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Real time GlaI-PCR assay of regulation regions of human genes HDAC4, RARB and URB1.

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ABSTRACT

Real-time GlaI-PCR assay is developed to determine DNA methylation status of the regulation regions of HDAC4, URB1 and RARB genes in DNA preparations from human leukocytes. Real-time GlaI-PCR assay is DNA hydrolysis with methyl-directed site-specific DNA endonuclese GlaI followed by real-time PCR from the primers located upstream and downstream of the studied DNA region. The obtained data confirm a full methylation of the studied ACGT and GCGC sites in the regulatory regions of the HDAC4 and URB1 genes and a complete hydrolysis of these sites with GlaI. A first exon of RARB gene is slightly methylated in the leukocytes DNA preparations and according to the results of GlaI-PCR assay we don't observe GlaI hydrolysis of ACGCG site in RARB gene. The data obtained correspond to the literature data. The proposed method of real-time GlaI-PCR assay may be used to determine the methylation status of any unique parts in human and mammalian genomes.

Keywords: DNA methylation, DNA endonucleases, real-time PCR

Abbreviations: 5mC – 5-methylcytosine, MD-endonuclease – methyl-directed site-specific DNA endonuclease

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INTRODUCTION

One of the main mechanisms of epigenetic regulation of genes activity in mammals is the methylation of their regulatory regions that is the promoter region and/or the first exon. As a result the transcription of modified gene is blocked and gene becomes inactive [1]. DNA methylation in mammalians genomes is mostly DNA methylation of CG dinucleotides with formation of 5-methylcytosine (5mC) in both DNA strands. In most cases methylation occur not in a single CG dinucleotide, but in several consequent ones, forming so-called CpG-Islands [2].

Mammalian DNA-methyltransferases DNMT1, DNMT3a and DNMT3b catalyze a reaction of DNA methylation [3]. DNMT1 maintains DNA methylation pattern in vivo modifying a new strand after replication, i.e., this enzyme methylates the cytosine in the CG-dinucleotide in the newly formed chain in accordance with the methylated CG-dinucleotide in the original chain. DNMT3a and DNMT3b are responsible for DNA methylation de novo and they modify a CG-dinucleotide in the sequence RCGY with formation of 5'-R(5mC)GY-3'/3'-YG(5mC)R-5' sequence[4].

At the same time, recently discovered methyl-directed site-specific DNA endonuclease GlaI recognizes and cleaves 5'-R(5mC)GY-3'/3'-YG(5mC)R-5' sequences [5], which makes it a convenient tool to detect de novo methylated sites in human and mammalian DNA. Previously for the determination of the DNA fragment's methylation status, we developed GlaI-PCR assay [6]. GlaI-PCR assay includes DNA cleavage by GlaI with subsequent amplification of the studied fragment with primers flanking this DNA region. Sites 5'-R(5mC)GY-3'/3'-YG(5mC)R-5' are cleaved by GlaI and there is no DNA amplification, whereas unmethylated DNA fragment remains uncut and amplicons in this case are formed successfully. PCR products are analyzed by electrophoresis in agarose gel. Based on GlaI-PCR assay, we developed a method of epigenetic typing of human malignant cell lines, which allows to distinguish tumor cell lines due to the different methylation patterns of the regulatory regions of several tumor suppressor genes [7].

In this work we have developed real-time GlaI-PCR assay for analysis of amplification products and determined the methylation status of the regulatory regions of HDAC4, RARB, and URB1 genes in genomic DNA from human leukocytes.

MATERIALS AND METHODS

Materials: saline (JSC "Krasfarma", Krasnoyarsk), trypsin solution (Trypsin-Versen) (LLC "Biolot", St. Petersburg), phenol (JSC "Reagent", Novosibirsk), chloroform (JSC "Reagent", Novosibirsk), isoamyl alcohol (JSC "Reagent", Novosibirsk), RNase A (OOO "Samson-Med", St. Petersburg), SDS ("Helicon", Moscow). Fosfamidit (Biosset, Novosibirsk, Russia).

