New enzymes
for DNA technologies

SibEnzyme

VECTOR

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

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Early cancer detection

- Nowadays 25-50% of all patients are diagnosed with cancer at stage III or IV.
- It's difficult to reach a positive result in the cancer treatment at these stages.
- At the same time early cancer detection dramatically improves a treatment of disease and the patient cure.
- Epigenetic diagnostics seems to be the most perspective for early cancer detection because epigenetic changes in genome take place at the beginning of illness.

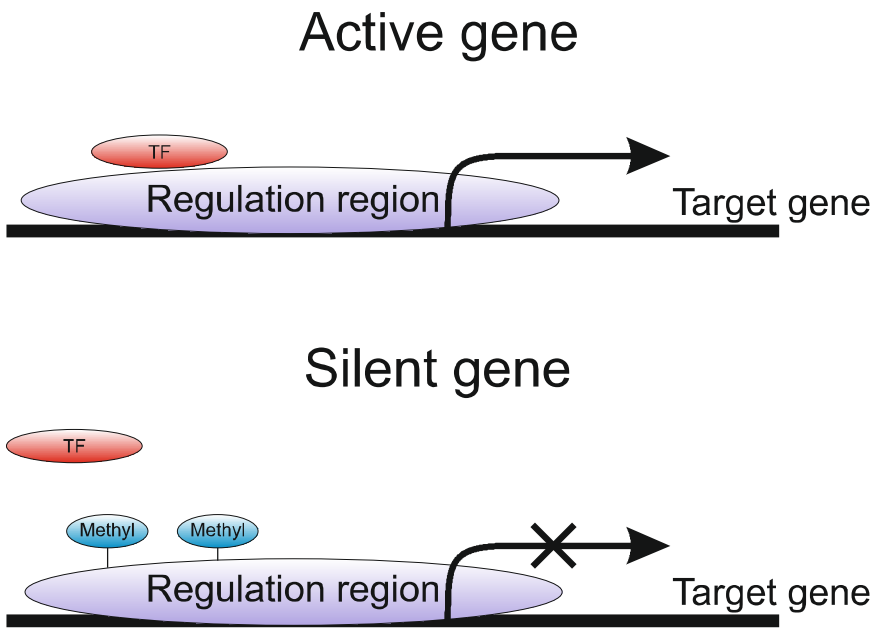
Epigenetics in Cancer

- It is well known that aberrant methylation of cytosines in regulatory regions (promotor and first exon) of tumor suppressor genes (TSGs) takes place at early stage of cancer in case of the most sporadic, nonheritable types of cancers.

- Modified TSGs are differing in various tumors, so the methylated TSGs may be the epigenetic markers for diagnostics and differentiation of the cancer nosologies.

DNA methylation

- DNA methylation in mammalians genomes is mostly 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.
  - DNMT1 maintains DNA methylation pattern in vivo modifying a new strand after replication.
  - DNMT3a and DNMT3b are responsible for DNA methylation *de novo*.
    This modification in regulation region (promotor and first exon) of gene results in the gene silencing.



