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GLAD-PCR assay of DNA methylation markers associated with colorectal cancer

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New enzymes for DNA technologies

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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 outcome 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.

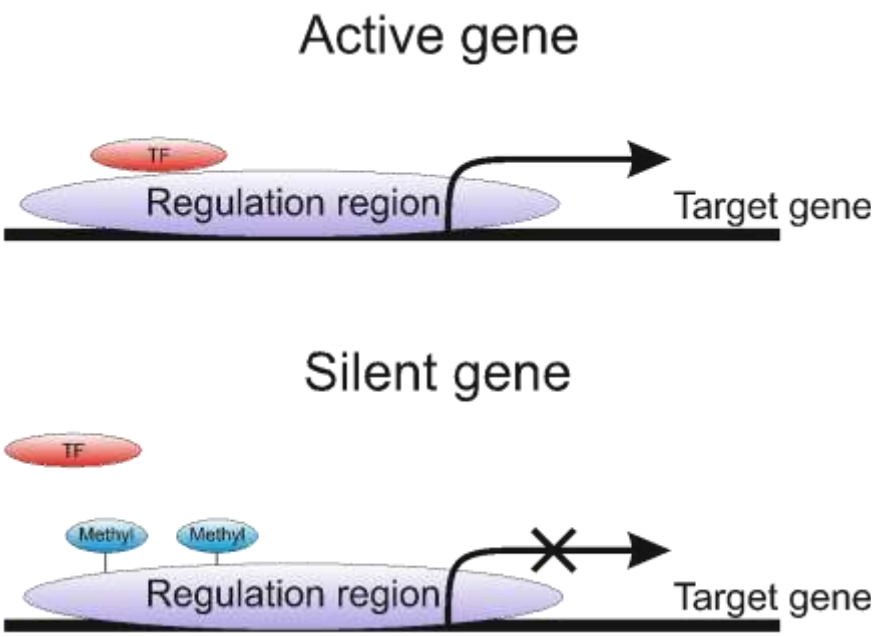
Epigenetics in Cancer

- It is well known that aberrant methylation of cytosines in regulatory regions (promotor and the 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 different in various tumors, so the methylated TSGs may be used as 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 the first exon) of gene results in the gene silencing.



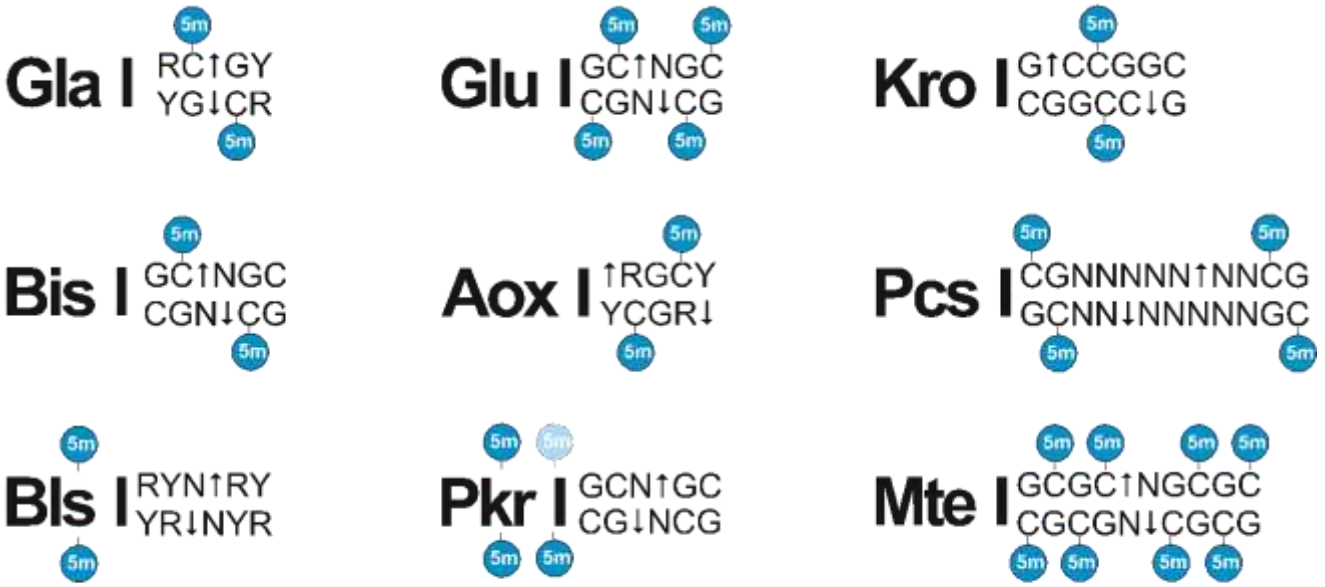
DNA Bisulfite Conversion

- DNA bisulfite conversion is the most frequently used 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.

