DEVELOPMENT OF METHODICAL APPROACH TO THE IDENTIFICATION OF THE FEATURES OF THE GENETIC POLYMORPHISMS AND GENE EXPRESSION IN CHILDREN UNDER INFLUENCE OF CHEMICAL ENVIRONMENTAL FACTORS ON THE EXAMPLE OF STRONTIUM

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Methodological approaches evaluating the features of genetic polymorphism associated with exposure to chemical etiology factors for the identification of genetic susceptibility markers were developed. The technologies, methodological aspects of the use of polymerase chain reaction, DNA sequencing fragments, studies of spontaneous and induced strontium expression of candidate genes that help identify changes in the genome and transcriptome in order to identify early disorders of adaptation processes in chronic environmental burden to prove the injury and assessment of individual exposure risk chemical factors were suggested.
Keywords: sensitivity markers, genetic polymorphisms, gene expression, sequencing, strontium

Background

The development of new approaches to the detection of adaptability of an individual and population as a whole to the impact of chemical mutagens associated with gene polymorphism is a topical issue. An organism's susceptibility to man-induced chemical factors mainly depends on the genetic associations that determine the following: the activity of enzymes in the xenobiotic detoxification system; factors involved in the pathogenesis of man-induced disorders in target organs; the state of the proteins of predisposition to cancer-proliferated states and immune response factors. In this regard, genetic make-up and genetic polymorphism are of particular interest. Genetic polymorphism is the recurrence within a population of two

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or more discontinuous genetic variants of a specific trait in such proportions that they cannot be maintained simply by mutation. According to scientific literature, the prevalence of the minor allele in the population takes an average of 10% on most significant SNPs. SNP (a variation in a single nucleotide) identification is used in the assessment of a qualitative polymorphisms. Detection of the structure and characteristics of gene expression that code all the protein molecules as well as the use of new diagnostic methods in the testing of various genetic polymorphisms (two or more variants of the same gene within a population) can predict the risk of certain diseases. In particular, genetic testing can reveal the predisposition to some disorders including detoxification of the medical and other heterologous compounds, carcinogenesis, cardiovascular diseases, and high-risk pregnancy.

In order to ensure early diagnostics and effective prevention of deadaptation in children exposed to man-induced factors, it is necessary to detect the specifics of identification of the genetic indicators disorders [1–23].

The development of research and methodological basis on this topic is needed to ensure the protection and stability of human genome under the exposure to adverse environmental factors.

The **purpose of the present study** was to develop methodological approaches to the identification of the specific aspects of the genetic polymorphism as a marker of early adaptation disorders (immune, metabolic, physical, and proliferative processes) in children under chronic exposure to chemical environmental factors on the example of strontium.

Materials and Methods. The design of the assessment of the genetic polymorphism under exposure to the chemical factors included a comparative analysis of the population at risk (exposed population) and the control group (non-exposed population). It also included an individual and population analysis conducted at various stratification levels (cellular and molecular).

In order to identify the risk and health effects, methodological approaches based on targeted instrumental database on genotyping (sequencer, Real-time Thermocyclers) and the study of cellular and gene expression (flow cytometry, cell sorter) were used as exemplified by the child population exposed to strontium.

The methodological procedure for SNP identification was based on the basic guidelines of the METHODOLOGICAL RECOMMENDATIONS "The List of gene polymorphism markers responsible for man-made features of chemical factors' mutagenic activity" MR 4.2.0075- 13 of 20.08.2013.

Personified genotyping was performed. It was based on an individual gene panel that reflected the professional, environmental, and social (bad habits) conditions and the presence of chronic pathologies.

Verification of the genetic polymorphism (sensitivity markers) included an assessment of the groups of genes that reflected the peculiarities of the exchange, topical (organ), immune and detoxification processes:

• identification of gene mutations of enzymes of the 1 and 2 phases of detoxification;

• gene polymorphism of proteins involved in the pathogenesis of man-induced disorders in the target organs (elastase gene, endothelial growth factor) and metabolic processes;

• genotyping of the predisposition to cancer-proliferating conditions;

• definition of immunogenetic markers.

To diagnose genetic polymorphism at the DNA level under exposure to various factors, we have selected the following *genes and their regions as sensitivity markers of potential risks of environmentally-induced health disorders*: Cytochrome P-450 CYP1A1 (rs4646421 and rs1048943), coproporphyrinogen oxidase CPOX (rs1131857), methylenetetrahydrofolate reductase (rs1801133) MTHFR, the endothelial NO-synthase eNOS (rs1799983), the protein apolipoprotein E ApoE (rs429358), matrix proteases MMP9 and MMP12 (rs17576 and rs652438) , sulfotransferase SULT1A1 (rs9282861), oncogenes BRCA1, BRCA2 and TP53 (rs3950989, 1,801,439, 1,042,522), ESR1 estrogen receptor gene (rs2228480) and the promoter region TNFA gene (rs1800629) tumor necrosis factor, GSTA4 (glutathione transferase), SOD2, ZMPSTE24 (zinc-metallopeptidase), TERT, DRD2, SIRT1, TLR4 (toll-4 receptor), PPAR, FAS, FOXP3, VEGF, APO-E, NO-synthase, ACE.

