Biology and Medicine
ISSN: 0974-8369

Evdokimov *et al.*, Biol Med (Aligarh) 2016, 8:7
DOI: 10.4172/0974-8369.1000342

Research Article Open Access

GLAD-PCR Assay of DNA Methylation Markers Associated with Colorectal Cancer

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Received: Aug 4, 2016; **Accepted:** Sep 1, 2016; **Published:** Oct 7, 2016

Abstract

Hypermethylation of the gene regulatory regions is documented for many cancer diseases. Such an aberrant DNA methylation in cancer cells is catalyzed by DNA methyltransferases Dnmt3a and Dnmt3b, which predominantly recognize and methylate RCGY sequences with formation of R(5mC)GY sites. Recently, based on a new methyl-directed DNA endonuclease GlaI, we developed a GLAD-PCR assay, which allows determining R(5mC)GY site in a defined position of the genomic DNA. In this work we applied GLAD-PCR assay for identification of the methylated RCGY sites in the regulatory regions of some downregulated genes associated with colorectal cancer (CRC). This list includes *ADHFE1*, *ALX4*, *CNRIP1*, *EID3*, *ELMO1*, *ESR1*, *FBN1*, *HLTF*, *LAMA1*, *NEUROG1*, *NGFR*, *RARB*, *RXRG*, *RYR2*, *SDC2*, *SEPT9*, *SFRP2*, *SOCS3*, *SOX17*, *THBD*, *TMEFF2*, *UCHL1*, and *VIM* genes. GLAD-PCR analysis of selected RCGY sites within the regulatory regions of some of these genes demonstrates a good prognostic potential with relatively high sensitivity and specificity of CRC detection in tumor DNA.

Keywords: Colorectal cancer; DNA methylation; Epigenetics; GLAD-PCR assay; Methyl-directed endonuclease GlaI

Introduction

Colorectal cancer (CRC) is one of the major malignancies leading to a high incidence of cancer death worldwide [1,2]. However, early disease detection significantly decreases CRC-related mortality [3]. Nowadays, the detection of epigenetic biomarkers is one of the most promising diagnostic and prognostic tools [4]. It is well known that hypermethylation of CpG-islands in regulatory regions of promoter and/or first exon in a variety of genes often occurs at early stages of sporadic carcinogenesis. This leads to downregulation of the genes expression in tumor cells, whereas in a healthy tissue the corresponding genes remain to be active [5]. In particular, such an aberrant methylation had been reported for about fifty genes in tumor tissues, blood and stool samples from CRC patients [4,6-11].

In mammals, *de novo* DNA methylation, including abnormal hypermethylation in cancer cells, is performed by DNA methyltransferases Dnmt3a and Dnmt3b, which predominantly recognize RCGY site and modify internal CG-dinucleotide to form 5'-R(5mC)GY-3'/3'-YG(5mC)R-5' sequence [12]. Subsequently, such a *de novo* methylation events are maintained during DNA replication by DNA methyltransferase Dnmt1 [13]. Meanwhile, recently discovered and characterized methyl-directed site-specific DNA endonuclease GlaI recognizes and cleaves DNA sequence R(5mC)↓GY as indicated by the arrow with formation of blunt ends [14]. Due to this unique substrate specificity, GlaI is a convenient tool for identification of *de novo* methylated sites in the human and mammalian DNA. On the basis of this enzyme, we developed a GLAD-PCR assay (GlaI hydrolysis and Ligation Adapter Dependent PCR) allowing quick and inexpensive estimation of 5'-R(5mC)GY-3' sequence in a definite position of human genome without bisulfite DNA conversion [15]. Briefly, GLAD-PCR assay is carried out in three steps. At the first step, GlaI cleaves R(5mC)GY sites in genomic DNA. Then the obtained DNA fragments are ligated with the unique oligonucleotide adapter. The last step is a PCR with TaqMan probe and a genomic primer, which are complementary to target DNA fragment, and a so-called hybrid primer, which is complementary to the adapter and partially to the genomic sequence at the cleavage point of the site of interest. As a result, despite the presence of a huge number of different DNA fragments obtained after GlaI hydrolysis and a ligation step, PCR takes place specifically from the target region of DNA.

Recently we applied this new assay to determine the methylated RCGY sites in the promoter region of *ESR1* gene and the first exon region of *ELMO1* gene in tumor tissues from CRC patients [16]. In the present study we expand our research on a larger number of DNA samples and CRC-associated, epigenetically downregulated genes in order to identify in human genome RCGY sites with the most prognostic potential.

Materials and Methods

Patients and samples

The study group included twenty-one CRC patients (8 male and 13 female) who had undergone surgery between September 2014 and August 2015. The age of patients was from 46 to 82 years. Fifteen patients had CRC without distant metastases (stage I to III), while the other six had metastatic stage IV disease. A total of thirty fresh-frozen surgical resection samples were studied, including colorectal adenocarcinomas of varying degree of differentiation ($n = 21$) and several paired normal colon mucosa controls ($n = 9$). The samples were collected at the Seversk Biophysical Research Centre (Seversk, Russia). All participating patients voluntarily joined this study with the written informed consent to have their biologic specimens to be analyzed, and the work had been approved by the Ethical Committee of the Seversk Biophysical Research Centre, consistent with the WMA Declaration of Helsinki. A colorectal adenocarcinoma

cell line SW837 was obtained from SRC VB "Vector," Novosibirsk, Russia.

DNA isolation

Samples of SW837 cells, fresh tumor and normal mucosa tissues were stored at –20°C until processing. The samples were ground in liquid nitrogen and DNA preparations were isolated by standard phenol-chloroform method [17]. Nucleic acid concentrations were estimated by UV spectrophotometry using NanoVue Plus (GE Healthcare, UK).

Selection of genes and RCGY sites for GLAD-PCR assay

We carried out a literature search of epigenetically downregulated genes involved in colorectal carcinogenesis. The selection criterion was a different methylation of the gene regulation regions in CRC tissues or cell lines and noncancerous controls. In addition the findings of ENCODE/HudsonAlpha project (https://www.encodeproject.org) [18] rendered by UCSC Genome Browser (http://genome-euro.ucsc.edu) were taken into consideration. The main criterion of RCGY sites choice was a rather long distance (50 bases or more) between the two RCGY sites to place a genomic primer and TagMan probe herein.

Primers, probes, and oligonucleotide adapter

To design the primer and probe, we used the GenBank database, Vector NTI 11.5 software (Invitrogen, USA), and NCBI BLAST resource (http://blast.ncbi.nlm.nih.gov). The sequences were calculated to allow uniform PCR conditions with an annealing temperature of 61°C. A list of primers and probes is provided in Table 1. The primers are indicated "g" (direct genomic primer) and "r" (reversed genomic primer) and serve to monitor a successful PCR amplification of target regulatory region and for normalization of tissue DNA concentration. In GLAD-PCR assay experiments, a reversed genomic primer was replaced by a "hybrid" primer, which corresponds to the methylated RCGY site in the studied DNA region. In detail, the determination of hybrid primers for GLAD-PCR assay is described in the Results section. As an adapter we used oligonucleotide duplex 5'-CCTGCTCTTTCATCG-3'/3'-pGGACGAGAAAGTAGCp-5', where "p" means phosphate.

