

Blsl- and Glal-PCR assays – a new method of DNA methylation study

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Blsl- and Glal-PCR assays have been developed to study DNA methylation. A new method includes DNA hydrolysis by methyl-directed site-specific DNA endonucleases Glal or Blsl with subsequent routine PCR. Study of DNA methylation in regulation region of human tumor suppressor genes has been performed for a new method evaluation. Blsl- and Glal-PCR assays have revealed different methylation patterns in promoter region of DAPK1, in promoter and first exon region of RARB and in first exon region of RASSF1A tumor suppressor genes in malignant cell lines HeLa, Raji, U-937, Jurkat and control L-68 cells. Glal-PCR assay has shown methylation of RARB promoter and first exon region in DNA from all malignant cell lines, but not in control L-68 cells. Glal- and Blsl-PCR assays have displayed DNA methylation of RASSF1A first exon region in Raji and Jurkat cells only. Blsl-PCR assay of DAPK1 promoter region has demonstrated an additional DNA methylation in Raji cells only. Glal- and Blsl-PCR assays may be useful both in determination of malignant cells and their discrimination.

INTRODUCTION

Regulation of genes activity in mammals genomes is based on DNA methylation of CG dinucleotides with formation of 5-methylcytosine (5mC) in both DNA strands. Mammalian DNA-methyltransferases Dnmt1, Dnmt3a and Dnmt3b catalyze a reaction of DNA methylation [1]. Dnmt1 maintains DNA methylation pattern in vivo modifying a new strand after replication. Dnmt3a and Dnmt3b are responsible for DNA methylation de novo and, likely, differ in their function and preferable region of modification [1]. Study of Dnmt3a and Dnmt3b substrate specificity has shown that both enzymes methylate CG-dinucleotide mostly in DNA sequence PuCGPy [2].

At present time 5mC determination is represented mostly by a chemical treatment of DNA with sodium bisulphite, which results in cytosine transformation into uracil, whereas 5mC is resistant against this modification. A subsequent analysis of modified and native DNA allows to locate positions of methylated cytosines in studied DNA [3-6]. Method of bisulphite conversion is quite expensive, time-consuming and often results in obtaining false positive data. Because of substantial DNA degradation this method is

used for analysis of only short (100 – 150 bp) DNA sequences.

Among enzymatic methods of 5mC determination, so called methyl-sensitive PCR assay is the most popular. This method is based on inability of restriction enzymes, which contain CG dinucleotide in the recognition site, to cut this site if 5mC is present in the dinucleotide [7]. A subsequent PCR from primers, which are located around a chosen recognition site, produces a corresponding DNA fragment if there is a methylated CG-dinucleotide within this site. On the contrary, DNA fragment is not produced in PCR if there is no methylated CG-dinucleotide in a recognition sequence of restriction enzyme. Application of methyl-sensitive PCR assay is limited by a very short list of recognition sequences.

Recently we have discovered and characterized absolutely new enzymes BslI and Glal, which belong to the type of methyl-directed site-specific DNA endonucleases and cleave only methylated DNA. Glal recognizes DNA sequence 5'-Pu(5mC)GPy-3'/3'-PyG(5mC)Pu-5' [8], [9] whereas BslI hydrolyzes DNA sequence 5'-GCNGC-3' if at least one 5-methylcytosine (N isn't considering) is present in each DNA strand of the recognition site [10], [11].

In this work we have developed a new method of DNA methylation determination – BslI- and Glal-PCR assays, and have applied this method to study a methylation of regulation region of tumor suppressor genes in DNA from different human cell lines.

MATERIALS AND METHODS

Genomic DNA from human cell lines HeLa, Jurkat, L-68, Raji, U-937; restriction endonucleases FatI, HaeIII, TaqI; methyl-specific endonucleases BslI and Glal; HotStart Taq DNA polymerase were supplied by "SibEnzyme" Ltd. (Russia). DNA from *Drosophila melanogaster* was kindly provided by Dr. L.P.Zakharenko (Institute of Cytology and Genetics, Novosibirsk).

Genomic DNA digestion.

5 µg of each genomic DNA was hydrolyzed with 100 units of TaqI in 100 µl of the reaction mixture containing SE-Buffer Y at 65 °C for 2 hours (preliminary treatment). Phenol extraction and purification of digested DNA was carried out as described [12]. DNA was diluted in TE Buffer (10mM Tris-HCl pH 8,0; 1mM EDTA) at concentration 0.1 mg/ml.

