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

E.V. Dubinin, A.A. Evdokimov, N.A. Netesova, N.A. Smetannikova, M.A. Abdurashitov, A.G. Akishev, E.S. Davidovich, S.Kh. Degtyarev
Early cancer detection

- Nowadays about 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 significantly improves a treatment of disease and the patient cure.
- Lung cancer - the most deadly type
- Incidence/Mortality – 1,8M/1,6M
DNA methylation in cancer

- DNA hypermethylation results in genes silencing
- Such methylation of tumor-suppressor genes (TSGs) shown for the most types of cancer
- Occurs at the early stages when there are still no clinical indications of disease
- Destructed methylated DNA gets into the bloodstream where it can be detected
- Different types of cancer – different patterns of methylation.

Determination of TSGs methylation status allows to distinguish cancer types and detect it at the most early stages.

**Thus epigenetic diagnostics seems to be very perspective for early cancer detection**

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2 Xueguang Sun, Jill E. Petrisko, Lam K. Nguyen, Marc Van Eden & Xi-Yu Jia Epigenetic biomarker discovery, validation for diagnosis, and therapeutic intervention for Hepatocellular Carcinoma
DNA methylation

- DNA methylation in mammalian 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 this gene silencing.
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.

\[
\begin{align*}
\text{RCGY} & \xrightarrow{\text{DNMT3}} \text{RCGY} \\
\text{YGCR} & \xrightarrow{\text{AdoMet}} \text{YGCR}
\end{align*}
\]

\[
\begin{align*}
\text{RCGY} & \xrightarrow{\text{GlaI}} \text{RC} \uparrow \text{GY} \\
\text{YGCR} & \xrightarrow{\text{GlaI}} \text{YG} \downarrow \text{CR}
\end{align*}
\]
GLAD-PCR assay - effective alternative of bisulfite methods

GLAD-PCR Assay is the novel methylation detection method developed by SibEnzyme

- Simple
- Quick
- Sensitive
- Requires only standard real time PCR machine
- Cheap

Method works with any source of DNA like sputum, urine, smear, tissue samp but most versatile and convenient is blood

For details see http://md.sibenzyme.com/2GLAD-PCR%20assay.pdf
Method demonstration: http://sibenzyme.com/info7820.php
or our site http://www.epigene.ru/glad-pcr-assay/
GLAD-PCR-assay
GLAD-PCR-assay Vs Bisulfite conversion

<table>
<thead>
<tr>
<th>Feature</th>
<th>GLAD-PCR-assay</th>
<th>Bisulfite conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>&gt; 20 pg of DNA</td>
<td>&gt; 500 pg of DNA</td>
</tr>
<tr>
<td>Simplicity</td>
<td>3 stages</td>
<td>&gt; 5 stages</td>
</tr>
<tr>
<td>Usage</td>
<td>RCGY site of interest within genome, up to 4 in multiplex</td>
<td>DNA region excessive</td>
</tr>
<tr>
<td>Duration</td>
<td>4-6 hours</td>
<td>&gt; 12 hours</td>
</tr>
<tr>
<td>Mistakes</td>
<td>Less than 1%</td>
<td>15-20%</td>
</tr>
</tbody>
</table>

Blood sample

DNA extraction

RESULT
GLAD-PCR assay of RCGY sites in regulation regions of TSGs in lung cancer

In this work we applied GLAD-PCR assay for identification of the methylated RCGY sites in the regulatory regions of some downregulated TSGs associated with lung cancer. The study group included 40 lung cancer patients (36 male and 4 female). 31 patient had no distant metastases. A total of 65 freshfrozen surgical resection samples were studied, including 40 tumor samples and 25 paired normal controls. The samples were collected at the Seversk Biophysical Research Centre (Seversk, Russia).
Studied RCGY sites

From the list of 34 candidate TSGs on the first stage we selected 11 for further testing: LHX6, MYF6, NID2, OTX1, RASSF1, RARB, RXRG, RYR2, SIX6, SKOR1 and TERT. At this moment 13 RCGY sites from regulation regions of 11 TSGs were studied.