GLAD-PCR assay protocol

Methyl-directed DNA endonuclease GlaI, T4 DNA ligase, Hot Start Taq DNA polymerase, dNTPs, 10× GlaI reaction buffer (0.1 M Tris-HCl, pH 8.5, 0.1 M NaCl, 50 mM $MgCl_2$, 10 mM ß-mercaptoethanol), 10× GLAD-PCR buffer (0.5 M Tris-SO_4, pH 9.0, 0.1 M $[NH_4]_2SO_4$, 0.3 M KCl, 0.1% Tween 20, 30 mM $MgCl_2$), and 5× Stabilizer for GC-rich DNA amplification (2.7 M betaine, 6.7 mM DTT, 6.7% DMSO) were supplied by SibEnzyme (Novosibirsk, Russia). The normalization of the tissue DNA concentration for each studied region of genome was performed by real-time PCR using primers and probes listed in Table 1. The PCR conditions were the same as described below for the PCR step of GLAD-PCR assay.

We performed GlaI hydrolysis and adapter ligation in one step. The reaction mixture contained 1× GlaI buffer, 100 ng/μL BSA,

Gene[a] (region)	Gene name[a]	Chromosomal location[a]	Primer/probe sequence[b]
ADHFE1	Alcohol dehydrogenase, iron containing 1	8q12.3	g: GTGGGCACCCTGCGGTCC p: FAM-CCCGCCGGCCCCGCACTC-BHQ1 r: GGTCGCGTACTTGCTGAGGCA
ALX4	ALX homeobox 4	11p11.2	g: CGTCAACAACCTCTCATCC p: FAM-TCCATTTCTTATTTCAGTTTGCCACCA-BHQ1 r: GGACTCTGGTTTCTAAGATCAG
CNRIP1	Cannabinoid receptor interacting protein 1	2p13	g: GCCAGACCCTCGCCCAGACA p: FAM-CGAGGCCCGGCAGGTCCCCC-BHQ1 r: CGTCATTAGGCTGGATGCGCA
EID3	EP300 interacting inhibitor of differentiation 3	12q23.3	g: GCTCCGCGGGAAGACAGCC p: FAM-CCCGGCCCAGCCACAAGC-BHQ1 r: TTTGAAAAAGAATAGCTGTCCCCTGA
ELMO1	Engulfment and cell motility 1	7p14.1	g: GGGTCGCCGGAGCTCTGA p: FAM-AACCCTTGCCGCTGCTGTCCTGC-BHQ1 r: CGCCAGCCCAGGAAACTTTAC
ESR1	Estrogen receptor 1	6q24-q27	g: CGCAGGGCAGAAGGCTCAGAA p: FAM-TGCTCTTTTTCCAGGTGGCCCGCC-BHQ1 r: CGGGACATGCGCTGCGTC
FBN1(1)	Fibrillin 1	15q21.1	g: GCGGGGAGACTTTCAGGGCA p: FAM-ATGCTGAAGCCTCGCGGTCCCC-BHQ1 r: CACGGGTTGGGCTTGGGA
FBN1(2)	–	–	g: CAGCAGCCCCGGCCGATC p: FAM-CCTCCCGGGCCCCGCCAGA-BHQ1 r: GGGTACTTTGCGCCGCGCTC
FBN1(3.1)	–	–	g: GTAGCGGCCACGACTGGGA p: FAM-CAGCCGCCGCCGCCTCCTC-BHQ1 r: CCGGCTGCACCCACTGGA
FBN1(3.3)	–	–	g: GAGCCCGGCACCAAGAGC p: FAM-CCCTCCCCTGCCTGACAGCTTCC-BHQ1 r: CACCGGGGCTGGAGCTGC
HLTF	Helicase-like transcription factor	3q25.1-q26.1	g: AACAAAACACCGGCACCGCA p: FAM-CAGTCGCACTCCTGGGGCCTCGT-BHQ1 r: GTTCTTCCCCGCCCCCAA
LAMA1	Laminin subunit alpha 1	18p11.3	g: CCACCTTCTCTGCCCACCTCCTA p: FAM-CTGACCGCGGCCGCCTCCC -BHQ1 r: CCGCCACCCAGACCCCTC

NEUROG1	Neurogenin 1	5q23-q31	g: GTGCCTCGGCCGCTAATCG p: FAM-CCGACCCCGCCTCTGTTTCACTGC-BHQ1 r: CCGTAATTACCGCCGGCCAATC
NGFR	Nerve growth factor receptor	17q21-q22	g: TGGCTTCACCCAGCCTCTC p: FAM-CAGCCAGAGCGAGCCGAGCC-BHQ1 r: TCCAGCTCGGTCCGCTTTG
RARB	Retinoic acid receptor beta	3p24	g: TTCAGAGGCAGGAGGGTCTATTC p: FAM-TCCCAGTCCTCAAACAGCTCGCATGG-BHQ1 r: GGTTCCCAGAAAGATCCCAAGTTC
RXRG	Retinoid X receptor gamma	1q22-q23	g: GCCGCCGTCACCGCTACT p: FAM-CCACCGCCGTCGCTGCTGC-BHQ1 r: GTGCCACCCGGTAGGGACC
RYR2	Ryanodine receptor 2	1q43	g: GGGGACCACGGAGGCGACT p: FAM-TTTCCCCCAAGTCAAGGTGCTGCGAAA-BHQ1 r: CGGGGGTGATGGTGCAGGA
SDC2	Syndecan 2	8q22-q23	g: GCGATTGCGGCTCAGGCT p: FAM-CCCCGAGCCCGAGTCCCCG-BHQ1 r: GGGAGTGCAGAAACCAACAAGTGA
SEPT9	Septin 9	17q25.3	g: GCAGGAGGCTGTATTTGGG p: FAM-AGCCAAACAAAGTTCTCTGTCACCGCC-BHQ1 r: CGCTGCCGTTTAACCCTTG
SFRP2	Secreted frizzled-related protein 2	4q31.3	g: GCACAGCCAGAGTTTTCTTG p: FAM-TACCCTTCATTGGCTCCTCCCTTGCT-BHQ1 r: AGGCTTCTCTGTTTGTTGTTTAAAG
SOCS3	Suppressor of cytokine signaling 3	17q25.3	g: CAGTCCCGGGGGCCCTTCT p: FAM-TGCTCCCCACCCGGCCACACTCC-BHQ1 r: CAAGGGCGCAGCGTGGGA
SOX17	SRY-box 17	8q11.23	g: CGCCCTCCGACCCTCCAA p: FAM-TCCCGGATTCCCCAGGTGGCC-BHQ1 r: CAGTTCAGGGCCAAGGGTGCT
THBD	Thrombomodulin	20p11.21	g: GTTCGGGAAAAGGAAGGAAGTGC p: FAM-ATTGCTGGGTTCTCTGGCCGCC-BHQ1 r: TTACTCATCCCGGCGAGGTGA
TMEFF2	Transmembrane protein with EGF-like and two follistatin-like domains 2	2q32.3	g: GGTGGGCTACCCGCACACTCATA p: FAM-CCATTCGCCTCACTCTCCGCTCCA-BHQ1 r: CGCCGACTCGCCCCTCTC
UCHL1	Ubiquitin C-terminal hydrolase L1	4p13	g: GCAGAACCAAGCGAGGGGGAA p: FAM-CGTACCCATCTGGCCGCGACCGTC-BHQ1 r: GGGGCCCGGCCGTACCAC
VIM	Vimentin	10p13	g: TCCGCAGCCATGTCCACCA p: FAM-CCGTGTCCTCGTCCTCCTACCGCAG-BHQ1 r: GCTGCCCAGGCTGTAGGTGC

[a]Gene symbol, gene name, and chromosomal location are in accordance with the approved guidelines from the HUGO Gene Nomenclature Committee (http://www.genenames.org);
[b]g—direct genomic primer, p—probe, r—reverse genomic primer, FAM—6-carboxyfluorescein, BHQ1—Black Hole Quencher 1.