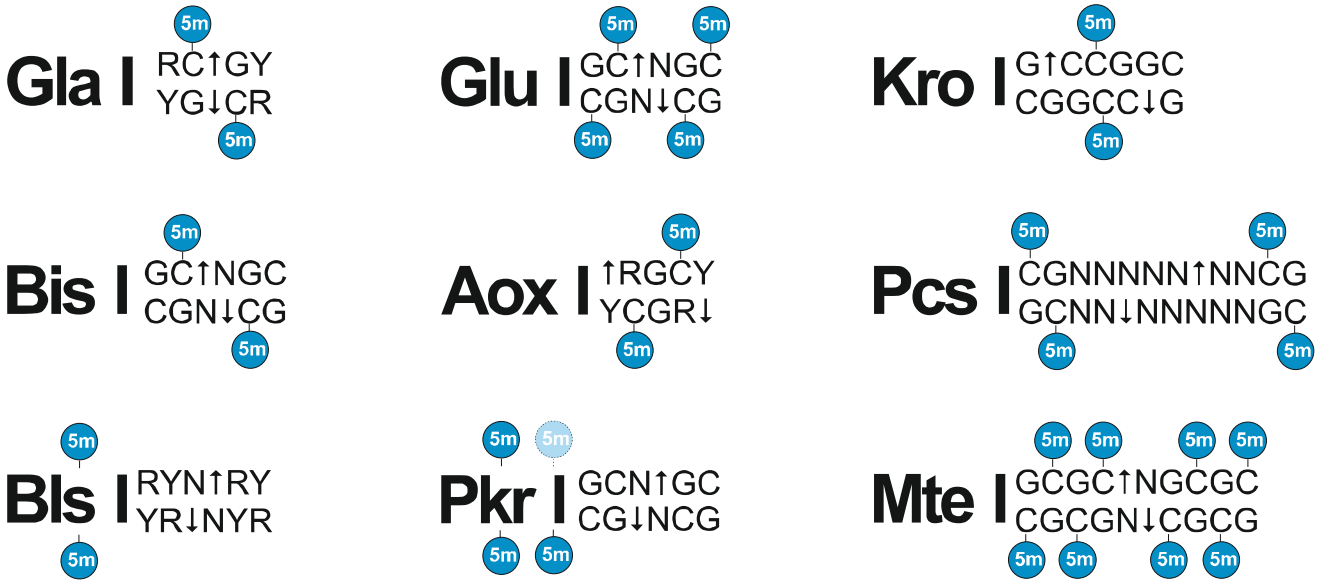
DNA Bisulfite Conversion

- DNA bisulfite conversion is the most frequently used (e.g. it is used in Human Epigenome Project) though it has a number of disadvantages

- A subsequent analysis of modified and native DNA allows to locate positions of methylated cytosines in studied DNA.
- Main disadvantage of method: it is quite sophisticated and often results in obtaining false data.

New type of enzymes

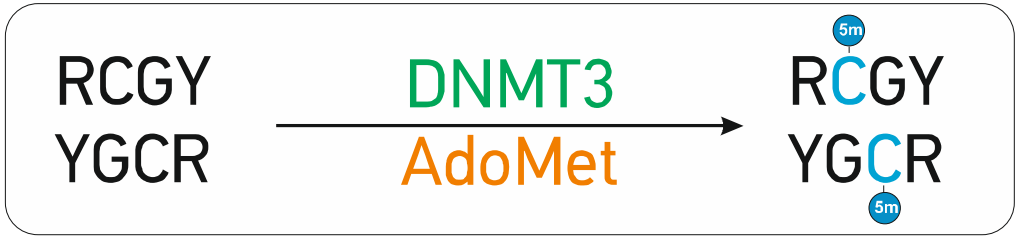
- Methyl-directed site-specific DNA endonucleases belong to a new type of enzymes discovered by SibEnzyme (Russia).
- These enzymes are very similar to restriction enzymes in biochemical properties and cleave DNA completely, but act in opposite way: they cleave only methylated DNA and do not cleave unmethylated DNA at all
- Now 9 different MD DNA-endonucleases are available



BlsI - at least one methyl group in each DNA strand
PkrI – at least 3 methyl groups in recognition sequence

Substrate specificity of DNMT3a, DNMT3b and GlaI

Study of DNMT3a and DNMT3b substrate specificity has shown that both enzymes methylate CG-dinucleotide mostly in DNA sequence RCGY.



One of new enzymes GlaI recognizes and cleaves site R(5mC)GY, which is product of *de novo* methylation.