0.1 µg of each TaqI-digested DNA was incubated in 20 µl of the reaction mixture containing either 100 units of HaeIII, or 10 units of FatI, or 16 units of BslI, or 16 units of Glal and 1 x SE-buffer, which was recommended by the manufacturer at 37 °C (HaeIII), 55 °C (FatI), or at 30 °C (BslI and Glal) for 2 hours. One microlitre of incubation mixture has been used in subsequent PCR.

PCR technique.

PCR was performed with GC-PCR kit (SE cat.number K007) and HotStart Taq DNA polymerase (SE cat.number E351) in 25 µl of reaction mixture in thermocycler "Tercyk" (DNA Technologies, Russia). The following primers from "SibEnzyme" Ltd. (Russia) have been used in the work:

- PK51 5' GAA CCG TGT TTC CCT AGA ACC CAG TC 3' and
- PK387 5' CGG TCC GGC TGT CCT CCT CAC 3' for *DAPK1*;
- AR118 5' CCG GGT AGG GTT CAC CGA AAG TTC ACT CGC 3' and
- AR941 5' TCA GCA AAG GGA ATC AAT ATG CAT GCC AGC 3' for *RARB*;
- SF458 5' GCC ATG TCG GGG GAG CCT GAG CTC A 3' and
- SF879 5' CTG TGG CCC AGA TAC GAG TGG AGT GCG AC 3' for *RASSF1A*.

Thermocycling profile of PCR has been optimized for each pair of primers.

Electrophoresis.

10 microlitres of PCR products were analyzed by 1.2 % agarose gel electrophoresis in TAE buffer (40 mM Tris-acetate, pH 8,0; 1 mM EDTA). After electrophoresis gel was stained with ethidium bromide and photographed in UV light. 100 bp DNA ladder ("SibEnzyme" Ltd., Russia) was used as a marker of DNA fragment lengths.

RESULTS AND DISCUSSION

Determination of methylated DNA regions by BslI- and Glal-PCR assays.

HpaII-PCR assay is the most known among methyl-sensitive PCR assays [13]. HpaII recognizes and hydrolyzes DNA sequence 5'-CCGG-3', whereas this enzyme can't cleave DNA sequence 5'-C(5mC)GG-3' [7]. So, PCR produces a corresponding DNA fragment if CG dinucleotide in HpaII recognition sequence is methylated and doesn't form a product in the absence of this CG dinucleotide methylation. However, application of HpaII-PCR assay is possible for sites 5'-CCGG-3' only, whereas mammalian DNA-methyltransferases Dnmt3a and Dnmt3b recognize and modify CG dinucleotide mostly in DNA sequence 5'-PuCGPy-3' [2].

Unlike HpaII, new methyl-directed site-specific DNA endonucleases BslI and Glal hydrolyze only methylated DNA. Moreover, DNA sequence 5'-PuCGPy-3'/3'-PyGCPu-5', a substrate of Dnmt3a and Dnmt3b, after methylation becomes a sequence 5'-Pu(5mC)GPy-3'/3'-PyG(5mC)Pu-5', which is, in turn, a recognition site of Glal. At the same time two recognition sites of Glal, separated by N, 5'-Pu(5mC)GCNG(5mC)GPy-3'/3'-PyG(5mC)GNCG(5mC)Pu-5' is a recognition site of BslI. Endonucleases Glal and BslI had been discovered recently but were already used for study of genomic and satellite DNA methylation [14], [15].

Thus, the suggested method of BslI- and Glal-PCR assays includes DNA hydrolysis with BslI or Glal with subsequent PCR from primers, which flank region of interest carrying site(s) 5'-PuCGPy-3'. BslI- and Glal-PCR assays give positive results - an absence of predicted PCR product in case of methylation, or negative results - a presence of PCR product if there is no methylation of studied site(s). A positive control is PCR of DNA digested with some methyl-insensitive restriction enzyme, which has recognition sites in a studied DNA fragment. Negative control is PCR of DNA without addition of endonucleases.

Structure of regulation region of the studied tumor suppressor genes.

Based on the suggested method of BslI- and Glal-PCR assays we have studied methylation of regulation region of three human tumor suppressor genes. These are a promoter region of *DAPK1*, a promoter and first exon region of *RARB* and a first exon region of *RASSF1A* genes. According to GenBank data base [17] a region of *DAPK1* (death-associated protein kinase 1) gene is located at 9q34.1, *RARB* (retinoic acid receptor beta) gene is positioned at 3p24 and *RASSF1A* (Ras association domain family 1A) gene is located at 3p21.3.

Schemes of DNA structure of *DAPK1* regulation region (reference assembly positions 19276988-19277344 in Chromosome 9), *RARB* regulation region (reference assembly positions 25409693-25410545 in Chromosome 3) and *RASSF1A* regulation region (reference assembly positions 50318239

– 50317790 in Chromosome 3), which have been studied in this work, are given in Figure 1 (parts A-C, respectively).