Table 1: The genes selected for the study and a list of specific primers and probes used

6 mM ß-mercaptoethanol, 0.5 mM ATP, 0.5 μM adapter oligonucleotide duplex, 0.04 U/μL GlaI endonuclease, and 33 U/μl T4 DNA ligase. Three nanograms of the tissue DNA or fifteen nanograms of SW837 DNA were used as the template. The reaction was conducted in 30 μl at 25°C for 1 h followed by inactivation of enzymes at 65°C for 20 min. Further the following components were added to the final concentration: 1× GLAD-PCR buffer, 1× Stabilizer, 0.2 mM of each of dNTPs, the mixture of genomic primer, hybrid primer and probe at 0.4 μM concentration of each, and 0.05 U/μL Hot Start Taq DNA polymerase. The hybrid primers, selected experimentally for each gene, are listed in Table 2. Real-time PCR was performed using the CXF-96 detection system (Bio-Rad Laboratories, Hercules, USA) in a final volume of 20 μl under the following conditions: preheating at 95°C for 3 min, then 5 cycles at 95°C for 10 s, 61°C for 15 s, 72°C for 20 s without detection followed by the another 50 cycles with detection of FAM fluorescence during the annealing step at 61°C. All the GLAD-PCR reactions were performed in triplicates.

Statistical analysis

The statistical analysis of results of GLAD-PCR assay was performed with the MedCalc 15.11 software (MedCalc Software, Ostend, Belgium). According to the quantification cycle (Cq) values obtained for each studied R(5mC)GY site, receiver operating characteristic (ROC) curves with 95% confidence interval (CI) were determined to assess the assay sensitivity and specificity with an area under the ROC curve (AUC) being estimated nonparametrically [19]. The optimal cutoff value was used to establish the marker methylation status (positive or negative).

Results

Selection of RCGY sites and corresponding hybrid primers for GLAD-PCR assay

In the first part of the work, we carried out a literature search of epigenetically downregulated genes involved in colorectal carcinogenesis. For the present study, we chose well-known genes,

Gene (region)	Target site	Site location[a]	Hybrid primer[b]
ADHFE1	GCGT	chr8: 66432544–66432547	CCTGCTCTTTCATCGGTAC
ALX4	GCGC	chr11: 44304711–44304714	CCTGCTCTTTCATCGGCGC
CNRIP1	GCGC	chr2: 68319361–68319364	CCTGCTCTTTCATCGGCGA
EID3	GCGT	chr12: 104303610–104303613	CCTGCTCTTTCATCGGTGT
ELMO1	GCGC	chr7: 37448622–37448625	CCTGCTCTTTCATCGGCCG
ESR1	GCGT	chr6: 151807784–151807787	CCTGCTCTTTCATCGGTGT
FBN1(1)	GCGT	chr15: 48645054–48645057	CCTGCTCTTTCATCGGCGC
FBN1(2)	GCGC	chr15: 48645174–48645177	CCTGCTCTTTCATCGGCTC
FBN1(3.1)	GCGC	chr15: 48645489–48645492	CCTGCTCTTTCATCGGCGG
FBN1(3.3)	GCGC	chr15: 48645489–48645492	CCTGCTCTTTCATCGGCCG
HLTF	GCGC	chr3: 149086718–149086721	CCTGCTCTTTCATCGGCGG
LAMA1	ACGC	chr18: 7117965–7117968	CCTGCTCTTTCATCGGCGG
NEUROG1	GCGC	chr5: 135536188–135536191	CCTGCTCTTTCATCGGCGG
NGFR	GCGC	chr17: 49495342–49495345	CCTGCTCTTTCATCGGCTC
RARB	ACGC	chr3: 25428374–25428377	CCTGCTCTTTCATCGGTTC
RXRG	GCGC	chr1: 165445276–165445279	CCTGCTCTTTCATCGGCCG
RYR2	GCGC	chr1: 237043053–237043056	CCTGCTCTTTCATCGGCGT
SDC2	GCGT	chr8: 96494057–96494060	CCTGCTCTTTCATCGGTTC
SEPT9	GCGC	chr17: 77372894–77372897	CCTGCTCTTTCATCGGCAG
SFRP2	GCGC	chr4: 153789220–153789223	CCTGCTCTTTCATCGGCGT
SOCS3	GCGC	chr17: 78359427–78359430	CCTGCTCTTTCATCGGCGG
SOX17	GCGC	chr8: 54458724–54458727	CCTGCTCTTTCATCGGCCG
THBD	GCGC	chr20: 23049816–23049819	CCTGCTCTTTCATCGGCGA
TMEFF2	GCGC	chr2: 192195257–192195260	CCTGCTCTTTCATCGGCGG
UCHL1	GCGC	chr4: 41256788–41256791	CCTGCTCTTTCATCGGCTG
VIM	GCGC	chr10: 17229549–17229552	CCTGCTCTTTCATCGGCGC

[a]Site locations are given in accordance with the recent human genome assembly GRCh38/hg38;
[b]3'-terminal tetranucleotide sequence of hybrid primer, which is complemented to the genomic sequence at the point of GlaI hydrolysis, is underlined.

Table 2: Studied DNA regions with indication of the target RCGY sites, their locations, and a structure of corresponding hybrid primers selected for GLAD-PCR assay

regulation regions of which were often hypermethylated at colorectal cancer: *SEPT9*, *FBN1*, *VIM*, *SDC2*, *THBD*, *SFRP2*, *ESR1*, *TMEFF2*, *NGFR*, *ALX4*, *HLTF*, and *NEUROG1* [20,21]. Less studied putative CRC biomarkers, such as *CNRIP1* [22], *ADHFE1* [23], *UCHL1*, *ELMO1* [24], *LAMA1* [25], and *SOX17* [26], were also examined. Three genes were selected on the basis of the recent genome-wide studies (*SOCS3*, *RYR2* [11], and *EID3* [27]). Finally we also analyzed the genes for retinoid receptors *RARB* and *RXRG*, which were found to be methylated in many types of cancer [28]. A list of the names and function of the studied genes, their chromosomal location and structure of probe, direct and reverse primers for PCR study of regulation regions is provided in Table 1.

At the second step we conducted screening of RCGY sites within the studied regulatory regions of genes in order to select the methylated sites for further GLAD-PCR analysis of clinical samples. To find these sites, we used the DNA preparation from colorectal adenocarcinoma cell line SW837. The screening was performed by the GLAD-PCR assay with hybrid primers corresponding to the RCGY sites located within ~200 bp distance from the probe position. A general DNA structure of hybrid primer is 5'-CCTGCTCTTTCATCGGYNN-3', where first 15 bases are complementary to the oligonucleotide adapter, whereas the underlined tetranucleotide is complementary to the genome sequence at GlaI cleavage point. This sequence corresponds to 32 possible variants of the hybrid primer depending on the structure of the blunt ends obtained after GlaI hydrolysis of sequence 5'-NNR(5mC)↓GY-3'.