Buffer A (1M EDTA, 1M NaCl, 1M Tris-HCl, pH 8.0, at 25 °C), proteinase K, BSA, 10x SE-buffer Y, 10x TNM buffer (10 mM Tris-HCl pH 7.9 at 25 °C, 5 mM $MgCl_2$, 25 mM NaCl), 50 mM $MgCl_2$, dNTP mix (concentration 10 mM each), 5x SE stabilizer PCR (2.7 M betaine, 6.7 mM DTT, 6.7% of DMSO), 10x SE the GLAD buffer (30 mM $MgCl_2$, 500 mM Tris-HCl pH 9.0 at 25°C, 300 mM KCl, 100 mM $[NH_4]_2SO_4$, 0,1% Tween 20), the restriction enzymes TaqI, HpySE526I, HspAI, BspFNI, methyl-directed site-specific endonuclease GlaI, T4 DNA ligase and Hot Start Taq DNA polymerase – produced by SibEnzyme Ltd. (Novosibirsk, Russia).

Obtaining human leukocytes DNA preparations:

Blood of 8 healthy donors was taken in the clinic in a standard way into K-EDTA vacuum tubes (vacutainers) (volume of 9 ml). Probes were taken in the morning on an empty stomach. The vacutainers with blood obtained from donors were gently turned over 7-8 times at intervals of no more than 30 minutes and centrifuged in a centrifuge ELMI CM-6MT (BioSan, Latvia) at 1000g for 10 min. The supernatant (blood plasma) was collected by pipette and removed. Saline was added to the precipitate blood cells in a volume equal to amount of removed supernatant. The sediment was suspended by cautious turning until it disappeared at the bottom of the tube. The obtained suspension was centrifuged 10 min at 1000 g. The supernatant was collected by pipette and removed. The cells were washed with saline twice.

After centrifugation the supernatant was collected by pipette and removed. A solution of trypsin (Trypsin-Versen 1:1) was added to the precipitate blood cells in a volume equal to amount of removed supernatant. The sediment was suspended by cautious turning until it disappeared and left for 10 min at room temperature. During this time the tube was carefully turned 2 more times and then centrifuged 10 min at 1000 g. The supernatant was collected by pipette (leaving the last 10 mm in height to avoid the roiling) and removed. The leukocytes from the boundary of two mediums, were carefully selected from the remaining 10 mm of the supernatant by pipette, taking care not to capture the red cells, and transferred to a 1.5 ml. microtube.

Then 1 ml of saline was added into a tube, the cells were gently suspended, then centrifuged 10 min in the centrifuge "Eppendorf-5415" at 1000 g and 6 °C and supernatant was removed. The procedure of the washing of the cells was repeated 2 times, after which the precipitate of cells was placed in a freezer at -20 °C.

At the next day the frozen sediment was added to 1.35 ml of buffer A, 75 µl of solution of 10% SDS and 20 µl proteinase K (20 mg/ml). The mixture was stirred softly turning the tubes and incubated in a thermostat at 55 °C within 1-2 hours to obtain a clear solution. Each sample was divided into 2 aliquots of ~675 µl each. To the contents of the tubes were added to 37.5 µl of 4M NaCl and then the resulting solution had to go through phenol deproteinization. To the DNA solution was added an equal volume of phenol impregnated with 10 mM Tris-HCl, pH 8.0. The mixture was stirred briefly by turning and centrifuged 3 min in the centrifuge "Eppendorf-5415" at 12,000 rpm. The upper aqueous phase was collected into a clean tube and 0.5 volume of phenol and 0.5 volume of chloroform-isoamyl alcohol (ratio 24:1) were added to it. The mixture was stirred briefly by turning and centrifuged 3 min in the centrifuge "Eppendorf-5415" at 12,000 rpm. The upper aqueous phase was collected in a clean tube and an equal volume of chloroform-isoamyl alcohol (ratio 24:1) was added to it. The mixture was stirred briefly by turning and centrifuged 3 min at 12000 rpm. The upper aqueous phase was collected in a clean tube and 0.1 volume of 3M potassium-acetate buffer (pH 7.0) and 2.5 volume of 96% ethanol were added to it. The mixture was stirred by turning 7-8 times and left for one hour at -20 °C. In an hour the mixture was centrifuged 5 min at 12,000 rpm and the supernatant was removed. The sediment was thoroughly suspended in 300 µl of 70% solution of ethanol and the mixture was centrifuged 5 min at 12000 rpm. The procedure of the washing of the sediment DNA was repeated 2 more times. Then the sediment was dried, removing the remaining liquid by pipette. A 1000 µl of TE buffer were added to the sediment, mixed by turning and kept at 4 °C overnight.

