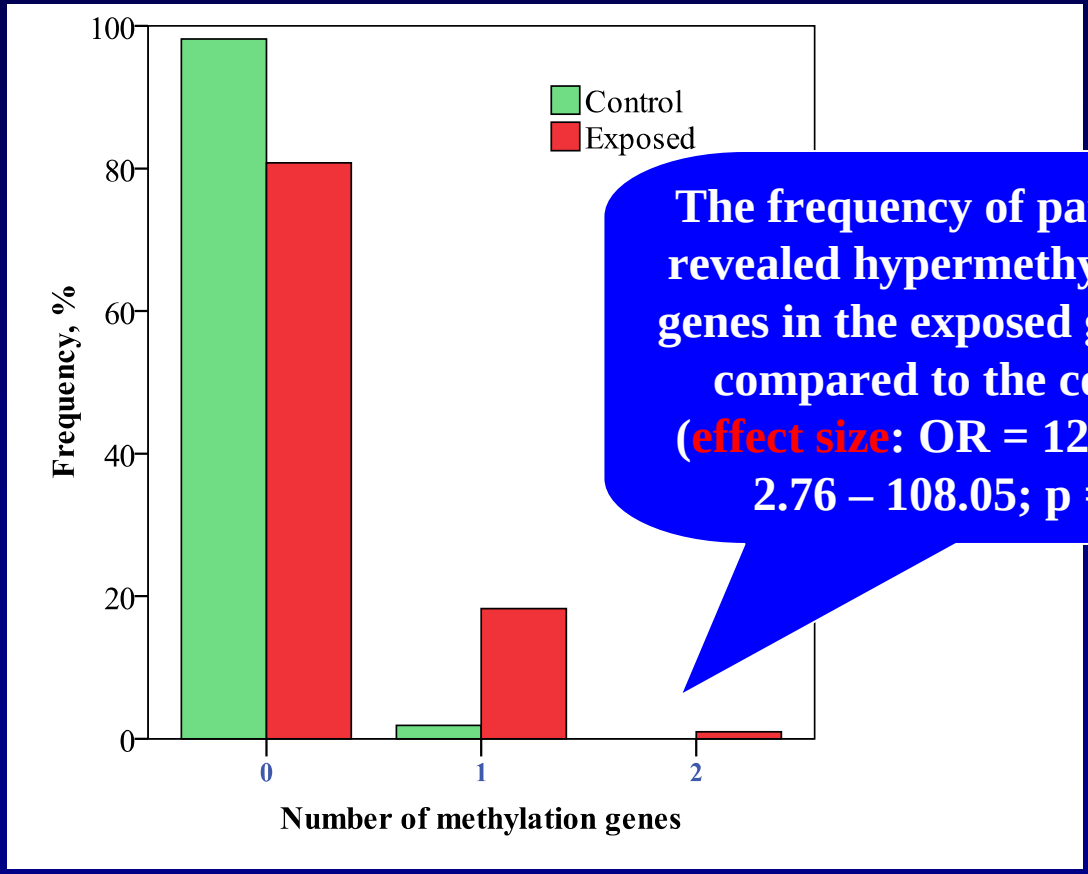
1 - 5: undigested DNA samples; 1a – 5a: digested DNA samples. A - *RASSF1A* gene, B - *p14/ARF* gene, C - *RAR-β2* gene (control of DNA preservation after restriction), D - *ING1* gene (positive control methylation), M - molecular ladder (step - 50 bp), K – water (no DNA). The arrows indicate the detected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

Revealed cases of hypermethylation of studied gene promoters in examined subjects

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**

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

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



The frequency of patients with the revealed hypermethylation of these genes in the exposed group is higher compared to the control group (effect size: OR = 12.02; 95% CI = 2.76 – 108.05; p = 5.3 x 10^{-5}

Correlation between age and gene methylation in examined subjects

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)

The multiple regression analysis of dependence of number of methylation genes on age and satus of subject (control/exposed)

	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			**$8{,}2\times10^{-6}$**

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

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

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

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

CONCLUSION

- Prolonged radiation exposure at small and medium doses is associated 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

- No CpG methylation of promoter regions of studed genes was found in non-exposed offsprings born from irradiated fathers, that requires further investigations

Practical significance

In the several wide-genome investigations the association between hypermethylation and chronological age (r = 0.80 - 0.95 for different tissues) was established (Day K. et al., Genome Biology, 2013; Johansson Asa et al., PLoS One, 2013; Horvath S., Genome Biology, 2013; Hannum G, et al., Mol Cell, 2013).

The accuracy of the age forecast based on hypermethylation estimation of 350 genes is +/- 3.6 years

Hypermethylation of some genes observed in malignant cells in patients with oncological diseases, was revealed in leukocytes peripheral blood of these subjects also (Al-Moundhri M. S. et al., PLoS One, 2010; Flanagan J. M., et al., Human Molecular Genetics, 2009; Tahara T. et al., Cancer Prev. Res., 2013)

Hypermethylation of some genes in leukocytes blood DNA samples was revealed in patients with nononcological age-related disease, above all with cardiovascular (Lakshmi Sana V. et al., Molecular and Cellular Biochemistry, 2013; Kim G. H. et al., Antioxid Redox Signal, 2013

Ionizing radiation may make additional contribution in the hypermethylation processes

Radiation-induced premature aging and age – related disease

«epigenetic clock» of irradiated organism

Thank you for attention!