An example of determination of the methylated site in the regulation region of *FBN1* gene is given in Figure 1. In case of *FBN1* gene, we studied four parts of its regulation region presented in Figure 1a, and they are indicated as *FBN1*(1), *FBN1*(2), *FBN1*(3.3), and *FBN1*(3.1). In the first three parts, a genomic primer is located before TaqMan probe, whereas in case of *FBN1*(3.1), a genomic primer and TaqMan probe lie in opposite direction. In the *FBN1*(3.1) region we have studied three RCGY sites closest to the probe annealing sequence: two GCGC sites and one ACGC site (Figure 1a). Figure 1b shows the result of GLAD-PCR assay of *FBN1*(3.1) regulation region and demonstrates that site GCGC in position 48645483 is methylated whereas sites GCGC and ACGC in positions 48645488 and 48645494, respectively, are not methylated. The results of the methylated site determination in all 26 studied regulation regions are presented in Table 2. As it follows from Table 2, *FBN1*(3.3) and *FBN1*(3.1) regulation regions show the same methylated site in position 48645483. So, we identified 25 methylated sites in regulation regions of 23 genes. In all cases we observed a presence of methylated and unmethylated sites, which were similar to a methylation scheme of *FBN1*(3.1) regulation region: there is one methylated site and other sites are not methylated or significantly less methylated. Earlier we observed a similar situation with *ELMO1* and *ESR1* genes where only one from three RCGY sites was significantly methylated in both cases [16]. As follows from Table 2, most of the target sites are GCGC (18 from 25), and other 7 target sites are GCGT or its complement ACGC. Surprisingly, there is no ACGT sequence among all target sites.

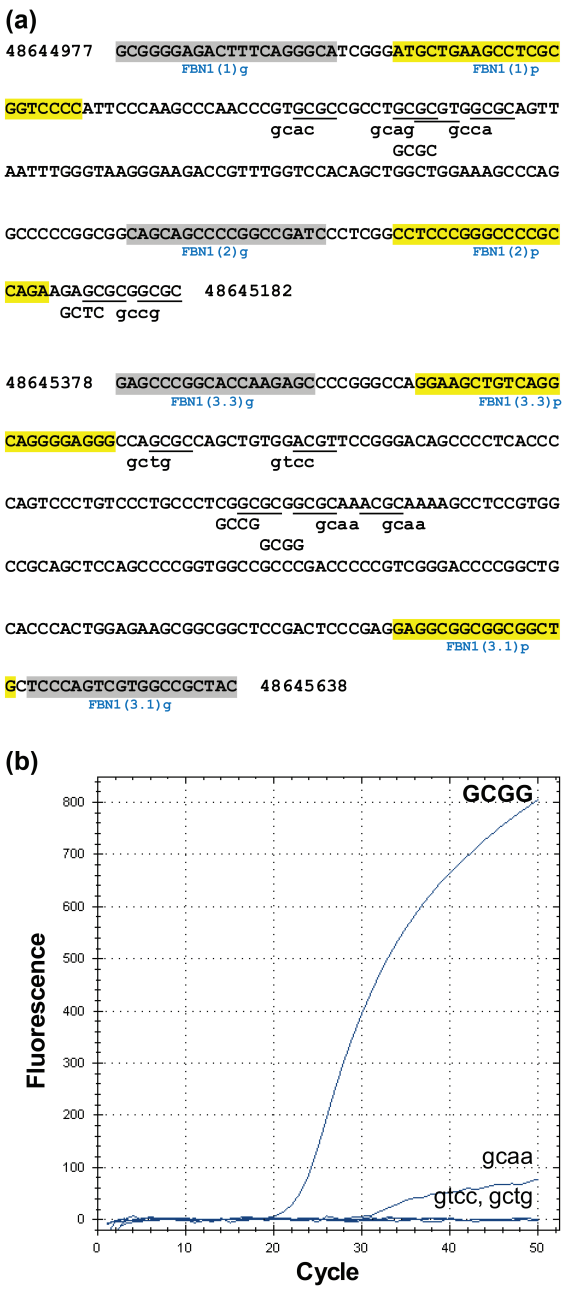


Figure 1: Selection of marker RCGY sites and corresponding hybrid primers for GLAD-PCR assay of *FBN1* gene. (a) Two fragments of *FBN1* regulatory region nucleotide sequence. The annealing sites for genomic primers and TaqMan probes are shown with gray and yellow background, correspondingly. RCGY sites are underlined. 3'-termini of the used hybrid primers are shown below the sequence. The selected termini for further GLAD-PCR analysis are indicated by capital letters. The location of region in chromosome 1 is given according to human genome assembly GRCh38/hg38. (b) Real-time PCR curves that were obtained using different hybrid primers for *FBN1*(3.1) variant

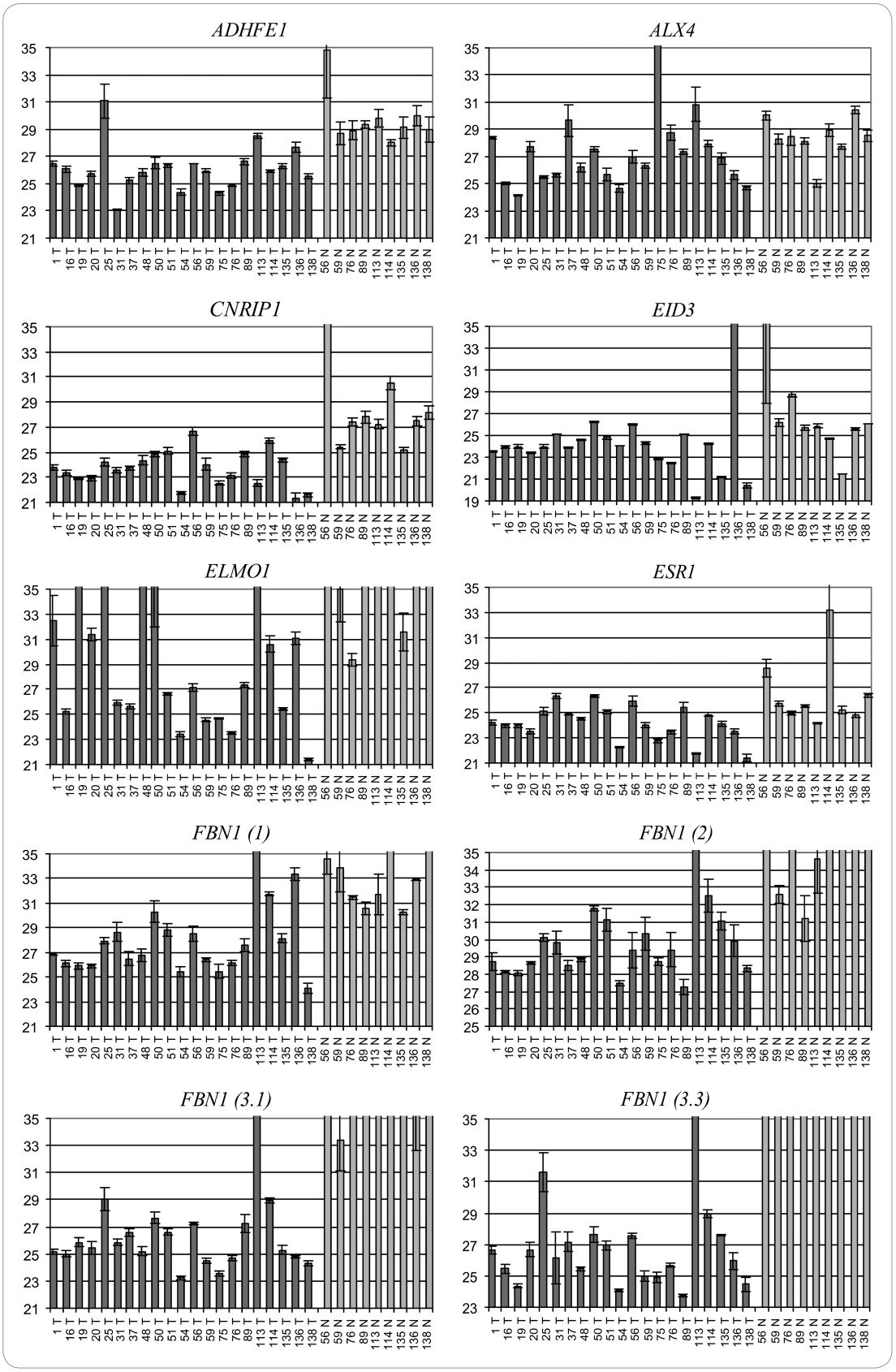
GLAD-PCR assay of selected RCGY sites in the clinical samples