In the morning the tube was placed in thermostat at 37 °C and periodically pipetted until complete dissolution of DNA (as indicated by absence of bands of DNA). A tube with a solution of RNase A (100 µg/µl) was heated for 15 minutes at a temperature of 100°C, then cooled to room temperature. The obtained DNA solution was added to 90 µl of RNase A, 120 µl 10x TaqI buffer and 4 µl of TaqI restriction enzyme. The tube was incubated at 60°C for 40 min. Then the purification of the reaction products with a phenol-chloroform extraction and subsequent precipitation with ethanol was performed as described above. Alcoholic sediment was washed with 300µl of 70% ethanol solution and centrifuged 5 min at 12000 rpm. The procedure of DNA preparation washing was repeated 2 more times.

The sediment was dried, removing the remaining liquid by pipette. Then the sediment was dissolved in 1000µl of TE buffer.

The concentration of the obtained blood leukocytes DNA preparations and the optical density at wavelengths 260 nm and 280 nm were determined on "Genesys 10UV" spectrophotometer (Thermo Electron Corporation, USA).

Hydrolysis of genomic DNA.

180 ng of leukocyte DNA were incubated in 50 µl reaction mixture containing either the 2.5 units of AgsI, or 5 units of HpySE526I, or 2.5 units of BspFNI, or 10 units of HspAI or 8 units of GlaI in 1x TNM buffer with addition of 0.1 µg/µl BSA, 3.3% of DMSO at a temperature of 30°C (GlaI) and 37°C (for AgsI, HpySE526I, HspAI, BspFNI) for 30 minutes.

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The structure of the analyzed DNA fragments, primers and TaqMan probes used in PCR.

Oligonucleotide primers and TaqMan probes were synthesized using amidophosphite method [8] on DNA-synthesizer ASM800 (Biosset, Russia). A reversed-phase chromatography on a PLRP-S 300A ("Polymer Laboratories Ltd", UK) or an oligonucleotides purification unit OPS-201 (Biosset, Russia) with subsequent gel filtration on "Sephadex G-25 DNA-grade" (Amersham-Pharmacia, USA) were used in the process of cleaning.

The determination of the number of copies of DNA fragments in the preparations of leukocytes DNA was performed by real time PCR. Four unique regions of DNA, located in different chromosomes and representing regulatory regions of the genes HDAC4, URB1, RARB, and a fragment of the HBB gene, which are widely used to determine the concentration of genomic DNA, were used in this test[9].

Table 1 represents data on the placement of the analyzed DNA in the human genome (according to the Assembly "GRCh38 Primary Assembly"), and their nucleotide sequences.

Table 1: The coordinates and the nucleotide sequence of the analyzed genomic regions.

Gene	Chromo-some	Coordinates	Nucleotide sequence*
RARB	3	25428290–25428403	TTCAGAGGCAGGAGGGTCTATTCTTTGCCAAAGGGGGGACCAGAATTCCCCCATGCGAGCTGTTTGAGGACTGGGATGCCGAGAACGCGAGCGATCCGAGCAGGGTTTGTCTGGGCACCG
URB1	21	32334235–32334314	GCGAAGGATGTCCCCGACACTCGGCTGCTGCCACAGTGACCTGCCCAGGCTCCGGCACGTCTTGTGATGCTCCACGGCCG
HDAC4	2	239189734–239189882	CCTGGGGCCCCAGCACACTCCGCGGAGGCAGGGCTGGAGTCACCGGGAGTTGAGGGTGCCGGAGGCCTGGCCCACCCGCAGCCCCGCACCGCGCCTCACCTTGATGTGCGTGGAGCTGCGCCTCGTGCTGCCGGGAGAGCTGCTCGT
HBB	11	5226723–5226934	CCATAACAGCATCAGGAGTGGACAGATCCCCAAAGGACTCAAAGAACCTCTGGGTCCAAGGGTAGACCACCAGCAGCCTAAGGGTGGGAAAATAGACCAATAGGCAGAGGAGTCAGTGCCTATCAGAAACCCAAGAGTCTTCTCTGTCTCCACATGCCCAGTTTCTATTGGTCTCCTTAAACCTGTCTTGTAACCTTGATACCAACCTGCC

* Underlines sequences are recognition sites of used restriction enzymes.

