# Integrated analysis of DNA methylation profiling and gene expression profiling identifies novel markers in lung cancer in Xuanwei, China

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Working
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ABSTRACT

**Background**: Aberrant DNA methylation occurs frequently in cancer. The aim of this study was to identify novel methylation markers in lung cancer in Xuanwei, China, through integrated genome-wide DNA methylation and gene expression studies.
**Methods**: Differentially methylated regions (DMRs) and differentially expressed genes (DEGs) were detected on 10 paired lung cancer tissues and noncancerous lung tissues by methylated DNA immunoprecipitation combined with microarray (MeDIP-chip) and gene expression microarray analyses, respectively. Integrated analysis of DMRs and DEGs was performed to screen out candidate methylation-related genes. Both methylation and expression changes of the candidate genes were further validated and analyzed.
**Results**: Compared with normal lung tissues, lung cancer tissues expressed a total of 6,899 DMRs, including 5,788 hypermethylated regions and 1,111 hypomethylated regions. Integrated analysis of DMRs and DEGs identified 45 tumor-specific candidate genes: 38 genes whose DMRs were hypermethylated and expression was downregulated, and 7 genes whose DMRs were hypomethylated and expression was upregulated. The methylation and expression validation results identified 4 candidate genes (*STXBP6*, *BCL6B*, *FZD10*, and *HSPB6*) that were significantly hypermethylated and downregulated in most of the tumor tissues compared with the noncancerous lung tissues.
**Conclusions**: This integrated analysis of genome-wide DNA methylation and gene expression in lung cancer in Xuanwei revealed several genes regulated by promoter methylation that have not been described in lung cancer before. These results provide new insight into the carcinogenesis of lung cancer in Xuanwei and represent promising new diagnostic and therapeutic targets.

EXTERNAL LINK

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THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

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PROTOCOL STATUS

**Working**

GUIDELINES

The first part of the process requires quality control of experimental (IP) and control (input) samples to verify quality prior to microarray hybridization. You then independently label these samples using a NimbleGen Dual-Color DNA Labeling Kit and co-hybridize them to a NimbleGen DNA Methylation array using a NimbleGen Hybridization System. Following hybridization, the arrays are washed, dried, and scanned using a NimbleGen MS 200 Microarray Scanner. Array data are extracted and analyzed using our NimbleScan software and SignalMap software.
Then various verification experiments were carried out.

MATERIALS

| NAME | CATALOG # | VENDOR |
|---|---|---|
| NimbleGen Arrays | | Contributed by users |

| NAME | CATALOG # | VENDOR |
|---|---|---|
| NimbleGen HX3 Mixer (for 3x720K arrays; includes mixer port seals) | 05 223 750 001 | Contributed by users |
| NimbleGen Dual-Color DNA Labeling Kit | 06 370 250 001 | Contributed by users |
| NimbleGen Hybridization Kit | 05 583 683 001 | Contributed by users |
| NimbleGen Sample Tracking Control Kit | 05 223 512 001 | Contributed by users |
| NimbleGen Wash Buffer Kit | 05 584 507 001 | Contributed by users |
| NimbleGen Array Processing Accessories | 05 223 539 001 | Contributed by users |
| β-Mercaptoethanol | M3148 | Contributed by users |
| Absolute Ethanol 500 ml | E702-3 | Contributed by users |
| Isopropanol 500 ml | I-9516 | Contributed by users |
| Water: reagent grade, ACS, nonsterile, type 1 | RC915025 | Contributed by users |
| QIAamp DNA Mini kit | | Contributed by users |
| QIAamp DNA Mini kit | | Qiagen |
| RNeasy Mini kit | | Qiagen |
| lysis of radio immunoprecipitation assay (RIPA) | | Contributed by users |
| phenylmethylsulfonyl fluoride (PMSF) | | Contributed by users |
| Transcriptor First Strand cDNA Synthesis kit | | Contributed by users |
| Epitect Fast DNA Bisulfite kit | | Contributed by users |
| unmethylated: EpiTect Control DNA | | Contributed by users |
| methylated: EpiTect Control DNA | | Qiagen |
| Epitect HRM PCR kit | | Contributed by users |
| Quantinova SYBR Green PCR Master Mix | | Contributed by users |
| sodium dodecyl sulfate–polyacrylamide gel | | Contributed by users |
| skimmed milk | | Contributed by users |
| TBST | | Contributed by users |
| Monoclonal rabbit anti-human STXBP6 | | Contributed by users |
| Monoclonal rabbit anti-human BCL6B | | Contributed by users |
| Monoclonal rabbit anti-human FZD10 | | Contributed by users |
| Monoclonal Mouse anti-human HSPB6 | | Contributed by users |
| Monoclonal chicken anti-human GAPDH | | Contributed by users |
| Peroxidase-conjugated goat anti-rabbit IgG(H L) | | Contributed by users |
| Peroxidase-conjugated goat anti-mouse IgG(H L) | | Contributed by users |
| Peroxidase-conjugated goat anti-chicken IgG (H L) | | Contributed by users |