DNAs isolated from the tissue samples ($n = 21$) of colorectal adenocarcinomas of varying degree of differentiation and nine paired normal colon mucosa samples were studied by GLAD-PCR assay using primers and TaqMan probes listed in Tables 1 and 2. Figure 2 presents the diagrams of the obtained Cq values for selected RCGY sites in all 26 studied DNA regions. According to Figure 2, the methylated selected sites (displaying a small Cq) in DNA preparations from tumor samples are presented in all regulation regions. We see the methylated target sites in *CNRIP1*, *ESR1*, and *SOX17* regulation regions in all DNA preparations from tumor samples. Target sites in *EID3* and *TMEFF2* regulation regions are methylated in all tumor DNA samples except one (136T and 75T, respectively). A maximal number of tumor DNA samples with unmethylated target RCGY sites are in *HLTF* and *SDC2* regulation regions (9 and 6, respectively). In case of *ELMO1*, *THBD*, and *VIM* regulation regions, we see five tumor DNA samples with unmethylated target sites. At the same time, GLAD-PCR assay of DNA preparations from normal colon samples shows an absence of the selected RCGY sites methylation in case of *SDC2*, *FBN1*(3.3), *FBN1*(3.1), *SEPT9*, *THBD*, and *VIM* genes. Surprisingly, we observed methylation of selected RCGY sites in all normal samples in case of *TMEFF2* and *SOX17* regulation regions and in most of the normal samples in *EID3* and *ESR1* genes. Based on Cq values obtained for each selected RCGY site, the receiver operating characteristic (ROC) curves were determined (Figure 3) to assess the assay sensitivity and specificity under the optimal cutoff values as well as an area under the ROC curve (AUC) with 95% CI. The results of ROC analysis of data are summarized in Table 3, where AUC presents the overall accuracy of the individual markers for distinguishing colorectal cancer from normal mucosa. The order of diagnostic performance for better markers (AUC > 0.8) looks as follows: *FBN1*(3.3) > *FBN1*(3.1) = *CNRIP1* > *ADHFE1* > *FBN1*(2) > *FBN1*(1) > *SDC2* > *UCHL1* > *SEPT9* = *VIM* > *THBD* > *SOCS3*.

Discussion

Earlier we used GLAD-PCR assay for DNA methylation study of regulation regions of *ELMO1* and *ESR1* genes in tumor tissues from CRC patients [16]. GLAD-PCR assay provides several advantages in comparison to the existing methods of locus-specific methylation analysis. GLAD-PCR assay is not based on DNA bisulfite treatment, which causes serious DNA degradation (up to 90% [29]). And this DNA damage may be especially important for currently developing diagnostic techniques, which use small amounts of free-circulating blood DNA for analysis. The unique degenerate recognition site of GlaI R(5mC)GY is more abundant in the human genome in comparison to the recognition sites of restriction enzymes used in epigenetic studies, such as HpaII, MspI, SmaI, and XmaI. Thus, GlaI usage allows to cover the putative methylation positions and to analyze sites that are not tested with other DNA endonucleases. A specificity of GLAD-PCR assay is rather high because it is based on DNA structure of genomic primer, TaqMan probe, and four complementary nucleotides of "hybrid" primer. A procedure of the sample DNA treatment in GLAD-PCR assay is simple and includes four steps in one tube: DNA cleavage with GlaI followed by heat inactivation, adapter ligation, and real-time PCR.