Polymerase chain reaction:

Below are the structures of primers and TaqMan probes used in the work:

HDAC4F 5' CCTGGGGCCCCAGCACACT 3'
HDAC4R 5' ACGAGCAGCTCTCCCGGCAG 3'
HDAC4Z 5' FAM-CCGGTGACTCCAGCCCTGCCTCC-BHQ1 3'
URB1F 5' GCGAAGGATGTCCCCGACACT 3'
URB1R 5' CGGCCGTGGAGCATCACAA 3'

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URB1Z 5' FAM-CTGCTGCCACAGTGACCTGCCCA-BHQ1 3'
RARbF 5' TTCAGAGGCAGGAGGGTCTATTCT 3'
RARbR 5' CCAGACAAACCCTGCTCGGATC 3'
RARbZ 5' FAM-TCCCAGTCCTCAAACAGCTCGCATGG-BHQ1 3'
HBBF 5' CCATAACAGCATCAGGAGTGGACA 3'
HBBR 5' GGCAGGTTGGTATCAAGGTTACAA 3'
HBBZ 5’ FAM-TAGACCACCAGCAGCCTAAGG-BHQ1 3’

The DNA preparation of sample No. 381 was used to construct a calibration graph. The amplification of DNA standards containing DNA sample No. 381 at concentrations of 15, 3, 0.75 and 0.19 ng/µl (which approximately corresponds to 5000, 1000, 250, 62 haploid genome copies in 1 µl solution) or 6, 3, 1.5, 0.75, 0.38 ng/µl (2000, 1000, 500, 250, 125 copies/µl) was carried out.

PCR for each DNA sample was performed tree times in 20 µl reaction mixture. The reaction mixture contained 1x buffer GLAD, 0.2 mM of each dNTP, 0.4 µM of each primer and TaqMan probe, 1 unit of Hot Start Taq-DNA polymerase, 9 ng hydrolyzed or not hydrolyzed lymphocytes DNA or 15, 3, 0.75, 0.19 ng DNA standard, or 9 ng of A/He mouse DNA lines (as negative control). In the case of amplification of HDAC4, RARB, and URB1 genes in the reaction mixture was added a 1/5 solution of 5x PCR SE stabilizer.

Real-time PCR was performed in the "CFX-96 Thermal Cycler Amplifier" (Bio-Rad, USA). Initiation was performed during 3 min at 95 °C (for HBB at 94 °C), then 45 cycles of amplification: the DNA melting - 95 °C (94 °C for HBB), 10 sec; annealing - 60 °C (URB1), 61 °C (for HBB), 62 °C (HDAC4), 63 °C (RARB) for 20 sec with detection of fluorescence in the FAM channel; elongation - 72 °C for 10 h (URB1) or 5 h (for RARB and HDAC4) or 68 °C - 15 sec (for HBB).

Data analysis and processing was carried out using the "Bio-Rad CFX Manager V. 2.1" software.

RESULTS AND DISCUSSION

Isolation of DNA from leukocytes and determination of DNA concentration:

Methods of DNA isolation from leukocytes are well known [10]. As a rule they include the following steps: blood sampling, separation of leukocytes from red blood cells, lysis of cells and DNA purification with phenol extraction or with commercial kits. In some cases the obtained preparations are treated with RNase A to get DNA free from RNA. After isolation the leukocyte DNA may be treated with DNA endonucleases or ultrasonic to achieve a partial fragmentation and avoid of DNA supercoiling fragments which hinder PCR. It is known that blood cells contain large amounts of DNA adsorbed on the surface of cells [11, 12]. Adsorbed DNA is extracellular and its presence complicates the characterization of extracted leukocyte DNA. In our work we pre-treated the blood cells with trypsin to remove DNA fragments from the external surface of cells [12]. Then cells were washed and lysed, DNA was purified with phenol deproteinization and partly hydrolyzed by TaqI restriction endonuclease, as described in "Materials and methods".