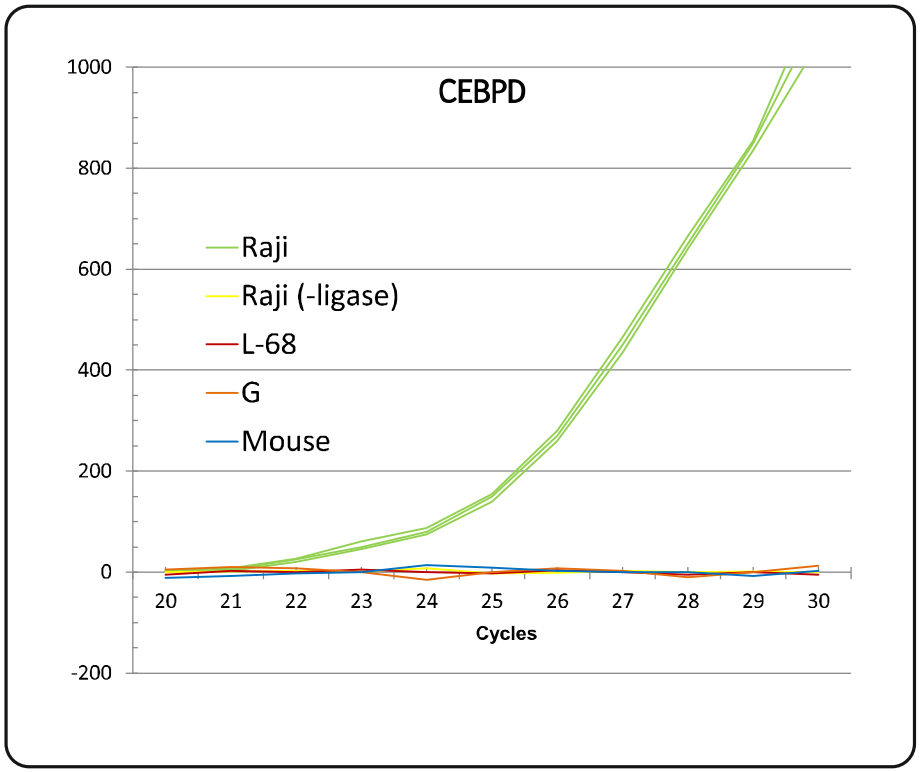
GLAD-PCR assay

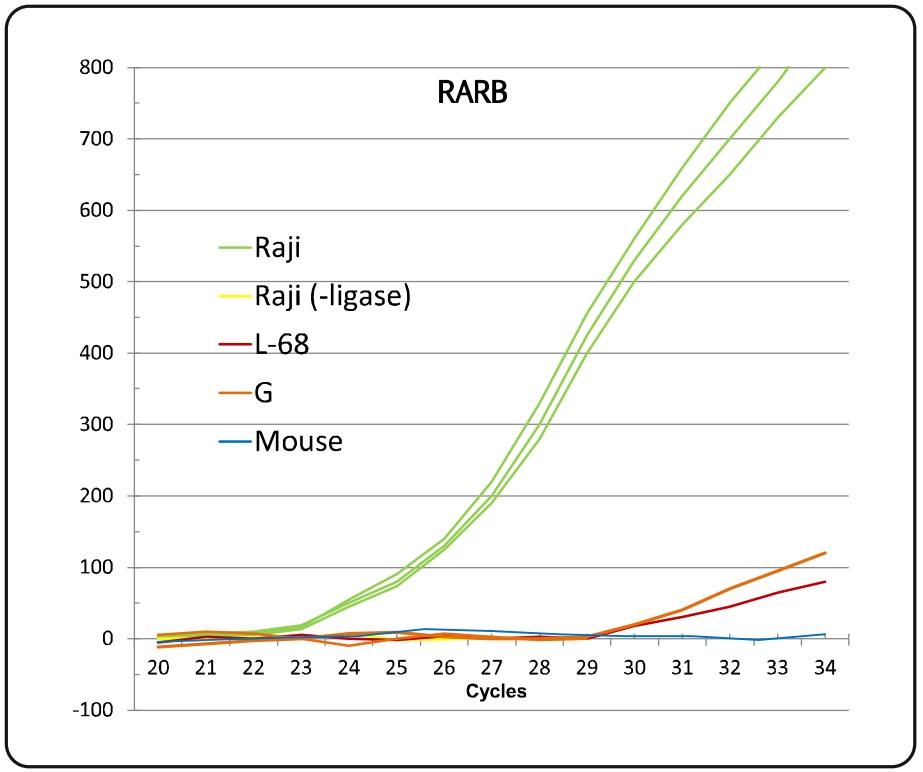
- **G**la**I** hydrolysis and **L**igation **A**dapter **D**ependent **PCR** (GLAD-PCR) assay is the novel method to determine R(5mC)GY sites produced by methylation with DNMT3A and DNMT3B.
- It has been developed to determine minimal quantities of methylated sites in presence of excess of unmethylated DNA.