In this work we applied GLAD-PCR assay for determination of R(5mC)GY sites in regulation regions of 23 CRC-associated genes in order to select sites with the most prognostic potential. GLAD-PCR assay of DNA from a colorectal adenocarcinoma cell line SW837 was used to reveal R(5mC)GY sites in a studied part of regulation region of 23 genes. In case of *FBN1* regulation region, we studied four parts of regulation region indicated as *FBN1*(1), *FBN1*(2), *FBN1*(3.3), and *FBN1*(3.1). In each studied DNA region, we revealed one methylated site, whereas other RCGY sites were not methylated or significantly less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CNRIP1
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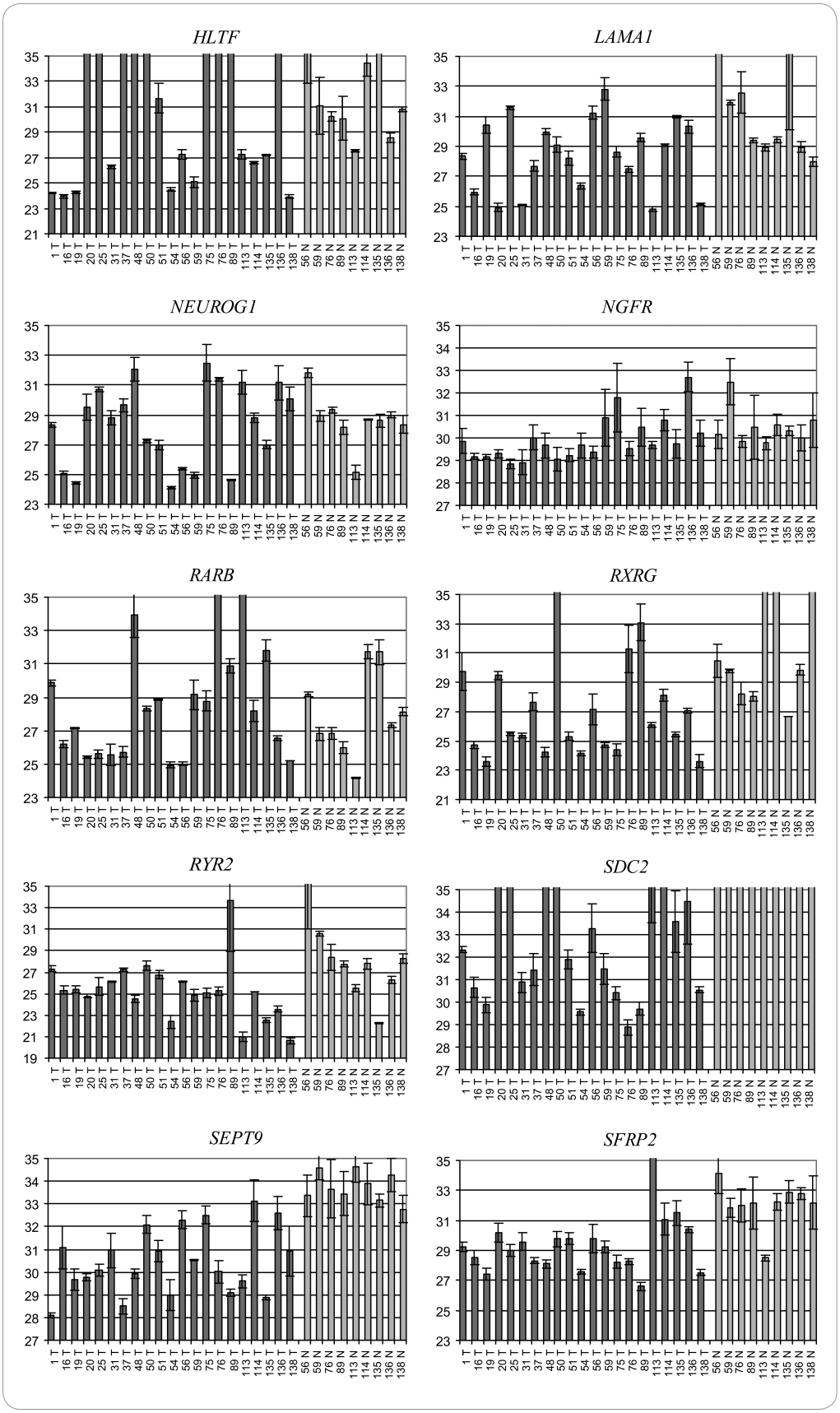
ELMO1
ESR1
FBN1 (1)
FBN1 (2)
FBN1 (3.1)
FBN1 (3.3)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NGFR
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SEPT9
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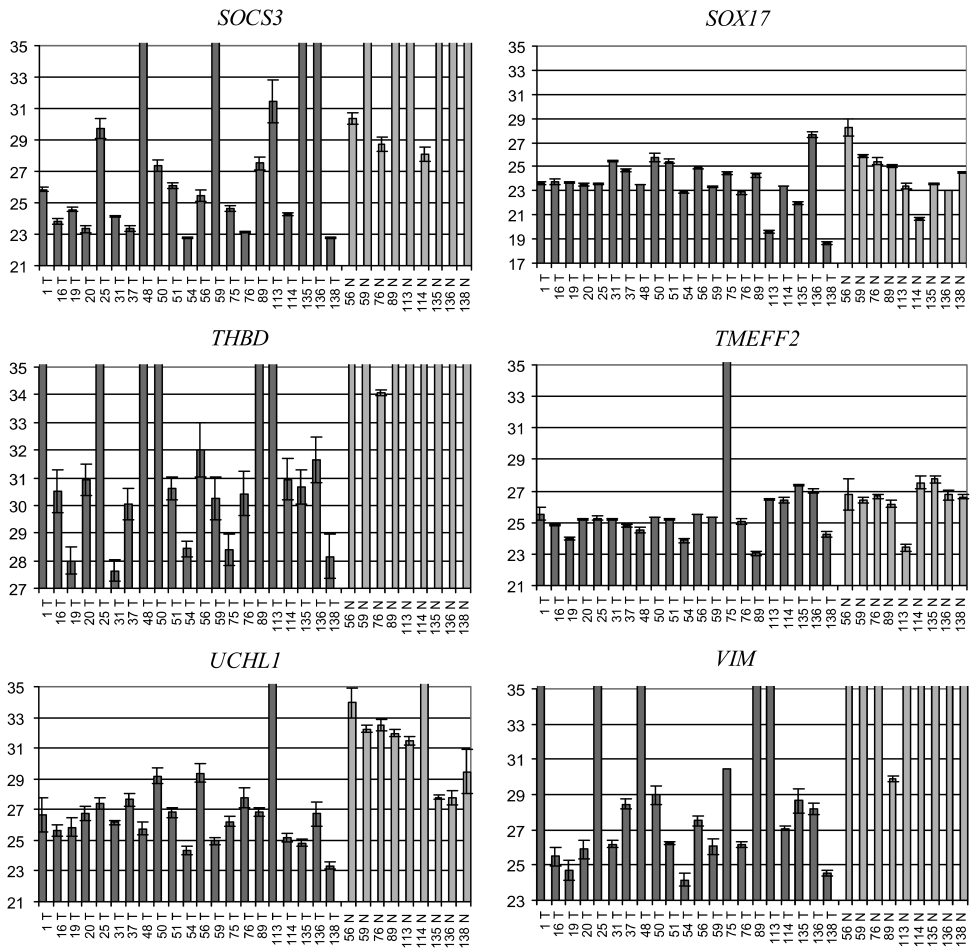
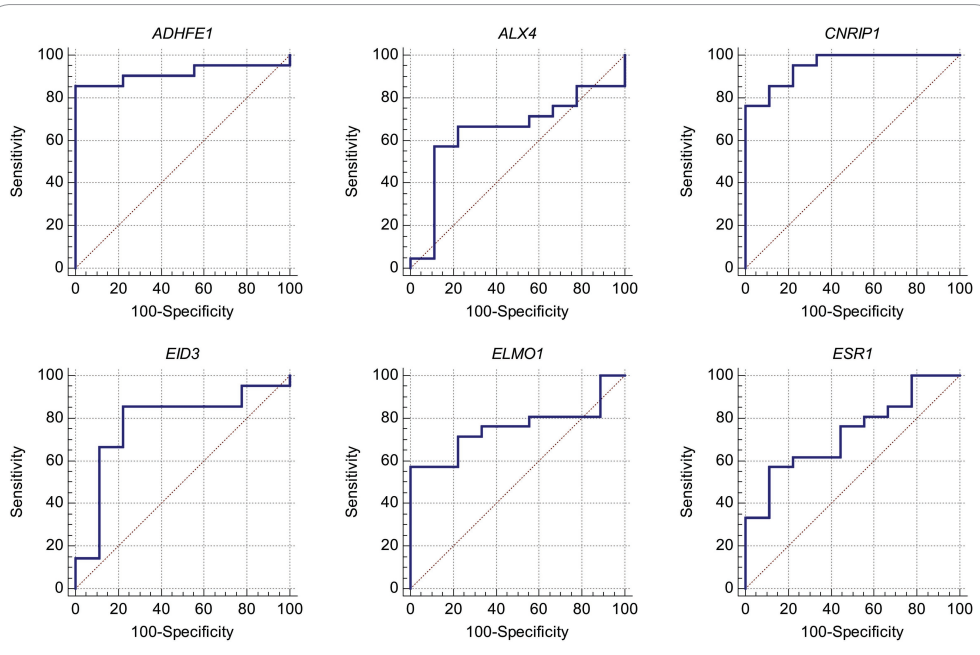
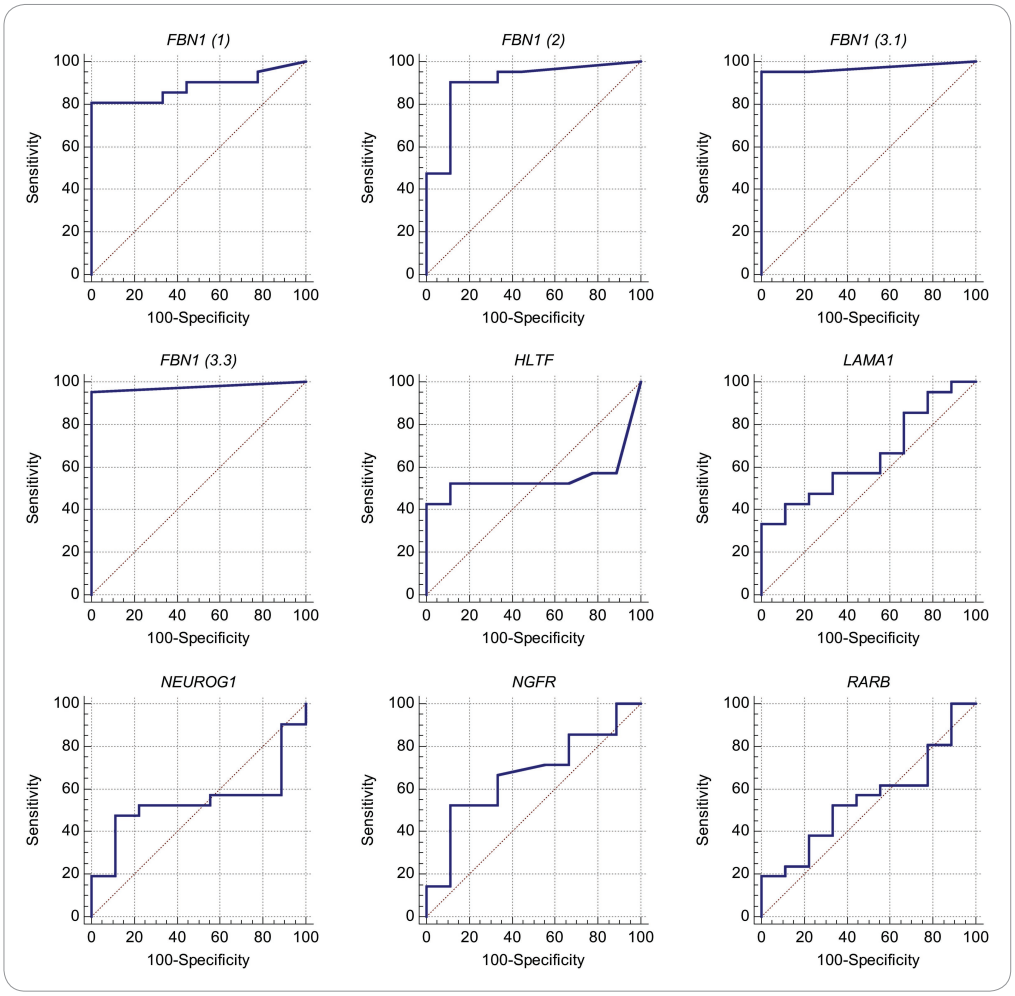
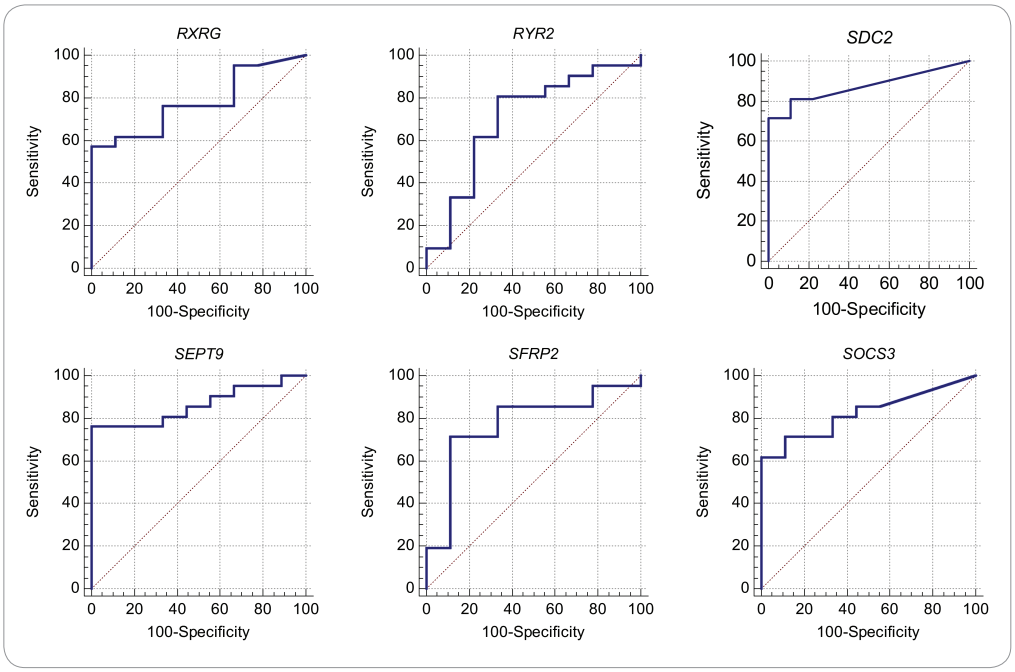


