Determination of R(5mC)GY sites in regulation regions of tumor-suppression genes by GLAD-PCR assay in colorectal cancer

A.A. Evdokimov, S.B. Oleynikova, N.A. Netesova*, N.A. Smetannikova, M.A. Abdurashitov, E.V. Dubinin**, A.G. Akishev, E.S. Davidovich, S.Kh. Degtyarev

Aberrant DNA methylation of regulatory regions of tumor-suppressor genes is shown for many types of cancer. DNA methyltransferase DNMT3 is responsible for hypermethylation and subsequent silencing of tumor suppressor genes (TSG) by converting RCGY sites into R(5mC)GY in the gene regulation regions (Handa V. et al, 2005).

Earlier, we developed a method of GLAD-PCR assay to determine R(5mC)GY site in a position of interest in human genome. This approach was used to define R(5mC)GY sites in regulation regions of ESR1 and ELMO1 tumor-suppressor genes in a panel of colorectal tumor tissues samples and SW837 cell line.

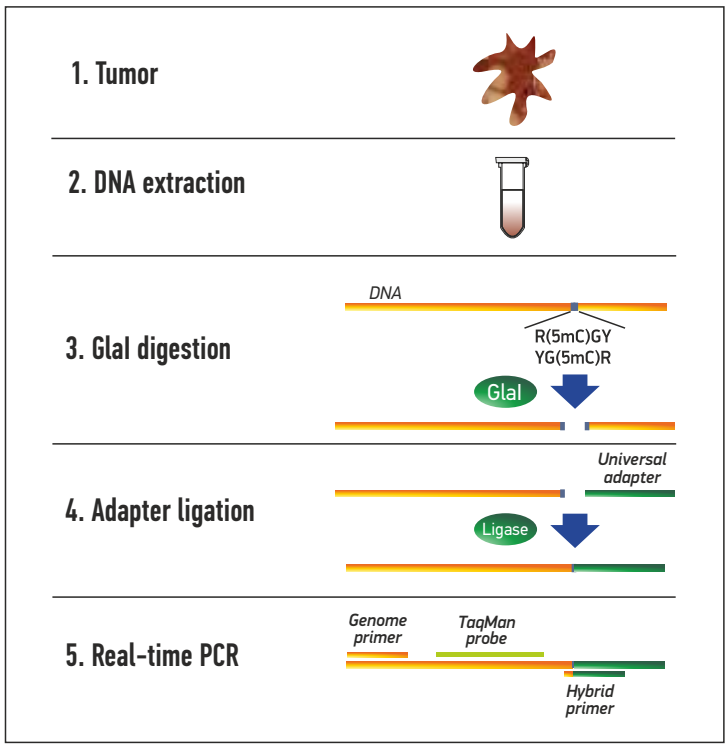
Targeted regions are localized in the first exon of ELMO1 gene and in the ESR1 promoter region. Four sites in each region were investigated, and as a result, two highly methylated sites: GCGC in the first exon of ELMO1 gene and GCGT in the promoter region of ESR1 gene were found in SW837 cell line and in cancer samples. In contrast, the poor methylation level of GCGT site of ERS1 gene and almost no methylation of GCGC site of ELMO1 gene were observed among healthy colorectal tissues samples. A possibility to use GLAD-PCR assay for cancer diagnostics is discussed.

MATERIALS AND METHODS

- Phenol-chloroform extraction was used for DNA isolation.
  Recently we have discovered and characterized a new DNA-endonuclease GlaI, which belongs to a novel type of site-specific methyl-directed DNA-endonucleases. These enzymes hydrolyze only methylated DNA.
  GlaI recognizes DNA sequence Pu(5mC)GPy which is identical to the modification site of DNMT3 (Tarasova G.V. et al, 2008).

- For GLAD-PCR assay was used 45 ng of DNA as described earlier (Kuznetsov VV at al., 2013).
- Tumor tissue samples were obtained from 15 patients with different stages of colorectal cancer: 2 samples (1 and 76) - stage 1 (T1N0M0); 3 patients (16, 20, and 37) - stage 2 (T2N0M0); 6 patients (2, 19, 31, 50, 54, and 56) - stage 3 and 4 (T3-4N1-2M0); 4 patients (25, 51, 59, and 75) had stage 4 with generalized CRC and metastasis in surrounding lymph nodes and to other areas of the body.
- As a positive control DNA from SW837 colorectal cancer cell line was used.

GLAD-PCR ASSAY



GlaI hydrolysis and Ligation Adapter Dependent PCR (GLAD PCR) is the novel method to determine R(5mC)GY sites produced by methylation with DNMT3A and DNMT3B. GLAD PCR analysis may be performed in one tube and includes 3 steps: DNA hydrolysis with site-specific methyl-directed DNA endonuclease GlaI, universal adapter ligation to the cleaved fragments, and Real-time PCR with Taqman probe. One genome primer is designed for DNA region of interest, structure of another hybrid primer is based on an adapter sequence.

Hybrid primer is a DNA sequence 5'-CCTGCTCTTTCATCGGYNN-3' wherein 5'-end of 15-dinucleotide primer corresponds with universal adapter and tetranucleotide part at the 3'-end (underlined) is complementary to the genomic sequence at DNA hydrolysis point by MD DNA-endonuclease GlaI. This structure implies the existence of 32 variants of hybrid primers corresponding to different possible terminal sequences after hydrolysis by GlaI of all possible variants of NNR(5mC)↓GY sequence.