Recently Kim et.al. [16] have analyzed DNA methylation in promoter region of several human tumor suppressor genes by bisulphite sequencing and have shown, that CG dinucleotide modification takes place mostly in DNA sequence 5'-PuCGC-3', which corresponds to a substrate specificity of human DNA-methyltransferases Dnmt3a and Dnmt3b. In the case of *DAPK1* we have studied a promoter region with 12 sites of methylation 5'- PuCGPy-3', which are recognition sites of Glal if internal CG dinucleotide is methylated (Figure 1A). Two these sites in positions 19277174 and 19277179 form DNA sequence 5'-GCGCCGCGC-3' (indicated below in Figure 1A), which is BslI recognition site if internal CG dinucleotides of both 5'- GCGC-3' sequences are methylated.

RARB promoter and first exon region includes only 4 sites of methylation (Figure 1B). A distance between any these sites is more than one nucleotide and BslI can't cleave a selected DNA fragment.

A regulation region of *RASSF1A* is more complex than *DAPK1* and *RARB* ones. In our work we have studied methylation of *RASSF1A* first exon region containing 19 sites 5'-PuCGPy-3', which are recognition sites of Glal if internal CG dinucleotide is methylated (Figure 1C). Two these sites in positions 50318146 and 50318141 form DNA sequence 5'-GCGCTGCGC-3' (indicated below in Figure 1C). If both CG dinucleotides of this sequence are methylated, the internal sequence 5'-GCTGC-3' is BslI recognition site.

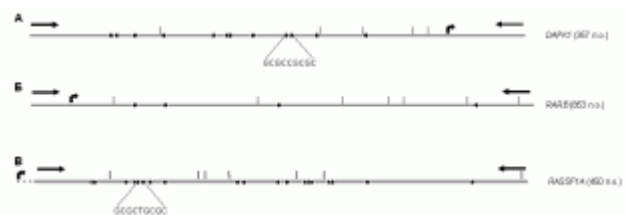


Fig. 1. Scheme of regulation region of *DAPK1* (A), *RARB* (B) and *RASSF1A*(C) genes. Positions of recognition sites of restriction endonucleases (HaeIII for A and C and FstI for B) are indicated by vertical bars. Sites 5'-PuCGPy-3' are indicated by circles. DNA sequence of two sites 5'-PuCGPy-3', which are separated by N and form BslI recognition site if methylated (underlined), are given under the schemes A and C. Transcription start is indicated by a bended arrow. A size of PCR fragment is given at right of each scheme. Primers locations are given by arrows

DNA preparations of human cell lines.

We have studied methylation of regulation region of tumor suppressor genes in DNA preparations from 5 human cell lines. These are four well known malignant cell lines HeLa (cervical cancer), Jurkat (T-Cell-leukemia), Raji (Burkitt's lymphoma), U-937 (leukaemic monocytic lymphoma) and DNA from fibroblast cell line L-68, which has been used as a control. At the beginning all DNA preparations have been hydrolyzed with restriction endonuclease TaqI, which doesn't have recognition sites in selected regulation region of *DAPK1*, *RARB* and *RASSF1A* genes (Figure 1).

Preparations of TaqI digested and purified DNA have been treated separately with a) control restriction enzyme, which has recognition sites in studied DNA fragment, b) Glal, c) BslI and d) no added enzyme.

After incubation aliquots from four reaction mixtures have been used as a template for PCR. DNA from *Drosophila melanogaster* at the same concentration has been used as a negative PCR control.

Methylation of regulation region of tumor suppressor genes.

In Figure 2 we have presented the data on study of *DAPK1* promoter region methylation by BslI- and GlalI-PCR assays. According to Figure 1A DNA fragment of 0.36 kb in length is produced as a result of PCR. Indeed, in Fig.2 we observe this DNA fragment in all control experiments (lanes 5, 9, 13, 19, 23) and after BslI cleavage of four DNA preparations (lanes 4, 8, 18, 22). PCR with all HaeIII DNA digestions (lanes 2, 6, 10, 16, 20) and BslI digestion of DNA from Raji cells (lane 12) doesn't produce 0.36 kb DNA fragment. PCR of GlalI DNA digestions results in either an absence of DNA fragment (lane 11) or a presence of the weak band (lanes 3, 7, 17, 21). So, our experiments have shown that GlalI-PCR assay gives a positive result and demonstrates a significant methylation of *DAPK1* promoter region in DNA preparations from all malignant cell lines and control L-68 cells. On the contrary, BslI-PCR assay clearly shows a presence of methylated sites in positions 19277174 - 19277179 with formation of sequence 5'-G(5mC)GCCG(5mC)GC-3'/3'-CG(5mC)GGCG(5mC)G-5' in DNA from Raji cells only. In other cell lines this DNA sequence is either nonmethylated or contains only one methylated 5'-GCGC-3' site.