Figure 2: Cq values for selected RCGY sites obtained using GLAD-PCR assay of tissue DNAs. Sample designations are given below each diagram (T—tumor tissue, N—normal tissue)




FBN1 (1)
FBN1 (2)
FBN1 (3.1)
FBN1 (3.3)
HLTF
LAMA1
NEUROG1
NGFR
RARB
Sensitivity
100-Specificity
0 20 40 60 80 100


RXRG
RYR2
SDC2
SEPT9
SFRP2
SOCS3
Sensitivity
100-Specificity
0 20 40 60 80 100

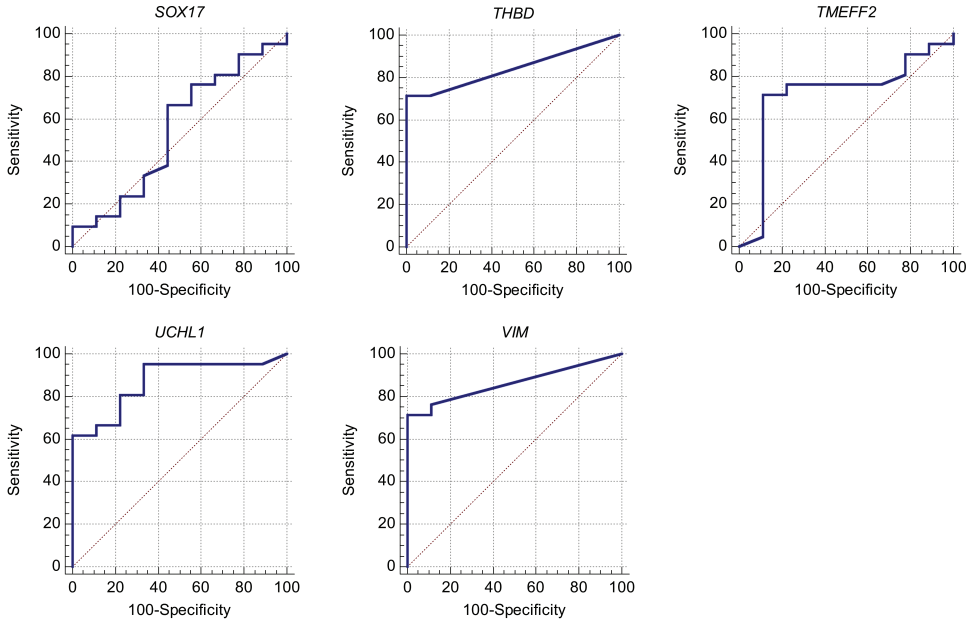


Figure 3: Receiver operating characteristic (ROC) curves for GLAD-PCR analysis of individual RCGY marker sites methylation in CRC versus normal mucosa tissues. Resulting sensitivity, specificity, area under the ROC curve (AUC) with standard error, and 95% confidence interval are summarized in Table 3