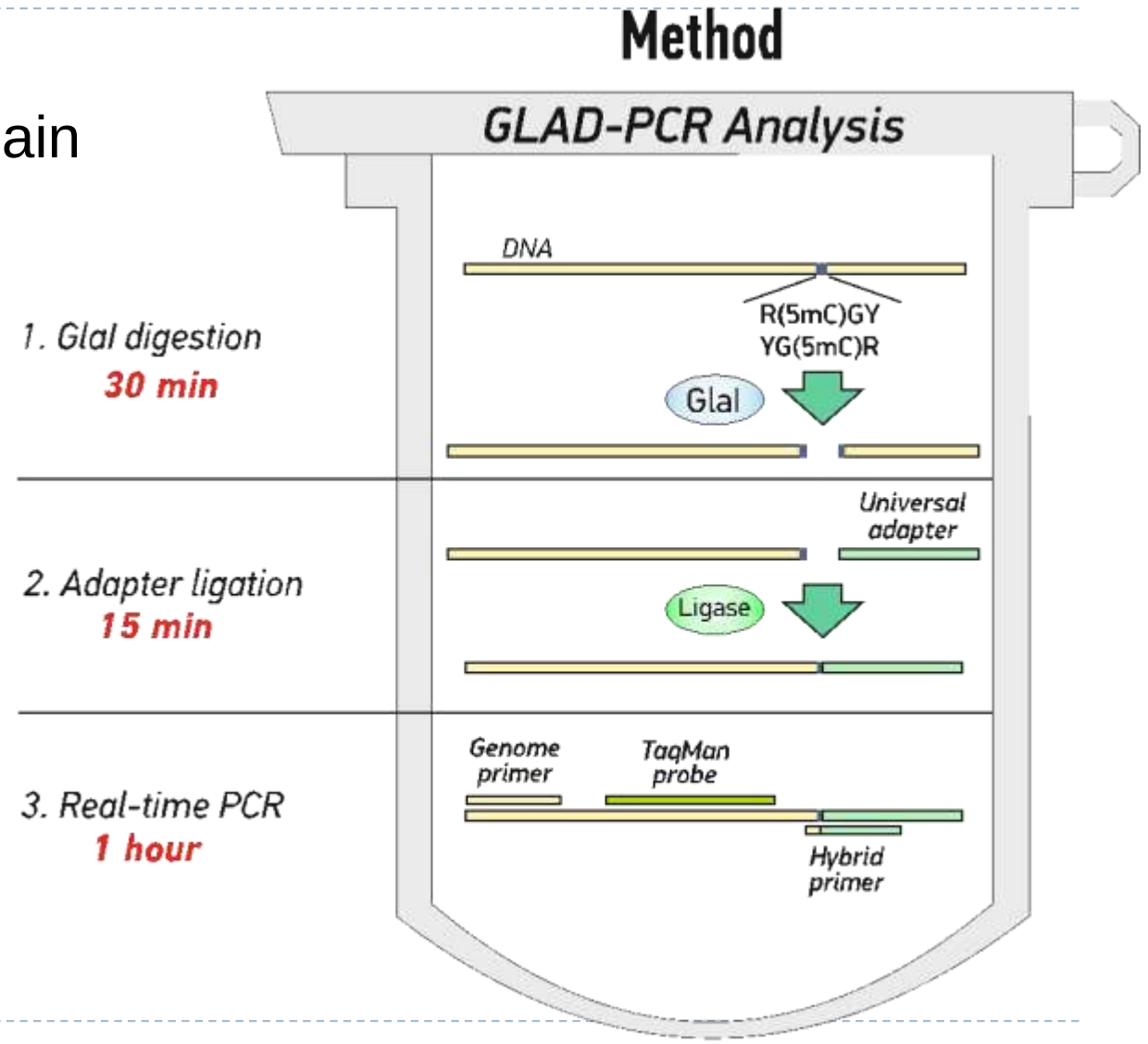
GLAD-PCR assay

- **G**lal 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.
- For this study we selected 23 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 assay