The concentration and purity of DNA preparations was determined by measuring their optical density at 260 nm and the $A_{260/280}$ ratio. In table 2 the third column represents data of the optical density ratio of the obtained DNA preparations at 260 nm and 280 nm ($A_{260/280}$), and the fourth column shows their concentration. As the table shows, for all the obtained preparations of DNA a $A_{260/280}$ ratio is 1,69-1,75, which indicates the absence of impurities in the DNA. It is known that if 1 ml of clean preparation of genomic DNA (with $A_{260/280}$ ratio in the range 1.7 to 1.8), has the optical density of 1, it has DNA concentration of 0.05 mg/ml, that is 1 OD_{260} unit = 50 µg/ml [13]. Meanwhile, 3 ng of human DNA correspond to approximately 1000 copies of a haploid set of human chromosomes [14] and, respectively, 1000 copies of any unique nucleotide sequence present in the genome. In the fifth column of table 2 shown the estimated number of copies of the HBB gene fragment in 1 µl of the obtained DNA preparations.

Table 2: The concentration of the obtained leukocytes DNA preparations

Sample No.	Sample No. of leukocytes DNA	$A_{260/280}$	DNA concentration, ng/µl	DNA concentration, 103 copies/µl (calculated)
1	374	1,69	149	49,7
2	375	1,735	150	50
3	381	1,733	228	76
4	382	1,75	250	83,3
5	383	1,75	136	45,3
6	384	1,755	143	47,7
7	390	1,733	160	53,3
8	391	1,718	150	50

Determination of the concentration of the fragment of the HBB gene in the DNA preparations:

We conducted PCR in the haemoglobin gene using 9 ng of DNA as a matrix, which corresponds to 3000 copies of the HBB gene. Figure 1 shows the curves of fluorescence accumulation for the analyzed fragment of the HBB gene. Threshold line to determine the number of cycles (Cq) were taken at the level of 150 conventional units of fluorescence (RFU). Obtained for each series of 3 experiments mean Cq and the standard deviation are shown in table 3. The obtained Cq for all samples are close enough (21,26 – 21,59) as can be seen from the table.

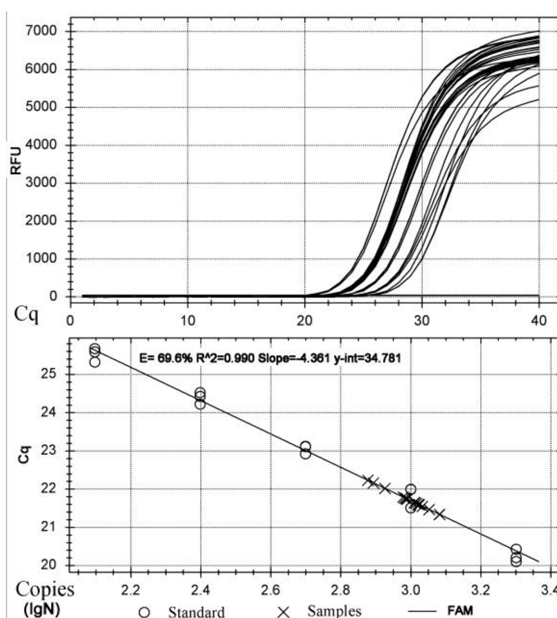

Fig 1: Curves of fluorescence accumulation (top) and threshold line (bottom) for HBB gene (DNA samples №№374, 375, 382, 383, 384, 390, 391 and standard solutions of sample No. 381).

Table 3: Obtained Cq (mean and standard deviation) for HBB, RARB, URB1, HDAC4 genes.

Gene / DNA sample	374	375	382	383	384	390	391
HBB	21.26±0.05	21.44±0.04	21.46±0.06	21.52±0.11	21.34±0.05	21.52±0.10	21.59±0.15
RARB	21.06±0.05	21.08±0.03	21.15±0.05	21.21±0.13	21.29±0.01	21.29±0.13	21.34±0.04
URB1	23.84±0.07	23.93±0.05	23.93±0.06	24.04±0.03	23.98±0.05	24.00±0.09	24.03±0.003
HDAC4	23.11±0.08	23.29±0.03	23.18±0.07	23.32±0.03	23.43±0.02	23.31±0.02	23.37±0.03

Figure 1 presents a graph of Cq linear dependence on log values of concentration of the standard dilutions of leukocyte DNA preparation No. 381 (see methods). This dependence was used to determine experimental values of the concentration of the fragment of the HBB gene in the examined DNA preparations shown in the fourth column of table 4. As can be seen from table 4, experimentally obtained values of the DNA concentration are close enough to the concentrations of DNA determined by optical density ratio $A_{260/280}$ (the third column of table 4), which indicates the purity of the obtained preparations of leukocyte DNA.