| NAME | CATALOG # | VENDOR |
|---|---|---|
| MassCLEAVE Kit | Part Number 10129 | Contributed by users |
| EZ DNA Methylation Kit | | Contributed by users |
| dd H2O | | Contributed by users |
| 10X Hot Star buffer | | Qiagen |
| dNTP mix | | Contributed by users |
| Hot Star taq | | Qiagen |
| Shrimp Alkaline Phosphatase | | Contributed by users |
| Polymerase buffer | | Contributed by users |
| T Cleavage Mix | | Contributed by users |
| DTT | | Contributed by users |
| T7 RNA & DNA Polymerase | | Contributed by users |
| RNase A | | Contributed by users |
| Clean Resin | | Contributed by users |

SAFETY WARNINGS

Wear gloves and take precautions to avoid sample contamination.

BEFORE STARTING

Cy dyes are light sensitive. Be sure to minimize light exposure of the dyes during use and store in the dark when not in use.

Cy dyes are ozone sensitive. Take the necessary precautions to keep atmospheric ozone levels below 20 ppb (parts per billion).

Cy dyes are humidity sensitive. Take the necessary precautions to keep humidity levels below 40%.

Roche NimbleGen has found that using VWR water and DTT for all post-hybridization washes results in higher signal from Cy dyes.

Roche NimbleGen recommends using a NanoDrop Spectrophotometer for quantifying and characterizing nucleic acid samples because this instrument requires only 1.5 µl of sample for analysis.

Perform all centrifugations at +15 to +25°C unless indicated otherwise.

## MeDIP-chip

1 Genomic DNA (gDNA) were extracted and purified from lung cancer and paired noncancerous lung tissues using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.
For genome-wide methylation detection, equal quantities of gDNA from each of the paired tumor and normal lung specimens from 10 patients were pooled. A total of 6 µg of the pooled gDNA from each tissue type was digested by *Mse*I, purified by using QIAquick PCR purification kit (Qiagen), and analyzed by agarose gel electrophoresis. *Mse*I-digested gDNA (1.25 µg) was subjected to immunoprecipitation with a mouse monoclonal anti-5-methylcytosine antibody (Diagenode, Liège, Belgium) and then was purified through Qiagen MinElute columns and amplified by using a GenomePlex Complete Whole Genome Amplification (WGA2) kit from Sigma-Aldrich (St Louis, MO, USA). The amplified DNA samples were purified with a QIAquick PCR purification kit. The NimbleGen Dual-Color DNA Labeling kit (Roche NimbleGen Systems, Inc., Madison, WI, USA) was used for labeling according to the manufacturer's guidelines. Briefly, 1 µg DNA of each sample was incubated for 10 min at 98°C with 1 OD of Cy5-9mer primer (immunoprecipitation sample) or Cy3-9mer primer (input sample). Then, 100 pmol of deoxynucleoside triphosphates and 100 U of the Klenow fragment (New England Biolabs, Ipswich, MA, USA) were added, and the mix was incubated at 37°C for 3 h. The labeled DNA was purified by isopropanol/ethanol precipitation and hybridized to NimbleGen Human Meth 720K CpG RfSq Prom according to the manufacturer's instructions.

You can see the attachment of NG_DNA_Methylation_Guide_v7p2 for the specific experimental process.

ng_dna_methylation_guide_v7p2.pdf

hb-0329-004-1102728-hb-qiaamp-dna-mini-blood-mini-0516-ww.pdf

## Gene expression microarray

2 Gene expression profiling was performed on the same 10 paired samples used in MeDIP-chip analysis by using the Agilent Oligo Microarray Kit 8×60 K (Agilent Technologies, Santa Clara, CA, USA) as described previously. Significantly differentially expressed genes (DEGs) were identified by using the mixed model analysis of variance with a false discovery rate (Benjamini–Hochberg test)–adjusted *q*-value of ≤0.05 and absolute fold-change values ≥2. Hierarchical clustering was carried out using cluster 3.0. GO analysis and Pathway analysis were performed to determine the roles of DEGs.