GLAD-PCR assay main advantages:

- Simple
- Reliable
- Fast
- Sensitive

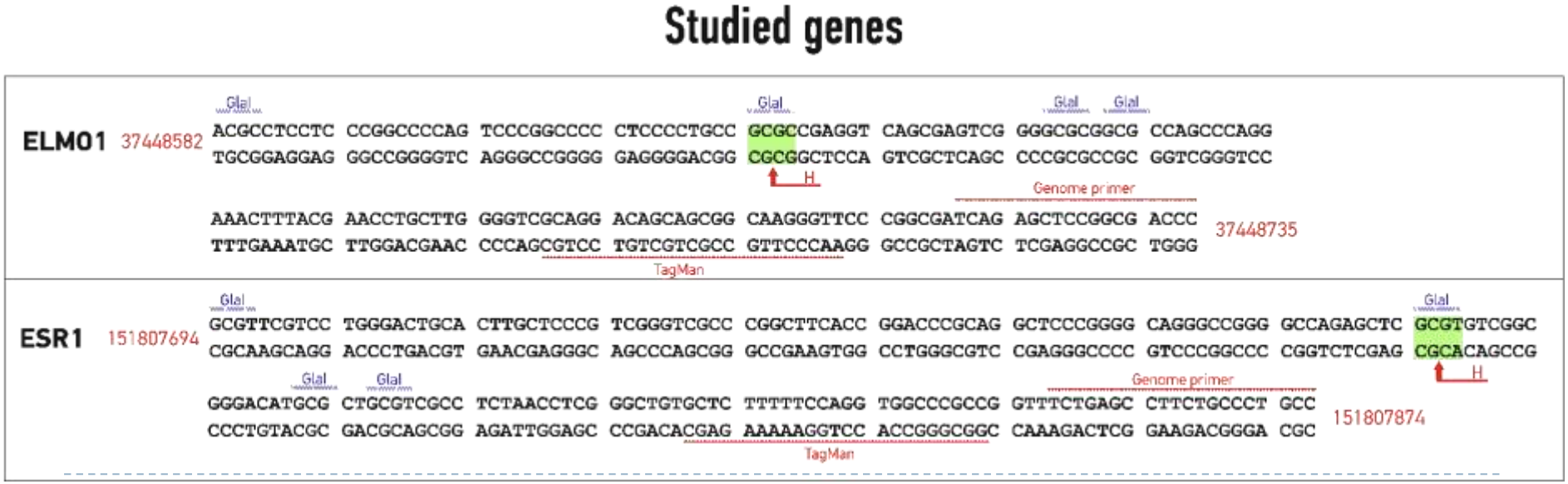


GLAD-PCR assay of RCGY sites in regulation regions of 26 TSGs

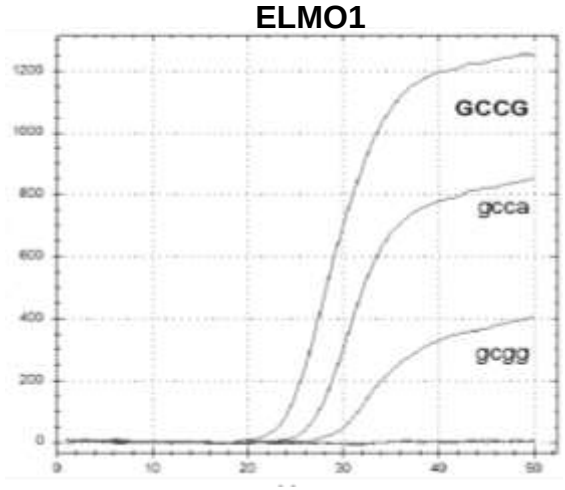
GLAD-PCR assay was used for determination of R(5mC)GY sites in 26 regulation regions of selected TSGs in SW837 cell line and tumor and healthy colorectal samples.