Gene (region)	Number of detected CRC samples/total number of CRC samples	Sensitivity, %	Number of negative controls/ total number of normal mucosa controls	Specificity, %	AUC (standard error)	95% CI
FBN1(3.3)	20/21	95.2	9/9	100	0.976 (0.024)	0.843-1.000
FBN1(3.1)	20/21	95.2	9/9	100	0.971 (0.029)	0.834-1.000
CNRIP1	16/21	76.2	9/9	100	0.952 (0.036)	0.806-0.997
ADHFE1	18/21	85.7	9/9	100	0.915 (0.055)	0.755-0.985
FBN1(2)	19/21	90.5	8/9	88.9	0.902 (0.065)	0.737-0.980
FBN1(1)	17/21	81.0	9/9	100	0.884 (0.062)	0.714-0.971
SDC2	15/21	71.4	9/9	100	0.873 (0.058)	0.701-0.966
UCHL1	13/21	61.9	9/9	100	0.870 (0.068)	0.697-0.964
SEPT9	16/21	76.2	9/9	100	0.862 (0.067)	0.688-0.960
VIM	15/21	71.4	9/9	100	0.862 (0.056)	0.688-0.960
THBD	15/21	71.4	9/9	100	0.841 (0.058)	0.662-0.948
SOCS3	13/21	61.9	9/9	100	0.825 (0.074)	0.644-0.939
RXRG	12/21	57.1	9/9	100	0.778 (0.085)	0.589-0.908
EID3	18/21	85.7	7/9	77.8	0.778 (0.103)	0.589-0.908
SFRP2	15/21	71.4	8/9	88.9	0.772 (0.099)	0.584-0.905
ELMO1	12/21	57.1	9/9	100	0.757 (0.089)	0.566-0.894
ESR1	12/21	57.1	8/9	88.9	0.730 (0.097)	0.538-0.875
TMEFF2	15/21	71.4	8/9	88.9	0.714 (0.114)	0.521-0.863
RYR2	17/21	81.0	6/9	66.7	0.704 (0.115)	0.510-0.855
NGFR	11/21	52.4	8/9	88.9	0.667 (0.108)	0.472-0.827
LAMA1	7/21	33.3	9/9	100	0.651 (0.106)	0.456-0.815
ALX4	12/21	57.1	8/9	88.9	0.646 (0.112)	0.451-0.810
HLTF	9/21	42.9	9/9	100	0.550 (0.106)	0.359-0.731
RARB	11/21	52.4	6/9	66.7	0.550 (0.114)	0.359-0.731
SOX17	14/21	66.7	5/9	55.6	0.548 (0.127)	0.356-0.729
NEUROG1	10/21	47.6	8/9	88.9	0.540 (0.110)	0.349-0.722

Table 3: Receiver operating characteristics for diagnosis of CRC versus normal mucosa determined by means of GLAD-PCR assay of selected RCGY sites (sorted by AUC values)

methylated. Methylated site in *FBN1*(3.3) and *FBN1*(3.1) regulation regions is the same and located in position 48645483 (Table 2). So, we studied 26 DNA regions and identified 25 R(5mC)GY sites in regulation regions of 23 genes. Results of GLAD-PCR assay of *FBN1*(3.3) regulation region (data are not shown) indicate that sites GCGC and ACGT, located in positions 48645433 and 48655447, respectively, are not methylated. Thus, a GLAD-PCR assay showed that in *FBN1*(3.1) and *FBN1*(3.3) regulation regions (positions 48645378-48655638, see Figure 1a), there is one methylated site G(5mC)GC in position 48645483, whereas other four RCGY sites (two RCGY sites upstream and two RCGY sites downstream this location) are not methylated.

At the next step, we performed GLAD-PCR assay of DNA preparations from 21 tumor tissues samples and 9 paired tissue samples from normal colon mucosa studying the abovementioned 26 DNA regions. The Cq data obtained for all studied DNA samples are shown in Figure 2. In all studied regulation regions, we see the methylated target sites in most of the tumor DNA samples. Comparing the obtained Cq data, we can see that *FBN1*(3.3) and *FBN1*(3.1) regulation regions give a similar pattern of selected RCGY site methylation in all 30 studied DNA samples. This coincidence corresponds to GLAD-PCR assay of two DNA regions but the same RCGY site located in position 48645483 of 15th chromosome. ROC curves and calculated AUC values are also identical for both regions (Figure 3 and Table 3).

We compared the data obtained in our study and published earlier. In most cases the results of our analysis confirm the high levels of methylation for many well-studied genes in CRC. All investigated genes with the highest AUC values also have shown sensitivity higher than 60% and specificity higher than 80% in the published literature. The sensitivity/specificity values for *FBN1* and *CNRIP1* biomarkers were determined as 79/99 and 94/95, correspondingly, in the study of DNA methylation in tumor and adjacent tissue samples [22]. Other tissue-based studies of *ADHFE1*, *SDC2*, and *UCHL1* methylation showed values of 82/92 [23], 75/83 [8], and 60/100 [24]. *VIM* gene methylation frequency was determined using DNA from stool and blood samples of CRC patients and healthy donors. In this case the sensitivity/specificity values were 73/87 [30] and 59/93 [31], correspondingly. *SEPT9* methylation is considered to be one of the most promising CRC biomarkers, so it was investigated in a number of works. For example, the cancer tissue DNA study resulted in 87/100 sensitivity/specificity values [8]. The changes in *SEPT9* methylation in blood samples of CRC patients compared to healthy donors were also significant and sensitivity/specificity values of 72/90 [32], 90/89 [33], 69/86 [34], and 73/91 [35] were reported earlier. *THBD* is also frequently methylated in the blood of CRC patients showing 71/80 sensitivity/specificity values [36].

It should be noted that the difference in sensitivity/specificity values for the same gene in different studies is not a rare case. For example, we can observe such results in literature for *SEPT9*, *ALX4*, *RASSF1*, and some other genes [20,21]. Probably the variability of the methylation data may be explained by the difference in the methodologies used in the experiments or by samples' selection. So, the experimental data mainly show the tendency and probability of the gene methylation in malignant cells.

Thus, the GLAD-PCR analysis allows identifying aberrantly methylated RCGY sites of gene regulatory regions in tumor tissues and cell line SW837. We are planning to continue a work with tissue samples to identify aberrantly methylated sites suitable for GLAD-PCR analysis in regulatory regions of other genes. A second group of scientists will analyze a methylation of established target sites in DNA preparations of peripheral blood of CRC patients.

Conclusion

In this study we applied a new GLAD-PCR assay to identify aberrantly methylated RCGY sites in a number of downregulated genes in the tissue DNA samples of CRC patients. The analysis of the methylation status of RCGY sites demonstrated good prognostic potential with relatively high sensitivity and specificity of CRC detection in the tissue DNAs. We believe that the selected RCGY sites may be used in GLAD-PCR assay of CRC determination in case of noninvasive blood and stool DNA analysis.

Conflict of Interests

The authors have not declared any conflict of interests.

Acknowledgment

The present study was supported by the Ministry of Science and Education of the Russian Federation under the Agreement No.14.604.21.0102 from 05.08.2014 (unique identifier RFMEFI60414X0102), concluded in the framework of the Federal Targeted Program "Research and development on priority directions of scientific-technological complex of Russia for 2014-2020 years." According to the results of this study, the application for patent "Method for determining the methylation sites PuCGPy in regulatory regions of genes-markers of colorectal cancer by a method of GLAD-PCR assay and a set of oligonucleotide primers and fluorescently labeled probes for the implementation of said method" was made, and the priority date is 21.07.16, number 2016129968.

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