GLAD PCR analysis allows to detect even several copies of methylated DNA and thereby may be used in routine laboratory and clinical practice in earlier cancer detection.

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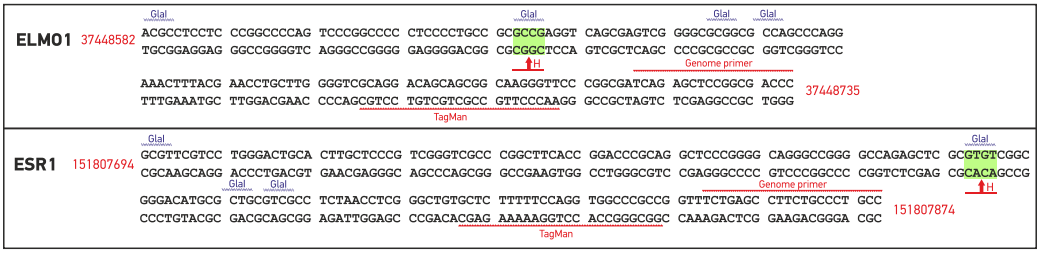
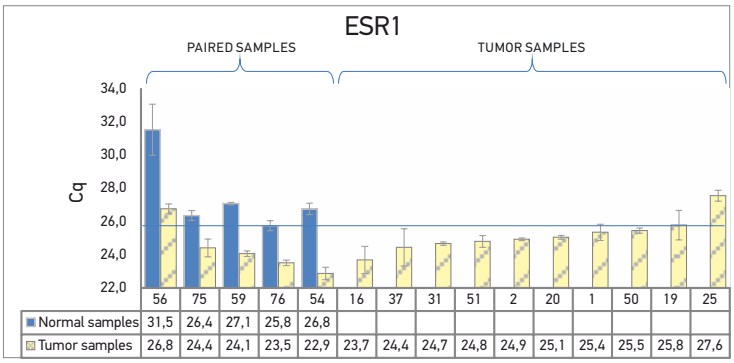
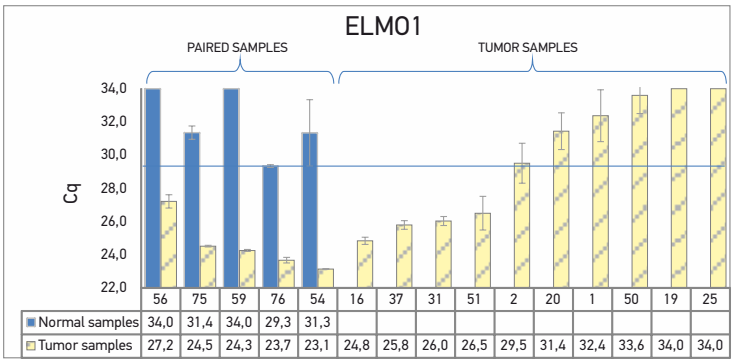
STUDIED GENES



RESULTS



	56	75	59	76	54	16	37	31	51	2	20	1	50	19	25
Normal samples	31,5	26,4	27,1	25,8	26,8										
Tumor samples	26,8	24,4	24,1	23,5	22,9	23,7	24,4	24,7	24,8	24,9	25,1	25,4	25,5	25,8	27,6



	56	75	59	76	54	16	37	31	51	2	20	1	50	19	25
Normal samples	34,0	31,4	34,0	29,3	31,3										
Tumor samples	27,2	24,5	24,3	23,7	23,1	24,8	25,8	26,0	26,5	29,5	31,4	32,4	33,6	34,0	34,0

GLAD-PCR assay of two selected sites was performed three times for each DNA sample containing 10^3 copies of the ELMO1 or ESR1 gene.
Tables demonstrate the results of GLAD-PCR assay where each number means the average number of amplification cycle (Cq) with standard deviation ($p<0.01$).

CONCLUSIONS

GLAD-PCR assay revealed that GCGT site in the promoter region of ESR1 gene is methylated in all tumor samples, but at the same time normal samples are also methylated, except samples 56.
Cq for GCGC site within the first exon of the ELMO1 gene in the normal tissue samples was around 30 and corresponds to no methylation. Same results were demonstrated for six tumor samples 1T, 2T, 19T, 20T, 25T, and 50T. Whereas for the rest nine tumor samples Cq was 23.14 - 27.21 which associated with significant level of methylation.

Based on our results GCGT site in the promoter region of ESR1 gene and GCGC site within the first exon of the ELMO1 gene seems to be perspective for diagnostic use. GCGT site is methylated in practically all tumor samples, whereas GCGC site is methylated in 60% of tumor samples. Thus, these sites might be included in multiplex TSG methylation analysis with GLAD PCR in a course of CRC diagnostics as indicated below.

PERSPECTIVES

We are planning to continue GLAD PCR analysis of RCGY sites in regulation regions of other tumor suppression genes and to obtain a panel of TSG regulation regions which are methylated in the most of tumor samples. At the next step the obtained panel of TSG regulation regions will be tested on the samples of the blood cell-free DNA in order to create the simple, reliable, and sensitive diagnostic test for CRC.

APPLICATIONS

GLAD-PCR assay may be used as a universal tool for methylation analysis of R(5mC)GY site of interest in the human genome instead of DNA bisulfite conversion. Therefore it can be used as epigenetic instrument for diagnostics of aging diseases, such as: the most kinds of cancer, coronary artery disease, type 2 diabetes (T2D) and others connected with epigenetic genome alterations. For such tests development a panel of reference genes should be comprised by examination of candidate genes based on their methylation status .

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PATENT IS PENDING

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