## Methylation-sensitive high-resolution melting assay (MS-HRM)

3 Genomic DNA (gDNA) were extracted and purified from lung cancer and paired noncancerous lung tissues using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.
gDNA was bisulfite-modified and purified by using the Epitect Fast DNA Bisulfite kit (Qiagen) according to the manufacturer's instructions, and its concentration was adjusted to 10 ng/L.
Methylation-sensitive high-resolution melting assay (MS-HRM) was performed on the 45 paired lung cancer and normal lung tissues to validate the methylation status of the candidate genes identified by the integrated analysis. A range of standards was included to control for bias in the sensitivity of the detection: 0% (unmethylated: EpiTect Control DNA; Qiagen), 100% (methylated: EpiTect Control DNA), and 50% (equal mixture of both templates). MS-HRM primers were designed to amplify a short amplicon size of less than 200 bp (http://www.urogene.org/cgi-bin/methprimer) and synthesized by Sangon Biotech (Shanghai, China) (S2 Table). MS-HRM assay was performed according to the instructions for the Epitect HRM PCR kit (Qiagen) on the RotorGene Q (Qiagen) in triplicate. A sample amplification curve between the 50% and 100% standard curves was defined as hypermethylation, and a sample amplification curve between the 0% and 50% standard curves was defined as hypomethylation.
You can see the attachments for the specific experimental process.

en-epitect-fast-bisulfite-conversion-handbook.pdf

en-epitect-hrm-pcr-handbook.pdf

hb-0329-004-1102728-hb-qiaamp-dna-mini-blood-mini-0516-ww.pdf

## Sequenom MassARRAY platform

4 Quantitative methylation analysis of DMRs in the candidate genes was performed on the 45 paired tumor and normal lung tissues by using the Sequenom MassARRAY platform (CapitalBio) as described previously. This platform employs RNA base-specific cleavage and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. PCR primers for MassARRAY were designed by using Epidesigner (http://www.epidesigner.com). The spectra methylation ratios were obtained via Epityper software version 1.0 (Sequenom, San Diego, CA, USA).
You can see the attachment for the specific experimental process.

sequenom massarray platform.pdf

## Real-time fluorescent quantitative PCR

5 Total RNA were extracted and purified from lung cancer and paired noncancerous lung tissues using RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. cDNA was synthesized by RNA reverse-transcription using the Transcriptor First Strand cDNA Synthesis kit (Roche, Mannheim, Germany).
Real-time fluorescent quantitative PCR (RT-qPCR) was performed on 39 of the paired tumor and normal lung tissues to validate the mRNA expression of the candidate genes; the samples in the other 6 pairs were insufficient for this analysis. The analysis was carried out on an Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Inc., Foster City, CA, USA) by using Quantinova SYBR Green PCR Master Mix (Qiagen). The relative expression values of tumor and normal lung tissues were calculated by the comparative Ct method. Primers for RT-qPCR are summarized in S4 Table. The expression of *β-actin* was used as a reference to normalize the other genes' expression. Each experiment was performed in duplicate.

You can see the attachments for the specific experimental process.

1076590_hb_quantinova sybr green pcr_1113_ww.pdf

rneasy-mini.pdf

transcriptor first strand cdna04379012001_en_06.pdf

## Western blotting

6 Total protein was isolated from lung tissues by lysis of radio immunoprecipitation assay (RIPA) and protected by phenylmethylsulfonyl fluoride (PMSF) following manufacturer's instructions (Beyotime, Shanghai, China).
Equal amounts of protein from each sample were separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride membranes (Merck Millipore, Billerica, MA, USA) by the semi-dry transfer protocol (Bio-Rad, Hercules, CA, USA), and blocked with 5% skimmed milk. The membranes were then probed with monoclonal antibodies against candidate genes and GAPDH overnight at 4°C. Antibody information is listed in S5 Table. The membranes were then rinsed with TBST and incubated with IgG conjugated to horseradish peroxidase at room temperature for 2 h. The protein bands were visualized on an ImageQuant LAS 500 (GE Healthcare, Pittsburgh, PA, USA) after applying electrochemiluminescent detection reagent (Millipore, Bedford, MA, USA). The intensities of the bands were quantified by using image J software. The band intensity for each candidate gene was subtracted from its own background intensity, normalized against the corresponding GAPDH intensity, and then compared with that of the paired sample. Each experiment was performed in duplicate.