GLAD-PCR assay

GLAD-PCR assay of GCGG and ACGC sites in regulation regions of CEBPD and RARB genes respectively in DNA from Raji cell line.

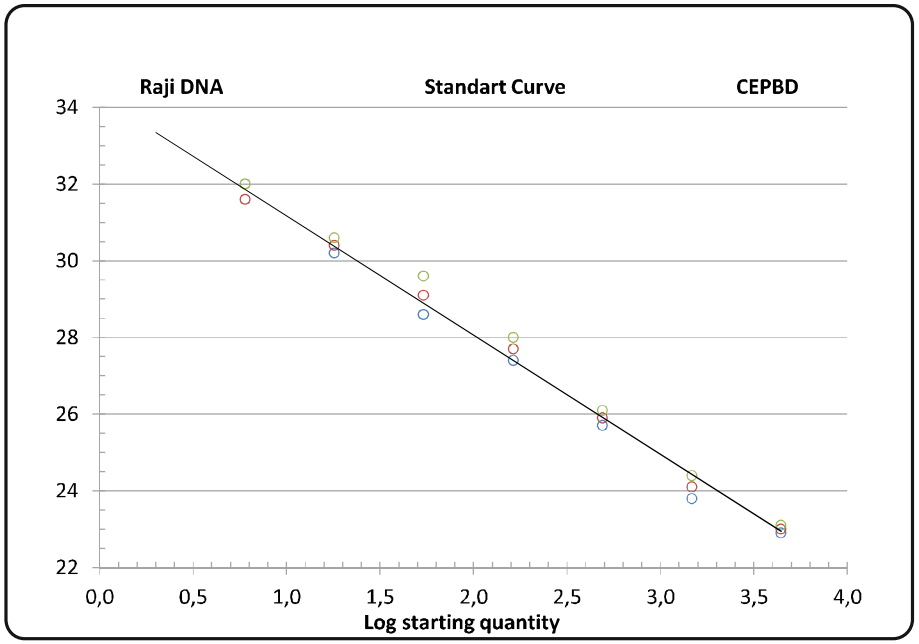
Amplification chart of GLAD PCR assay of 15 ng DNA per reaction using Bio-Rad CFX96.