To determine the genotype of a person, we used the method of allelic discrimination. The differences between heterozygous, homozygous wild and minor versions were established by the differences in the amplification reactions of corresponding primers.

The DNA detection procedure included the following: collection of the DNA samples from buccal mucosa; isolation of genomic DNA by phe-

nol-chloroform extraction; analysis of the polymorphic variants of the genes with the help of PCR in real time; amplification and detection of variant alleles using CFX96 thermocycler along with the primer structure and parameters of the temperature cycles described in the literature; processing of the obtained results by the method of allelic discrimination along with the identification of differences between the homozygous and heterozygotes substitution and normal homozygote.

The identification of the mutagen-induced genetic expression included the assessment of the transcriptome of the target cells of the studied factors in the exposed population using the criterion of spontaneous and induced expression of the test gene. The comparison material included control bioassays, with an acceptable level of contamination mutagen.

The estimation of the induced gene expression included the following:

1. Collection of the material and its transportation to the laboratory.

2. Determination of the blood mutagen.

3. Separation of the blood cells and their identification.

4. Sample preparation and isolation of total RNA.

5. Reverse transcription of mRNA into cDNA.

6. Amplification of cDNA with prepared primers and probes.

7. Calculation of a relative expression of the marker gene.

8. Statistical analysis

The results of the specific gene expression analysis allowed us through the allocation of specific cellular phenotypes of CD4 +, CD8 +, CD16 +, expressing alpha defensin gene, to predict the immune disorders associated with strontium exposure.

The minutes of the candidate gene expression studies, performed by the internal control (GAPDH gene), included:

1. The relative amount of protein (RNA) alpha defensin (spontaneous expression level of CD8 +)

2. Strontium-induced level of protein expression (RNA) of alpha defensin (induced expression level CD8 +).

The results were analyzed using the ratio of strontium-induced and spontaneous expression of the alpha defensin levels.

The specific inducibility capabilities were assessed by the magnitude and direction of expression variability induced by strontium exposure ex vivo in comparison with spontaneous expression given the base exposure (blood strontium content).

Statistical analysis of the genetic data survey was carried out using the accumulated data sets individually for each gene in two groups: experimental and control. We used the statistical methods to describe the equilibrium frequencies of genotypes and genetic alleles based on the Hardy-Weinberg equilibrium. The differences between the two populations were established using the odds ratio (OR) for various models of inheritance: additive, common multiplicative dominant and recessive, and were considered significant at p <0.05.

Results Table 1 below presents the results of the assessment of polymorphic variants of the candidate genes of different functional systems in strontium-exposed patients.

The structure of max polymorphism mutations had detoxification genes - 37.5% of the entire gene sample. The exchange genes and immune regulation genes are second with 27.5%. Substitutions in somatic genes expressed less. This is connected to the high polymorphism lability of functional proteins and metabolism neurotransmitters that ensure adaptability to the changing characteristics of the environment and high sensitivity of transcriptome-genomic relationships of immunoregulatory genes.

Specific inducibility was assessed by the magnitude and direction of expression variability induced by ex vivo strontium exposure in comparison with spontaneous expression given base exposure (blood strontium content).

The data obtained in the tests are given in Table 2.

The results of candidate gene sequencing

Chromosome	Gene	The number of polymorphisms as compared to the reference sequence					
		1 p	2 p	3 p	4 p	5 p	6 p
1	2	3	4	5	6		
1	MTHFR	13	13	13	14	23	23
1	CLCN6	0	1	0	0	1	1
1	ZMPSTE24	4	3	3	4	1	4
3	CPOX	1	8	7	8	8	10
5	TERT	9	5	14	3	6	5
5	IL17B	0	2	0	2	1	1
6	PPARD	4	6	4	5	5	1
6	VEGFA	2	4	3	2	8	5
6	IL17F	0	0	0	0	0	1
6	GSTA4	11	12	9	15	11	15
6	SOD	4	0	6	2	0	6
6	HLADRB1	45	19	6	0	15	10
7	NOS3	19	14	12	14	12	14
9	TLR4	0	1	1	1	2	2
10	ACTA2	1	1	1	2	0	0
10	FAS	0	4	8	5	7	9
11	SIRT3	9	12	8	12	8	1
11	TH	17	16	15	8	16	18
11	DRD2	19	12	16	14	19	20
13	IL17D	3	1	2	1	1	1
15	CYP1A2	5	3	3	4	3	4
16	SULT1A1	41	33	37	10	39	37
16	IL17C	3	3	2	3	4	2
17	TP53	7	7	6	6	7	6
17	ACE	4	28	24	22	21	25
19	APOE	3	3	3	2	2	5
X	FOXP3	3	3	4	0	2	2
14 chromo-somes	27 genes	227	214	207	159	222	228