<table>
<thead>
<tr>
<th>Gene (region)</th>
<th>TARGET SITE</th>
<th>Site location(^1)</th>
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</tr>
</thead>
<tbody>
<tr>
<td>LHX6 (1)</td>
<td>GCGC</td>
<td>chr9: 122219596–122219599</td>
<td>RXRG</td>
<td>GCGC</td>
<td>chr1: 165445276–165445279</td>
</tr>
<tr>
<td>LHX6 (2)</td>
<td>GCGT</td>
<td>chr9: 122219886–122219889</td>
<td>RYR2</td>
<td>GCGC</td>
<td>chr1: 237043053–237043056</td>
</tr>
<tr>
<td>MYF6</td>
<td>GCGC</td>
<td>chr12: 80708733–80708736</td>
<td>SIX6 (1)</td>
<td>GCGC</td>
<td>chr14: 60509991–60509994</td>
</tr>
<tr>
<td>NID2</td>
<td>GCGC</td>
<td>chr14: 52069060–52069063</td>
<td>SIX6 (2)</td>
<td>GCGT</td>
<td>chr14: 60509827–60509830</td>
</tr>
<tr>
<td>OTX1</td>
<td>GCGC</td>
<td>chr2: 63057659–63057662</td>
<td>SKOR1</td>
<td>ACGC</td>
<td>chr15: 67821744–67821747</td>
</tr>
<tr>
<td>RASSF1</td>
<td>GCGC</td>
<td>chr3: 50340715–50340718</td>
<td>TERT</td>
<td>GCGT</td>
<td>chr5: 1295645–1295648</td>
</tr>
<tr>
<td>RARB</td>
<td>ACGC</td>
<td>chr3: 25428374–25428377</td>
<td></td>
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\(^1\) Site location are given in accordance with the recent human genome assembly GRCh38/hg38
Top-five studied RCGY sites

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<tr>
<th>Gene (region)</th>
<th>Number of detected LC samples /total number of LC samples</th>
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<th>Specificity, %</th>
<th>AUC (standard error)</th>
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<td>SIX6 (2)</td>
<td>23/30</td>
<td>76.7</td>
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<td>93.8</td>
<td>0.888 (0.049)</td>
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EpiGene, Novosibirsk, Russia, 2017
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<td>RXRg</td>
<td>23/40</td>
<td>57.5</td>
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<td>LHX6 (1)</td>
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<td>RASSF1</td>
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EpiGene, Novosibirsk, Russia, 2017
Results

- Based on our results five sites in the regulatory regions of SIX6, MYF6, RXRG, LHX6 and RASSF1 genes seem to be perspective for diagnostic use. **The panel** including these five top sites results in AUC = 0.936

- Thus, these sites may be considered as preliminary candidate sites in GLAD PCR assay for lung cancer diagnostics

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<tr>
<td><strong>Panel of five top genes</strong></td>
<td><strong>33/40</strong></td>
<td><strong>82.5</strong></td>
<td><strong>23/25</strong></td>
<td><strong>92.0</strong></td>
<td><strong>0.936 (0.030)</strong></td>
<td><strong>0.847–0.982</strong></td>
</tr>
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Perspectives

- We are planning to widen the list of candidate TSGs which methylation correlates with formation of lung cancer.
- We will continue a study of methylation of RCGY sites in regulation regions of other genes to obtain the better panel. Such panel will include TSGs which are methylated in the most of tumor DNA samples and nonmethylated (or minimally methylated) in controls.
- 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 for lung cancer detection similar to the previously developed colorectal cancer detection test which was presented at CGS-2015.

Candidate genes
- 30-40 genes from open sources
- Additional genes from epigenetic sequencing

Tumors and tissues tests
- 40 tumor and normal tissue samples
- 10 RCGY top sites selected

Blood cfDNA tests
- 200-300 blood cfDNA samples
- 3 RCGY sites selected for final Dx panel
Colorectal cancer detection kit

- GLAD-PCR assay was recently used for development of epigenetic test for colorectal cancer screening and early detection
- Colorectal cancer test is ready for usage and it is on its way to Russia market
Government Support

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«Development of epigenetic tests for lung, breast and stomach cancer detection»
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Thank You!

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