Table 4: The concentration of HBB, RARB, URB1, and HDAC4 genes fragments in the leukocytes DNA.

Sample No	Sample No. of leukocytes DNA	DNA concentration, 10^3 copies/μl (calculated)	HBB (10^3 copies/μl)	RARB (10^3 copies/μl)	URB1 (10^3 copies/μl)	HDAC4 (10^3 copies/μl)	Mean value for 4 genes
1	374	49,7	55,0	47,8	51,4	45,9	50,0±5,0
2	375	50	49,2	52,8	54,2	49,0	51,3±2,9
3	382	83,3	81,1	85,0	90,0	86	85,5±4,5
4	383	45,3	42,3	47,9	48,8	46,6	46,4±4,1
5	384	47,7	50,1	42,7	47,5	41,4	45,4±4,7
6	390	53,3	49,6	56,2	61,8	57,6	56,3±6,7
7	391	50	44,5	51,5	56,8	52,6	51,4±6,9

Real-time GlaI-PCR assay of regulatory regions of HDAC4, RARB and URB1 genes:

GlaI-PCR assay of gene regulatory regions (promoter and first exon) allows to identify the presence of 5'-R(5mC)GY-3'/3'-YG(5mC)R-5' sites and determine, thus, the methylation status of the gene. The method of GlaI-PCR analysis was previously developed to determine the methylation of tumor suppressor genes in DNA from the human cell lines, though the analysis of the obtained PCR products by electrophoresis in agarose gel [6]. In the present work we determined the methylation status of gene's regulatory regions in leukocytes DNA of healthy individuals using real-time PCR. To select genes, we analyzed data of RRBS-sequencing of 96 genomic DNA from various tissues and cell lines, as malignant and amalignant, using the UCSC Genome Browser (http://genome-mirror.duhs.duke.edu/cgi-bin/hgTracks tracks group DNA Methylation ENCODE/HAIB Methyl RRBS) [15]. It was found that a region of the genome on chromosome 21 (position 32334235-32334314, the coding part of URB1 gene) is methylated in all studied samples. Analysis of the HDAC4 gene sequences showed that the CpG island located upstream of the alternative transcription start site in the area 239189527-239189982 of chromosome 2, is also methylated in healthy and in tumor cells. These methylated DNA fragments, representing the regulatory regions of the genes HDAC4 and URB1, as well as a fragment of RARB gene, which is almost unmethylated in healthy cells [13], were chosen for a further work.

Table 3 presents the values of Cq obtained by rtPCR of the studied fragments of HDAC4, RARB for URB1 genes in seven obtained DNA preparations, and table 4 shows the concentrations of DNA in the preparations, calculated from the Cq values. As the table shows, the concentrations of studied fragments in the examined DNA preparations fairly well correspond to the earlier determined concentrations of the HBB gene. And the standard deviation for all four fragments for each of the seven DNA preparations, does not exceed the of 15% value of the measuring error specified by the device manufacturer.

According to the structure of DNA (table 1) studied fragment of the HDAC4 gene contains a GCGC site (hydrolyzed by HspAI restriction enzyme), which in healthy cells is methylated and forms G(5mC)GC (cleaved by GlaI and not cleaved by HspAI).

The URB1 gene fragment contains the sequence ACGT (hydrolyzed by Hpy526I), which in healthy cells is methylated and forms A(5mC)GT (cleaved by GlaI and not cleaved by Hpy526I).

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The fragment of RARB gene contains in healthy cells the sequence 5'-ACGCG-3'/3'-TGCGC-5' (cleaved by BspFNI within CGCG site), which upon methylation of RCGY site is converted into 5'-A(5mC)GCG-3'/3'-TG(5mC)GC-5'. This methylated sequence is a recognition site of GlaI enzyme, but it should not be cleaved by BspFNI restriction enzyme. In Fig. 2 shows the results of real-time GlaI-PCR assay data of three above mentioned fragments, before and after hydrolysis by the corresponding enzymes. As a control, we incubated the DNA with AgsI restriction enzyme (recognizes the DNA sequence 5'-TTSAA-3'), which has no recognition site within the analyzed fragments.