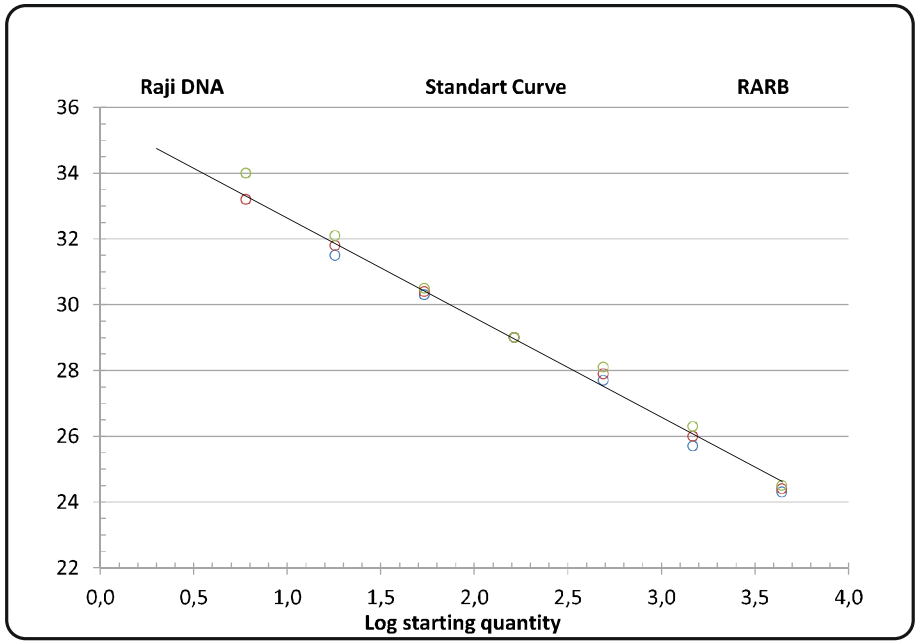
GLAD-PCR assay sensitivity

CEBPD



DNA Raji, pg	15 000	5 000	1 666	555	185	62	21	7
Equivalent of normal DNA copies	4 412	1 471	490	163	54	18	6	2
Cq Mean	23	24,1	25,9	27,7	29,1	30,4	31,8	-