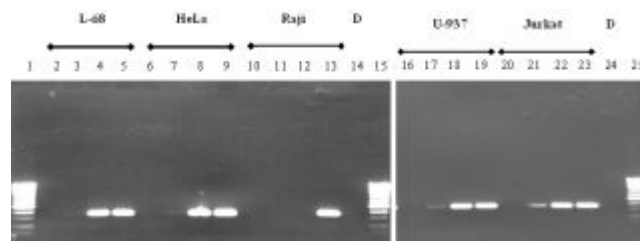


Fig. 2. BslI- and GlalI-PCR assay of *DAPK1* promoter region (PCR fragment 357 bp)

Pretreated DNA from L-68 (2-5); HeLa (6-9); Raji (10-13); U-937 (16-19); Jurkat (20-23); DNA from *Drosophila melanogaster* (14 and 24). HaeIII (2, 6, 10, 16, 20), GlalI (3, 7, 11, 17, 21), BslI (4, 8, 12, 18, 22). 100 bp DNA Ladder (1, 15, 25).

Methylation of *DAPK1* promoter region in DNA samples from tumors has been investigated in many publications [18, 19, 20 and references within them]. Raval et.al. studied in detail methylation of *DAPK1* regulation region in DNA from Jurkat and Raji cell lines. They observed more than 80% methylation in the promoter region of Raji cells, whereas DNA from Jurkat cells was methylated for 20% only [21]. Data of BslI- and GlalI-PCR assays correspond to these data.

In Figure 3 we have provided the results of study of RARB regulation region methylation. In accordance with data of Figure 1B PCR should produce DNA fragment of 0.85 kb in length. We observe this DNA fragment in all control experiments (lanes 5, 9, 13, 19, 23), in results of BslI-PCR assay (lanes 4, 8, 12, 18, 22) and GlalI-PCR assay of DNA from L-68 (lane 3). BslI-PCR assay provides a negative result due to absence of BslI recognition sites in the studied DNA fragment (Figure 1B). There are no bands in GlalI-PCR assay of DNA from HeLa and U-937 cells, and there are weak bands in GlalI-PCR assay of DNA from Raji and Jurkat cells. According to the obtained data GlalI cleaves RARB regulation region in DNA from all malignant cell lines. At the same time GlalI-PCR assay of DNA from L-68 cells (Figure 3, lane 3) produces a target band of an intensity, which is similar to control one (Figure 3, lane 5). So,

Glal-PCR assay has shown a significant methylation of RARB regulation region in DNA from HeLa, Jurkat, Raji, and U-937 cell lines, but not in DNA from L-68 cells.

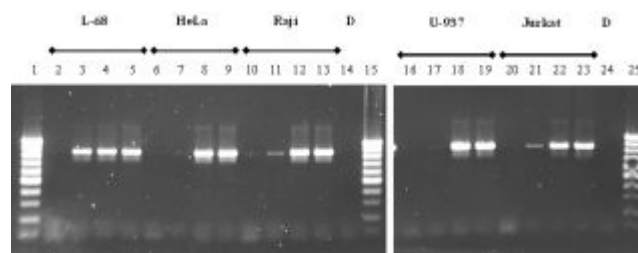


Fig. 3. BlsI- and Glal-PCR assay of RARB promoter and first exon region (PCR fragment 853 bp) Pretreated DNA from L-68 (2-5); HeLa (6-9); Raji (10-13); U-937 (16-19); Jurkat (20-23); DNA from *Drosophila melanogaster* (14 and 24). FatI (2, 6, 10, 16, 20), Glal (3, 7, 11, 17, 21), BlsI (4, 8, 12, 18, 22). 100 bp DNA Ladder (1, 15, 25).

Methylation of RARB promoter and first exon region in DNA from tumors has been observed in many works [18, 19 and references within them]. Moreover, determination of methylation of RARB regulation region was already used for early detection of breast cancer [22]. This is, probably, because RARB tumor suppressor gene is responsible for a growth and cell cycle regulation [3]. In this case methylation assay of RARB regulation region is supposed to be one of the most suitable methods of cancer detection at early stages. Our results have confirmed methylation of RARB regulation region in malignant cells only and support an idea of this DNA methylation assay as a possible method of early cancer diagnostics.