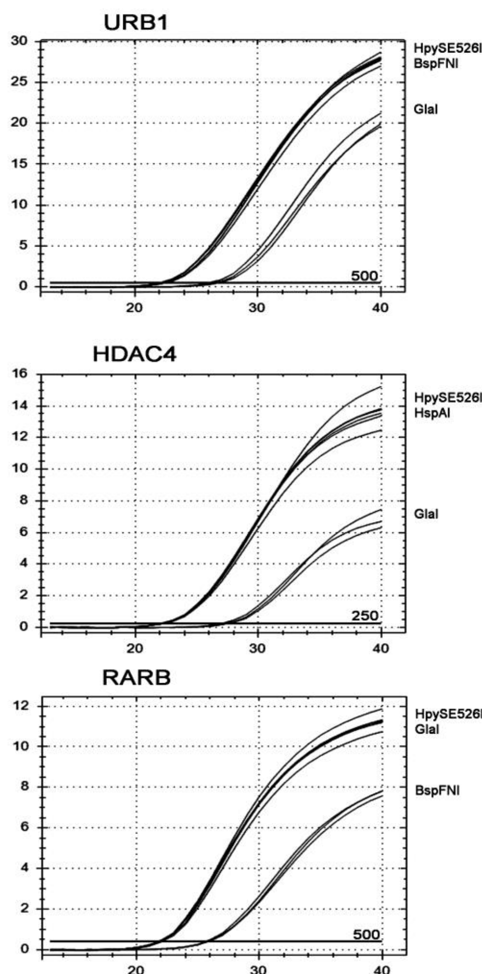

Fig 2: Curves of fluorescence accumulation for URB1, RARB and HDAC4 genes after incubation of genomic DNA with endonucleases. The abscissa axis - number of cycles, the ordinate axis is arbitrary units of fluorescence (RFU×10^3). Below are the threshold lines for determine the number of cycles (Cq) in RFU. To the right of the curves shown the names of the corresponding endonucleases

Table 5 presents the concentrations of DNA fragments, calculated from the Cq values obtained in experiments (Fig. 2). From the table 5, it is seen that the analyzed fragments of the HDAC4 and URB1 genes are cleaved by GlaI and not hydrolyzed by HspAI and Hpy526I, respectively. Whereas the RARB gene fragment is not cleaved by GlaI, but hydrolyzed by BspFNI. The obtained data confirm a complete methylation of the studied ACGT and GCGC sites in the regulatory regions of the HDAC4 and URB1 genes respectively. The lack of hydrolysis of these sites by appropriate restriction enzymes is also consistent with their methylation. Site ACGCG in RARB gene is cleaved by BspFNI enzyme in accordance with the literature data about its unmethylated state in healthy cells. The percent of remaining (not hydrolyzed) DNA is different for the analyzed DNA regions. In case of HDAC4 and URB1 regulatory regions about 2-4% of DNA remains uncleaved, while BspFNI cleaves the RARB gene less efficiently, which is probably associated with a small level of CGCG site methylation in DNA from healthy cells.

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Table 5: The concentration of RARB, URB1, and HDAC4 genes fragments, determined by real-time GlaI-PCR assay

Sample No. of leukocytes DNA	URB1				HDAC4				RARB			
	HpySE526I hydrolysis	GlaI hydrolysis	Negative control (AgsI hydrolysis)	Unhydrolyzed DNA by GlaI, %	HspAI hydrolysis	GlaI hydrolysis	Negative control (AgsI hydrolysis)	Unhydrolyzed DNA by GlaI, %	GlaI hydrolysis	BspFNI hydrolysis	Negative control (AgsI hydrolysis)	Unhydrolyzed DNA by BspFNI, %
374	3590	64	3450	2	3140	75	3080	2	3770	261	3210	8
375	3290	70	3250	2	3140	107	2940	4	3590	359	3170	11
382	3650	101	3240	3	3110	121	3110	4	3500	215	3060	7
383	3380	66	3020	2	2960	83	2880	3	3350	409	2960	14
384	3430	51	3150	2	2900	78	2740	3	3190	280	2830	10
390	3060	92	3040	3	2850	111	2910	4	3340	235	3840	6
391	2830	52	3150	2	2720	57	2820	2	3250	279	2760	10