RARB

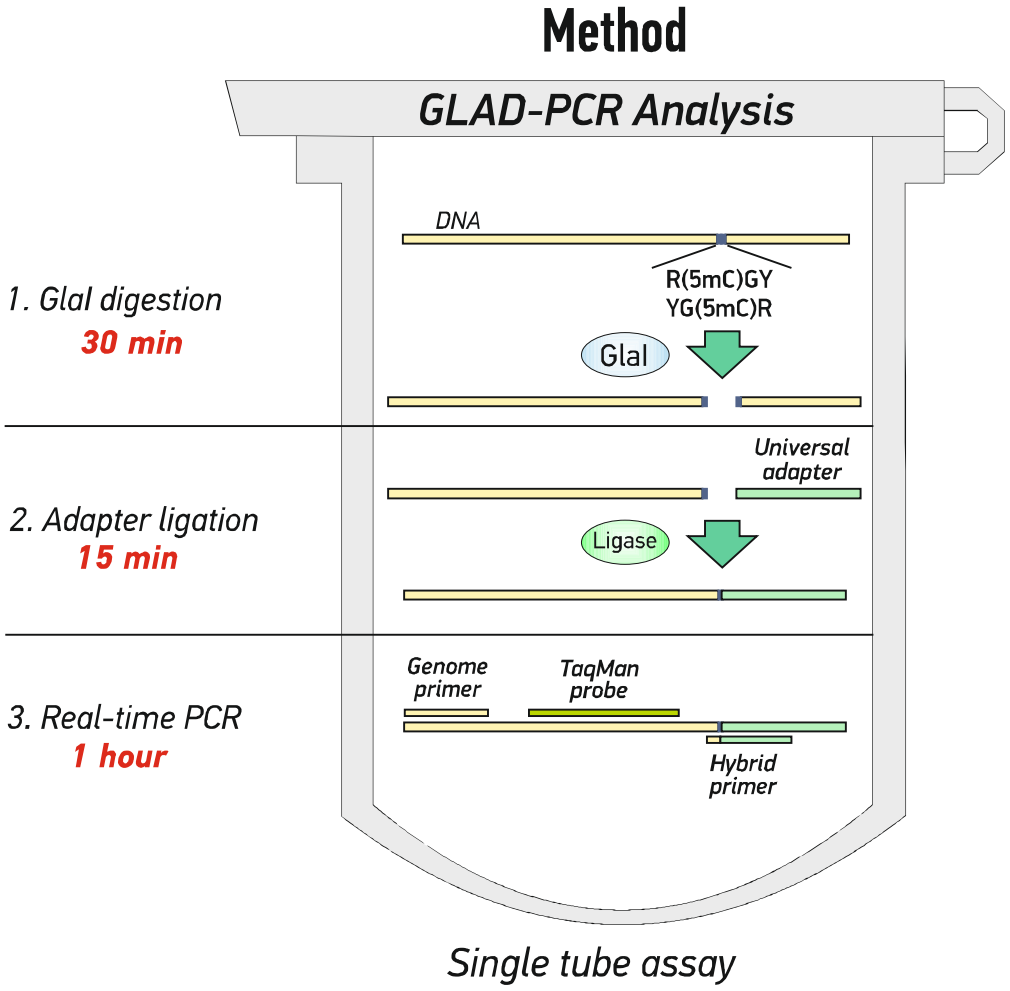


DNA Raji, pg	15 000	5 000	1 666	555	185	62	21	7
Equivalent of normal DNA copies	4 412	1 471	490	163	54	18	6	2
Cq Mean	24,4	26	27,9	29	30,4	31,8	33,6	-

GLAD-PCR assay

GLAD-PCR assay main advantages:

- Simple
- Reliable
- Fast
- Sensitive



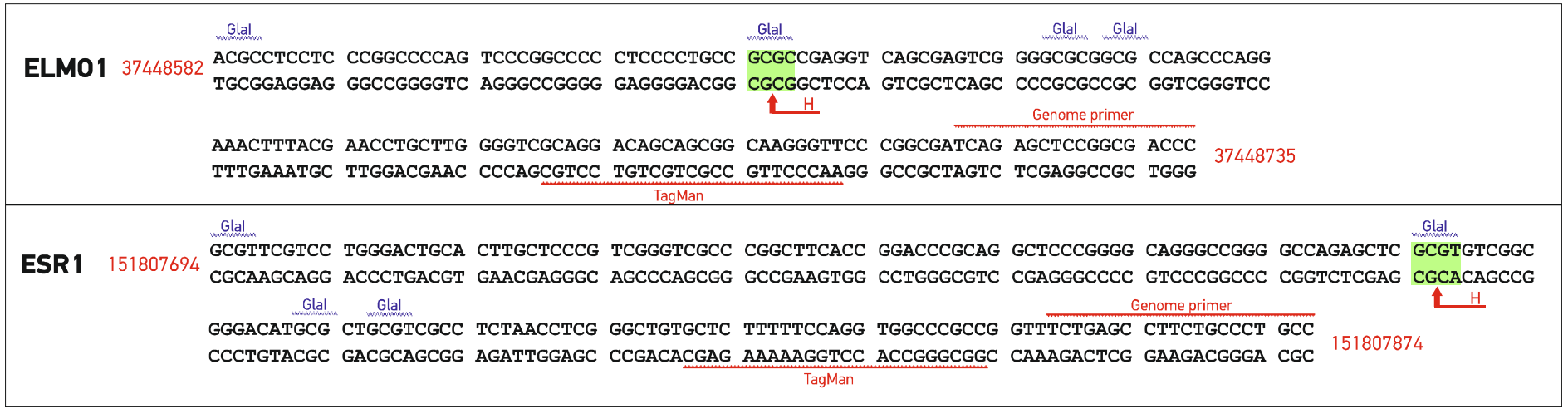
GLAD-PCR assay of RCGY sites in regulation regions of ESR1 and ELMO1 TSGs

GLAD-PCR assay was used for determination of R(5mC)GY sites in regulation regions of ESR1 and ELMO1 TSGs in SW837 cell line and tumor and healthy colorectal samples.

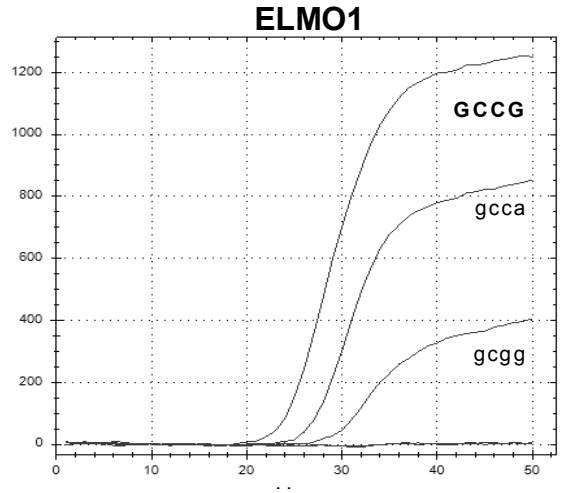
We analyzed the following fragments:

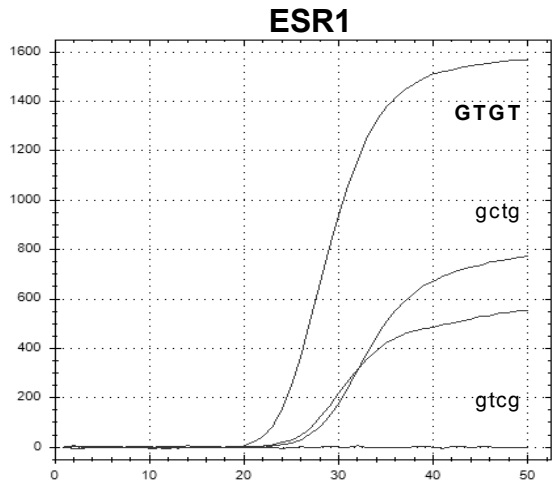
- 154 nucleotide fragment in the first exon of the ELMO1
- 183 nucleotide fragment in promotor of the ESR1

Studied genes



Selection of candidate RCGY sites for GLAD-PCR assay





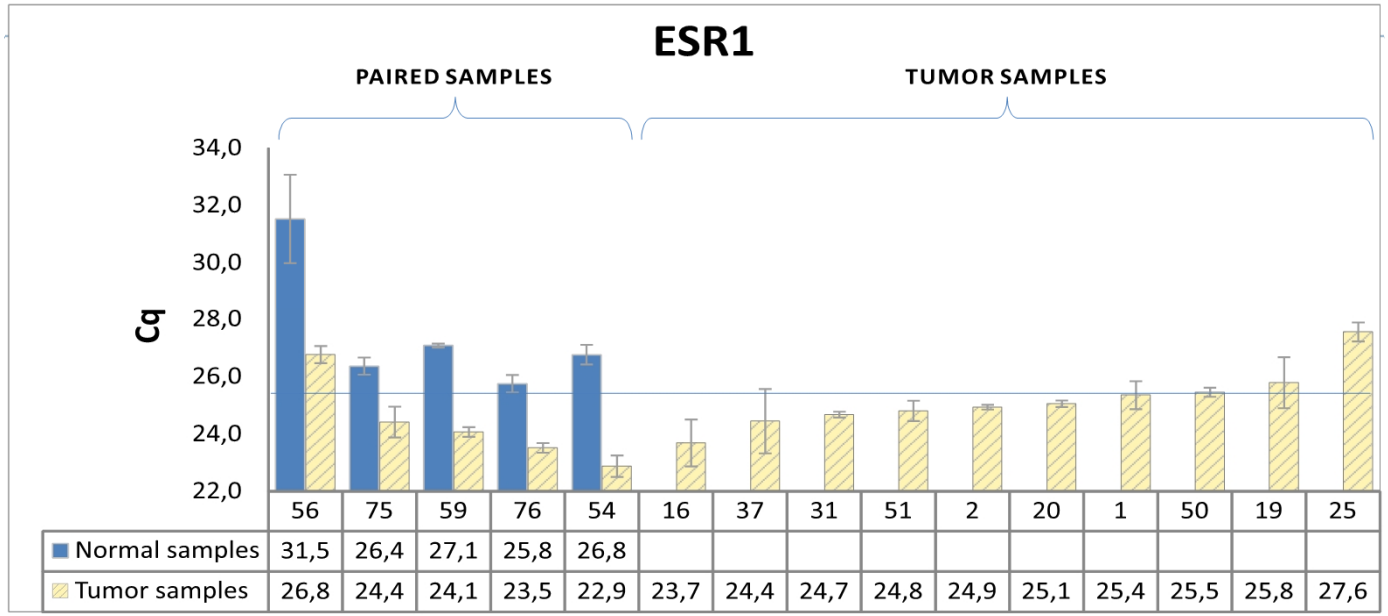
On the first stage preparation of DNA from SW837 cell line was analyzed.