Figure 4 demonstrates the obtained results of BlsI- and Glal-PCR assays of RASSF1A first exon region. In accordance with data of Fig.1C PCR of RASSF1A first exon region should produce DNA fragment of 0.45 kb in length. We observe this band in PCR of all control DNA (lanes 5, 9, 13, 19, 23) and in case of Glal (lanes 3, 7, 17) and BlsI digested DNAs (lanes 4, 8, 18) from all cell lines, except Raji and Jurkat. BlsI- and Glal-PCR assays give a positive result in case of DNA from Raji and Jurkat cells, which are completely cleaved by both Glal and BlsI producing no fragments in PCR (lanes 11, 12, 21, 22).

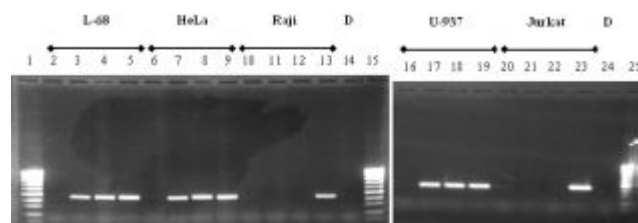


Fig. 4. BlsI- and Glal-PCR assay of RASSF1A first exon region (PCR fragment 450 bp) Pretreated DNA from L-68 (2-5); HeLa (6-9); Raji (10-13); U-937 (16-19); Jurkat (20-23); DNA from *Drosophila melanogaster* (14 and 24). HaeIII (2, 6, 10, 16, 20), Glal (3, 7, 11, 17, 21), BlsI (4, 8, 12, 18, 22). 100 bp DNA Ladder (1, 15, 25).

Data of BlsI- and Glal-PCR assays of RASSF1A first exon region show, that methylated sites in

positions 50318146 and 50318141, which form DNA sequence 5'-G(5mC)GCTG(5mC)GC-3'/3'-CG(5mC)GACG(5mC)G-5', are present in DNA from Raji and Jurkat cells only. According to the data of Glal-PCR assay both sites 5'-GCGC-3' in DNA sequence 5'-GCGCTGCGC-3' are not methylated in other cell lines (lanes 3, 7, 17).

Methylation of RASSF1A regulation region was investigated thoroughly [23-25]. A detailed study of RASSF1A promoter and first exon regions methylation in DNA from breast cancer cell lines was undertaken by Yan et.al. [26]. The authors have observed a significant methylation of DNA in a region, which is close to DNA sequence 5'-GCGCTGCGC-3'. Based on these data and results of our work it's possible to conclude that a significant methylation of definite position(s) in a RASSF1A first exon region indeed takes place in some malignant cell lines, whereas other cell lines don't have modification of a first exon region.

Summarizing the obtained results we should notice that, BslI- and Glal-PCR assays have shown an absence of methylation of RARB and RASSF1A regulation regions in DNA from control L-68 cells. At the same time RARB regulation region is methylated in DNA from all malignant cell lines studied in this work. It's interesting that according to the data of BslI- and Glal-PCR assays RASSF1A first exon region isn't methylated in DNA from two malignant cell lines (U-937 è HeLa) and is completely methylated in DNA from two other malignant cell lines (Raji è Jurkat). In a case of DAPK1 promoter region BslI-PCR assay has revealed an additional methylation of this region in DNA from Raji cells only. Surprisingly, a study of methylation of regulation region of three tumor suppressor genes displays different methylation patterns in all three studied leukemia cell lines U-937, Raji and Jurkat, but we have received the same methylation patterns for cervical cancer (HeLa) and leukaemic monocytic lymphoma (U-937).

Methylation of RARB and RASSF1A regulation regions and an additional methylation of DAPK1 promoter region, which are observed in different combinations in studied malignant cell lines, likely, result in inactivation of corresponding oncosuppressor genes and proliferation of different tumors. DNA methylation study of regulation region of other tumor suppressor genes should reveal a difference in methylation patterns of HeLa and U-937 cells. So, experimental data on BslI- and Glal-PCR assays of regulation region of a whole set of tumor suppressor genes may be used both in detection of malignant cells and their discrimination.

In fact, the suggested method of Glal- and BslI-PCR assays is a version of simple, well known and widely used PCR assay (even not a real-time PCR assay) and requires just an additional treatment of studied DNA with endonuclease before PCR. This analysis may be done in standard laboratories in hospitals and universities, and its simplicity allows using Glal- and BslI-PCR assays for massive screening of DNA samples in clinics.

SibEnzyme Ltd.: Patent RU 2413773 C1 (2011).

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