CONCLUSION

In this work we have developed a method of real-time GlaI-PCR assay to determine the methylation status of the human genes regulatory regions. The proposed method has been applied for determination of the methylation status of the regulatory regions of HDAC4, RARB and URB1 genes. GlaI-PCR assay showed that in leukocytes cells of healthy people regulatory regions of HDAC4 and URB1 genes are fully methylated, while the first exon of RARB gene is slightly methylated in accordance with the literature data. The proposed method for determination of the methylation status of the regulatory regions of genes is simple and requires only DNA hydrolysis by GlaI with subsequent standard real-time PCR with primers flanking the studied DNA fragment. The proposed method may be used to determine the methylation status of any unique parts of the genome of humans and other mammals.

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REFERENCES

[1] Kass S.U., Pruss D., Wolffe A.P. How does DNA methylation repress transcription? // Trends. Genet. – 1997 – Vol. 13. – P. 444-449.

[2] Deaton A.M., Bird A. CpG islands and the regulation of transcription // Genes Dev. – 2011. – V. 25. – P. 1010–1022.
[3] Okano M., Bell D.W., Haber D.A., Li E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development // Cell. – 1999. – Vol. 99. – P. 247–257.
[4] Handa V., Jeltsch A. Profound flanking sequence preference of Dnmt3a and Dnmt3b mammalian DNA methyltransferases shape the human epigenome // J. Mol. Biol. – 2005. – Vol. 348. – N 5. – P. 1103–1112.
[5] Tarasova G.V., Nayakshina T.N., Degtyarev S.Kh. Substrate specificity of new methyl-directed DNA endonuclease GlaI // BMC Mol. Biol. – 2008. – Vol. 9:7.
[6] Gonchar D. A., Akishev A. G., Degtyarev S. Kh. BlsI - and GlaI - PCR analysis – a new method for studying methylated DNA // Bulletin of biotechnology and physico-chemical biology. Yu. A. Ovchinnikov. – 2010. – T. 6. – No. 1. – P. 5-12.
[7] Akishev A. G., Gonchar D. A., Abdurashitov M. A., Degtyarev S. Kh. Epigenetic typing of human malignant cell lines with the help of BlsI - and GlaI-PCR analysis // Bulletin of biotechnology and physico-chemical biology. Yu. A. Ovchinnikov. – 2011. – T. 7. – No. 3. – S. 5-16.
[8] Beaucage S.L., Caruthers M.H. Deoxynucleoside phosphoramidites – a new class of key intermediates for deoxypolynucleotide synthesis // Tetrahedron Lett. – 1981. – Vol. 22. – P. 1859–1862.
[9] Fleischhacker M., Schmidt B., Weickmann S., Fersching D.M., Leszinski G.S., Siegele B., Stötzer O.J., Nagel D., Holdenrieder S. Methods for isolation of cell-free plasma DNA strongly affect DNA yield // Clinica Chimica Acta. – 2011. – Vol. 412. – P. 2085–2088.
[10] Lindblom B., Holmlund G. Rapid DNA purification for restriction fragment length polymorphism analysis // Gene Anal. Tech. – 1988. – Vol. 5. – P. 97–101.
[11] Laktionov P. P., Tamkovich S. N., Rykova E. Yu., Morozkin E. S., Vlassov V. V. Method for early diagnosis and monitoring of cancer // Patent RF № 2251696 C2. – 2005.
[12] Sitko V. L., Kuksova A. N., Evdokimova I.I., Netesova N. A., Degtyarev S. Kh. The development of a simple method of obtaining high-purity DNA associated with blood cells // Problems of Hematology/Oncology and immunopathology in Pediatrics. – 2014. – Vol. 13. – No. 4. - S. 50-55.
[13] Sambrook J., Russell D. Molecular cloning: A laboratory manual. 3rd ed. – New York: Cold Spring Harbor Laboratory Press, 2001. – Vol. 1–3. – 2222 p.
[14] International Human Genome Sequencing Consortium. Finishing the euchromatic sequence of the human genome // Nature. – 2004. – Vol. 431. – P. 931–945.
[15] The ENCODE Project Consortium. An integrated encyclopedia of DNA elements in the human genome // Nature. – 2012. – Vol. 489. – P. 57–74