The most methylated RCGY sites within selected fragments of regulatory regions of TSG were:

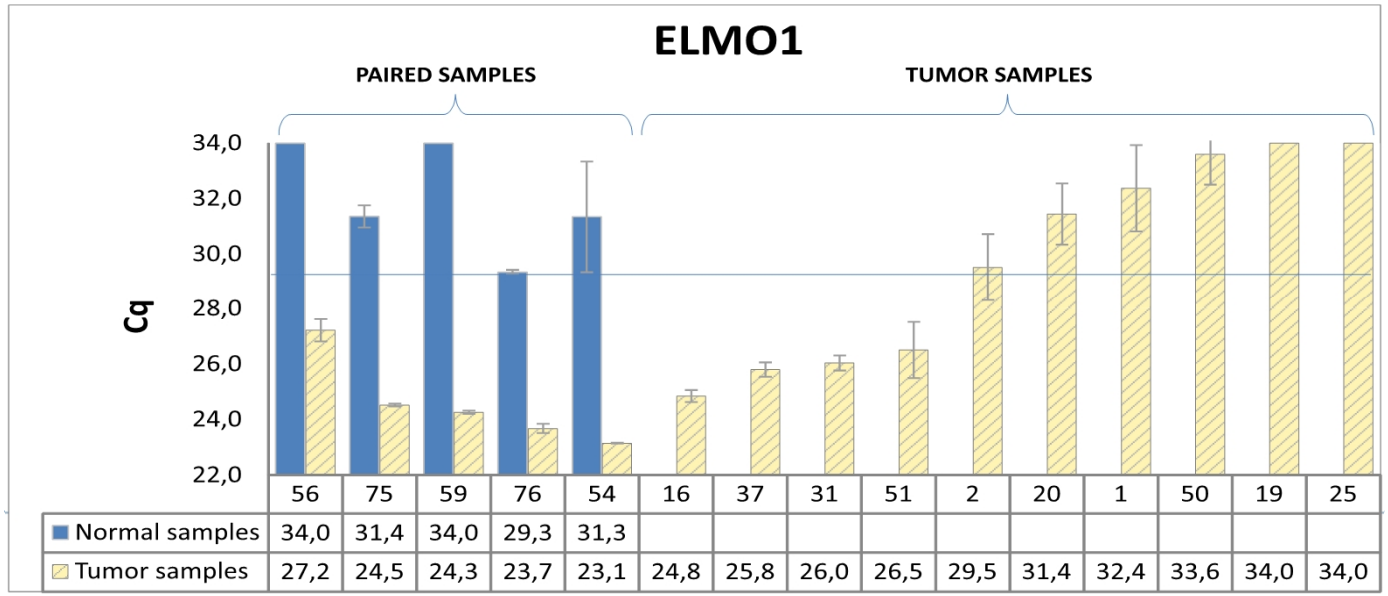
- GCGC site in first exone of ELMO1 (position 37448622 in 7th chromosome)
- GCGT site in promotor of ESR1 (position 151807784 in 6th chromosome)

The methylation of these sites in tumor samples were analyzed at the next stage.

GLAD-PCR assay of tumor DNA samples



	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

Conclusions

- 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 may be considered as the candidate sites in GLAD PCR assay for CRC diagnostics.

Perspectives

- We are planning to continue GLAD PCR assay of RCGY sites in regulation regions of other tumor suppression genes and to obtain a panel of sites located in TSG regulation regions which are methylated in the most of tumor DNA samples.
- At the next step the obtained panel of RCGY sites will be tested in GLAD-PCR assay of the blood cell-free DNA samples in order to develop the simple and cheap PCR test system for CRC diagnostics.

More 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 (CAD),
  - 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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