Study of DNA methylation associated with Lung Cancer: GLAD-PCR Assay of R(5mC)GY sites

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SUMMARY

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 5'-RCGY-3' sequences with formation of 5'-R(5mC)GY-3'/3'YG(5mC)R-5' sites (Handa V. et al, 2005).

RCGY / YGCR —DNMT3, AdoMet→ RCGY / YGCR

Recently discovered site-specific methyl-directed DNA-endonuclease GlaI recognizes exactly this DNA sequence 5'-R(5mC)GY-3'/3'YG(5mC)R-5' and cleaves it as indicated by arrows

RCGY / YGCR —GlaI→ RC↑GY / YG↓CR

Earlier, we developed a method of GLAD-PCR assay to determine R(5mC)GY site in a position of interest in human genome. Recently we applied GLAD PCR assay for development of the epigenetic test for colorectal cancer. In present work this approach was used to define R(5mC)GY sites in regulation regions of tumor-suppressor genes associated with lung cancer in a panel of lung tumor tissues samples and paired normal tissues controls.

In present work we studied four R(5mC)GY sites in regulation regions of HOXA5, LHX6, RASSF1 and RARB genes to identify aberrantly methylated RCGY sites.

The analysis of RCGY sites methylation with GLAD-PCR assay demonstrated a relatively good prognostic potential of LC detection for RCGY sites in regulation regions of LHX6 and RASSF1 genes. We believe that these RCGY sites may be used for LC determination by GLAD PCR assay of DNA samples from blood and sputum.

A possibility to use GLAD-PCR assay for cancer diagnostics is discussed.

MATERIALS AND METHODS

- GLAD-PCR assay for R(5mC)GY detection (Kuznetsov VV at al., 2013);
- 40 tumor tissue samples from 40 patients with different stages of lung cancer:
  - 5 patients - stage 1 (T1-2aN0M0);
  - 12 patients - stage 2 (T1-3N0M0, T1-2N1M0);
  - 6 patients - stage 3 (T1-2N2M0, T3N1-2M0, T4N0-2M0, T1-4N3M0);
  - 4 patients - stage 4 with generalized lung cancer and metastasis in surrounding lymph nodes and to other areas of the body
- 25 normal paired controls from 25 patients during surgery;

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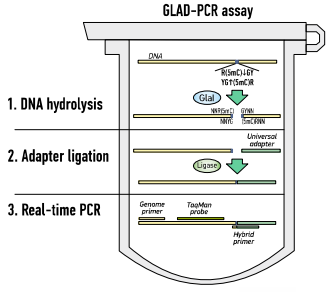
GLAD-PCR ASSAY



http://sibenzyme.com/glad_pcr_assay.php

GlaI hydrolysis and Ligation Adapter Dependent PCR (GLAD PCR) is a new and unique method for study of DNA methylation. It is developed 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:

1. DNA hydrolysis with site-specific methyl-directed DNA endonuclease GlaI;
2. universal adapter ligation to the cleaved fragments;
3. Real-time PCR with Taqman probe.

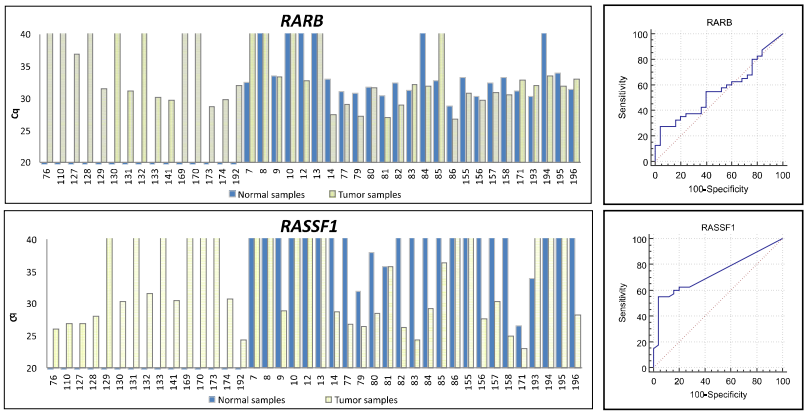
NO BISULFITE CONVERSION!

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-nucleotide 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 assay allows to detect even several copies of methylated DNA and thereby may be used in routine laboratory and clinical practice in earlier cancer detection.

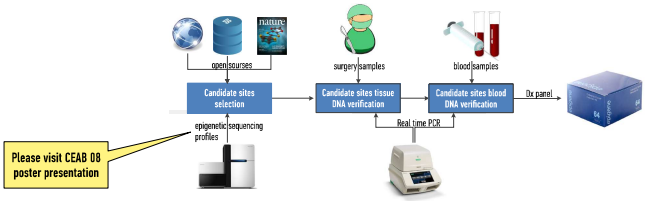
RESULTS



GLAD-PCR assay of two selected sites was performed three times for each DNA sample containing 10^3 copies of the *RASSF1* or *HOXA5* gene. Graphs demonstrate the results of GLAD-PCR assay where each number means the average number of amplification cycle (Cq) with standard deviation ($p<0.01$). Right graphs shows corresponding ROC curves. Result of ROC analysis is given in the table below.

Site (region)	Sequence	Coordinates	TP/(TP+FN)	Sensitivity, %	TN/(TN+FP)	Specifity, %	AUC (st.error)	95% c.i.
LHX6	GCGC	chr9: 122219596—122219599	31/40	77.5	15/25	60	0.746 (0.061)	0.622—0.845
RASSF1	GCGC	chr3: 50340715—50340718	22/40	55	24/25	96	0.733 (0.056)	0.608—0.835
HOXA5	GCGT	chr7: 27143017—27143020	28/39	71.8	16/25	64	0.697 (0.069)	0.570—0.806
RARB	ACGC	chr3: 25428374—25428377	11/40	27.5	24/25	96	0.558 (0.072)	0.429—0.681

TEST DEVELOPMENT WORKFLOW



PATENT IS PENDING

CONCLUSIONS

GLAD-PCR assay revealed that there is no significant difference in methylation of selected ACGC site in case of *RARB* gene in tumor and normal samples. Diagnostic effectiveness is very low (AUC=55%).

At the same time selected GCGC site in case of LHX6 gene is methylated in most tumor samples , but normal samples are also methylated in 40% of cases. GCGC site in case of RASSF1 gene is unmethylated in most normal samples while it is methylated in 55% of tumor samples.

Methylation pattens for *LHX6* and *RASSF1* genes are different in the same samples. Thus, these sites might be included in multiplex TSG methylation analysis with GLAD PCR in a course of lung cancer diagnostics development.

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 DNA bound to the blood cells in order to create the simple, reliable, and sensitive diagnostic test for lung cancer.

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 may 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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