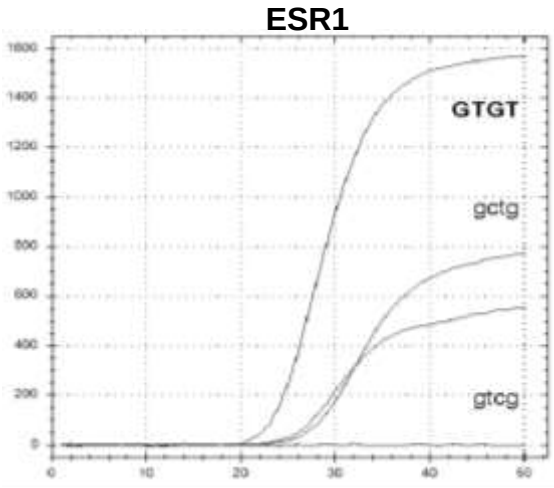
As an example in ELMO1 and ESR1 we analyzed the following fragments:

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



Selection of candidate RCGY sites for GLAD-PCR assay





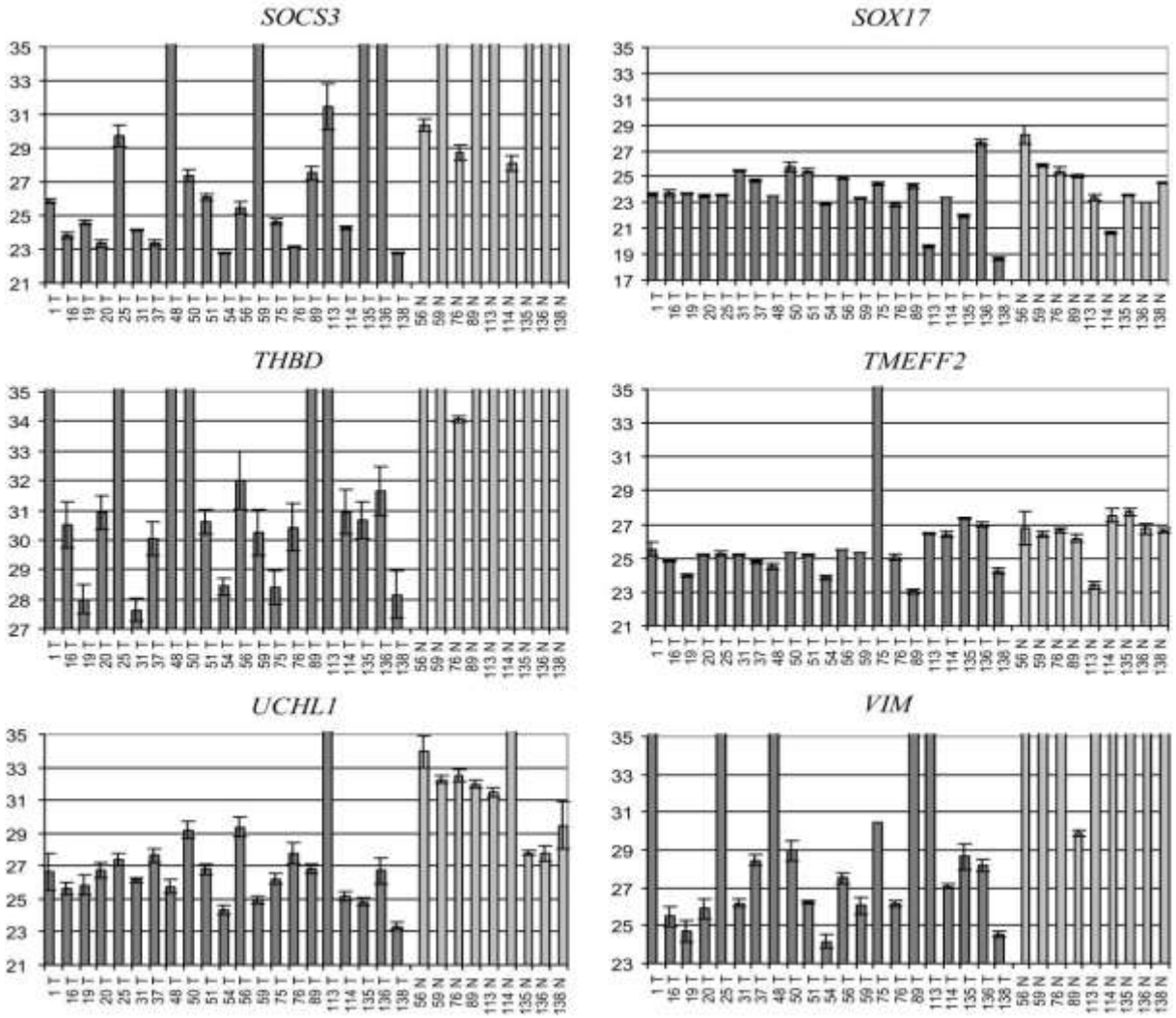
On the first stage preparation of DNA from SW837 cell line was analyzed.