Characteristics of the gene expression index in defensin alpha in patients with various strontium levels in blood

№ №	Expression			Level of CD8+, % Spontaneous	Storntium level in blood, µg/ml Strontium-induced
	Spontaneous	Strontium-induced	Expression index		
1	0.007	0.01718	2.26	24	0.0181
2	0.011	0.0121	1.05	26	0.012
3	0.062	0.2349	3.8	35	0.123
4	0.010	0.007	0.67	17	0.090
5	0.603	0.359	0.60	14	0.0862
6	0.128	0.085	0.66	21	0.0553
7	0.051	0.1429	2.8	28	0.0216
8	0.072	0.19541	2.71	29	0.0347

9	0.024	0.0394	1.63	32	0.032
10	0.033	0.05215	1.58	28	0.0201
	norm		0.7–3.0	28.0 ± 4.7	0.024 ± 0.007

The above values of the spontaneous and induced expressions and the proposed index in strontium exposed patients differed significantly from the values in these patients with the strontium content in blood below the reference level (0.077 mg/dm3) (p <0.05). To validate the liminality of the expression index which would characterize the immune disorders in a patient, the following principle were used. The liminality principle suggests a gap between the threshold (minimum current) levels of a factor and invalid levels by a factor of 3 or higher. Several authors (Trifonova EA et al Acta nature Vol.6, -.. №2 (31) -2014) consider the gene expression of more than 1.5 to be a significant alteration, although they identify its increased intensity in women with pre-eclampsia by 6-8 times. Therefore, we recommend that a 1.5 times decrease in the gene expression be considered a diagnostic criterion of significant alteration, a critical level of inhibition of gene expression. And Index 3.0 be considered a threshold of permitted increase in the gene expression which will be manifested in a range of optimum coefficients expression (expression index) of 0.7-3.0. The difference between the coefficients that limit the increase or decrease in the gene expression can be explained by the specific traits and intensity of these processes in eukaryotes. Conservation of the genetic material under suppression and initiation of synthesis when stimulated (achieved by repeated initiation of DNA synthesis) lead to a significant increase in the genetic material. In the analysis of cellular phenotypes responsible for immunoresistance, the deficit thereof was revealed. That in association with a high level of strontium contamination of the biological media and suppression of the alpha defensin expression indicates the presence of immune deficiency in the immune system of a patient associated with strontium exposure.

Analysis of induced gene expression is recommended to determine the expressiveness of the individual and population epigenetic changes in the population groups exposed to chronic hapten intoxication in the algorithmic sequence that follows the sequencing and PCR identification of candidate genes.

Thus, we have proposed a methodology for genotyping nucleotide substitutions. It includes an algorithmic sequence of sequencing, PCR-typing and candidate gene expression. The results of the analysis of genetic polymorphism under high strontium exposure indicate excessive variability of the following gene: CYP1A2, TERT, FAS, FOXP3, TP53, HLADRB1, MTHFR, GSTA, SULT1A1, NOS, SIRT, and ACE responsible for immune regulation and detoxification. The above approaches to and principles of the identification of de-adaptation genes can be used in selecting a combination of genetic markers for certain conditions (the environment area, nosology) for the identification of health problems.

Conclusions Identification of sensitivity markers with the help of the latest genetic methodologies should include an algorithmic sequence of biosample testing using polymerase chain reaction, sequencing, and assessment of the candidate gene expression.

Genetic markers can serve to evaluate the environmentally-induced health effects and facilitate early diagnosis of health impact associated with chemical exposure.

The approaches (as exemplified by strontium) developed to identify genetic polymorphism in children exposed to adverse environmental factors are based on the following:

- The adequacy of the sample; its homogeneity; lack of congenital anomalies, the adequacy of the selected control population living outside the impact area;
- An integrated approach to the analysis of polymorphic variants of candidate genes using PCR analysis in real time, their sequencing, and tandem repeats calculation and gene transversions with the evaluation of spontaneous and gene expression;
- Analysis of the key SNPs and variant alleles of candidate genes ("immune" exchange; somatic, cancer-proliferated, and detoxification genes) responsible for the metabolism of the current factor or for the state of the effector organs and systems (target tissue);
- Analysis of the transcriptome by the largest relative expression of candidate genes matrix RNA of the separated target cells of the analyzed factors.

- Analysis of gene-receptor-ligand association with the analyzed condition of both the gene and the expressed protein in various forms: intracellular (transcription factors), membrane (receptor), and extracellular (ligand);

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