As an example in ELMO1 and ESR1 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 and target sites of the other genes in tumor samples were analyzed at the next stage.

GLAD-PCR assay using primers and TaqMan probes for selected 26 RCGY sites



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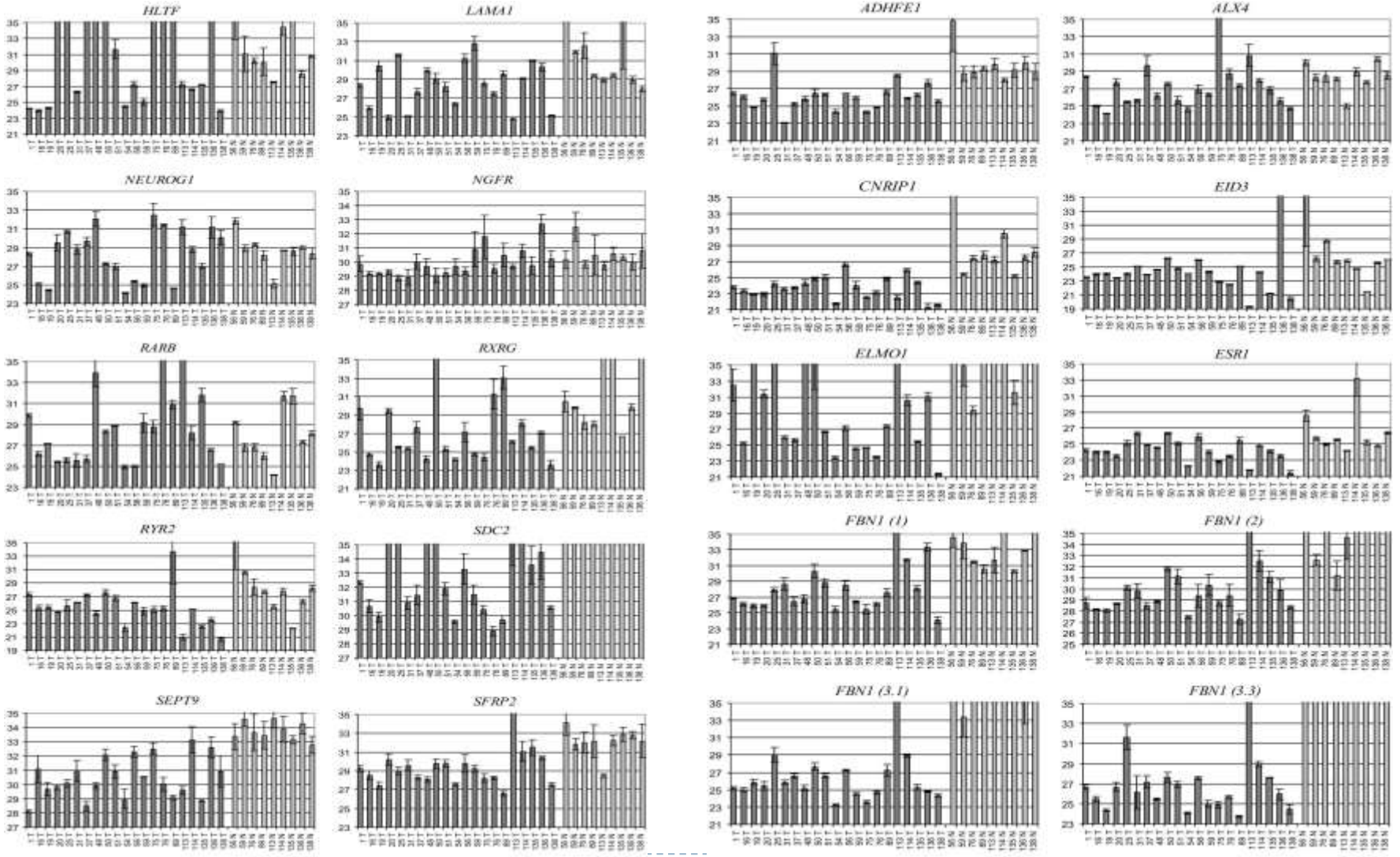
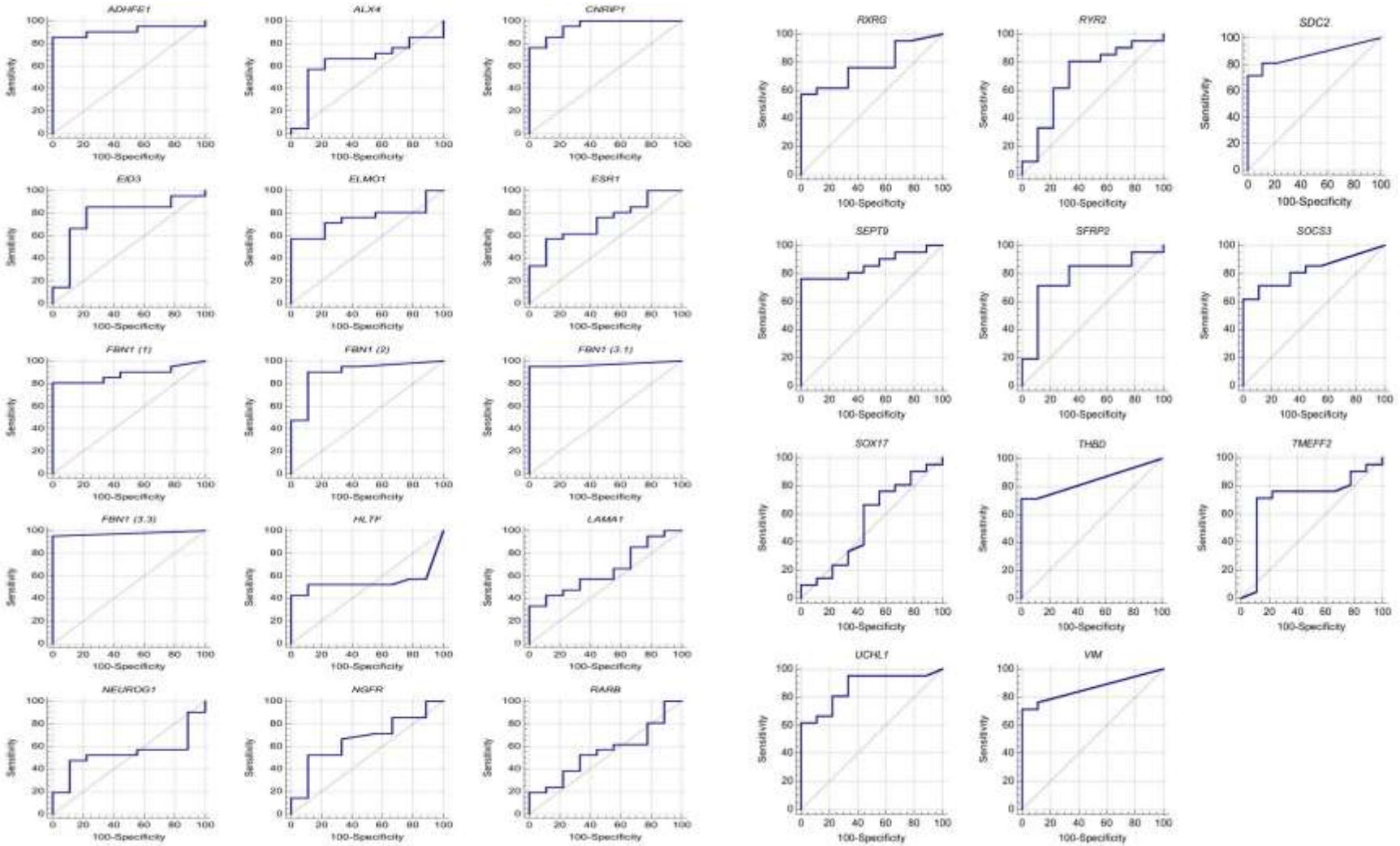


The receiver operating characteristic curves (ROC) for selected 26 RCGY sites



Conclusions

- In this study we applied a new GLAD-PCR assay to identify aberrantly *de novo* methylated RCGY sites in 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. Therefore these sites may be considered as the candidate sites in GLAD PCR assay for CRC diagnostics.
- As the next step, the obtained panel of RCGY sites will be tested in GLAD-PCR assay of the blood cell-free DNA and/or stool samples in order to develop the simple and cheap PCR test system for CRC diagnostics.

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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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Thank You!