Methods

Cell culture

Human lung carcinoma cell lines (CL1-5) had been described previously (Chu *et al.*, 1997). All of the cells were cultured at 37°C in 5% CO_2 and RPMI-1640, supplemented with 10% fetal bovine serum (FBS) and Gibco™ Antibiotic-Antimycotic (100X) (Yang *et al.*, 1992).

5-Aza-dC treatment

Cells were plated at 10^5 per 100-mm culture dish on one day before treatment. The cultures were treated for 48 h with 1 μM 5-aza-dC (Sigma, St Louis, MO, USA) as described previously (Cameron *et al.*, 1999). The cells were then harvested for an analysis of methylation specific PCR (MSP) and quantitative reverse transcription polymerase chain reaction (QRT–PCR) assays.

DNA extraction, bisulfite modification and MSP

For methylation specific PCR, genomic DNA was isolated using the QIAamp DNA mini kit (Qiagen, Hilden, Germany). The genomic DNA was modified by CpGenome™ Fast DNA Modification Kit (Chemicon International, CA, USA). Positive control samples with 5-aza-dC-treated DNA were also included for each set of PCR.

RNA extraction, quantitative reverse transcription polymerase chain reaction (QRT–PCR)

Total RNA was extracted from cells using Tri Reagent (Ambion, Austin, TX, USA) according to manufacturer's instructions. Reverse transcription of total RNA was performed with High Capacity cDNA RT Kit (Applied Biosystems, Foster City, CA) using random primers and 1μg total RNA as template. The reaction mixture was incubated at 25 °C for 10 min, at 37 °C for 2 h and at 85 °C for 5 sec. Quantitative RT-PCR is detected by SYBR Green (Sigma) and was performed using the ABI 7300 (Applied Biosystems, Foster City, CA). The reactions were performed using the following program: 95 °C for 10mins, 40 cycles of denaturing at 95 °C for 15 s and 1 min of annealing and elongating at 60 °C. For each PCR sample, the C_T (the cycle at which the amplification plot of the individual reaction passes a threshold line) was used to determine the relative amount of target sequence. Each measurement was made in triplicate and each was normalized to a glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) control to ensure comparable amounts of cDNA in all wells. The PCR primers are listed in the following table 1.

Table 1. MSP primers and quantitative PCR primers.

Gene	Sense primer	Antisense primer
MYCN-M	5'- TTTTATTTCGGTAAATTAGATTCGT-3'	5'- TTCCTAAAAAAAATAAACGTACGTT-3'
MYCN-U	5'- TTTTTATTTTGGTAAATTAGATTTGT-3'	5'- CTCTACTTCCTAAAAAAAATAAACATACAT-3'

NAR42-M	5'-TTTATTTATTTTTTTGTTTGGGATCGT-3'	5'- CGTAAAACGCACCATTATAACCGAA-3'
NAR42-U	5'-TATTTATTTATTTTTTTGTTTGGGATTGT-3'	5'-AAAAAACCATAAAACACACCATTATAACCA-3'
RAB42-M	5'- GTTATGGAGGTCGAGGGTTGTC-3'	5'- AAAAAATTCTAACCCCAATAAACCG-3'
RAB42-U	5'- TATGGAGGTTGAGGGTTGTTGT-3'	5'- AAAATTCTAACCCCAATAAACCAAA-3'
MYCN	5'-AAGAACCCAGACCTCGAGTTTGAC-3'	5'-GCA GCAGCTCAAACTTCTTCCAGA-3'
NAR42	5'- TACACCAAGCTGACCATGGACCTT-3'	5'- ATTTGGTACACGCAGGAAGGCTTG-3'
RAB42	5'-ATGCTTCAGCGCTGTTAGTCTCCT-3'	5'-ACTCCTGGCCAAGTTTCTCTCTCT-3'

RESULTS AND DISCUSSION

To confirm the accuracy of DBCAT, we first used the primers designed for genes on DNA from human lung cancer cell lines. The primers were designed to discriminate between methylated and unmethylated alleles following bisulfite treatment and to discriminate between DNA modified by bisulfite and that which had not been modified. To accomplish this, we chose methylated genes sorted out under the high criteria (l=400, r=3, d=3) and low criteria (l=400 r=2, d=3). For example, the rate of methylated genes sorted out only under the low criteria (*BAZ1A*, *EAF1*, *EMX2*, *EXOC7*, *MDM2*, and *76P*), 33.3% of genes were amplified with the methylated primers ; however, the rate of methylated genes sorted out under the high criteria (*CHORDC1*, *NAR42*, *MARK3*, *MYCN*, *PDSS2*, *RAB42*, *RPS19* and *SLC30A4*), 87.5% of genes were amplified with the methylated primers. It was known that methylation in 5' regulatory regions of genes can silence gene (Bird, 1986 ; Jones and Laird, 1999). To confirm whether methylation of these genes resulted in down -regulation of their gene expression, randomly selected genes with low expression levels in CL1-5 cells, including *MYCN*, *NAR42* and *RAB42*, were compared to those of these genes in CL1-5 cells treated with 5-aza-2'-deoxycytidine (5-aza-dC). As shown in Figure 1, treatment with 5-aza-dC successfully restored mRNA expressions and demethylated region of genes, which were originally methylated in CL1-5 cells. After 5-aza-dC treatment, they were de-methylated and the expression levels apparently increased. The results indicated that DBCAT is a reliable tool for determining functionally methylated genes.

(A)

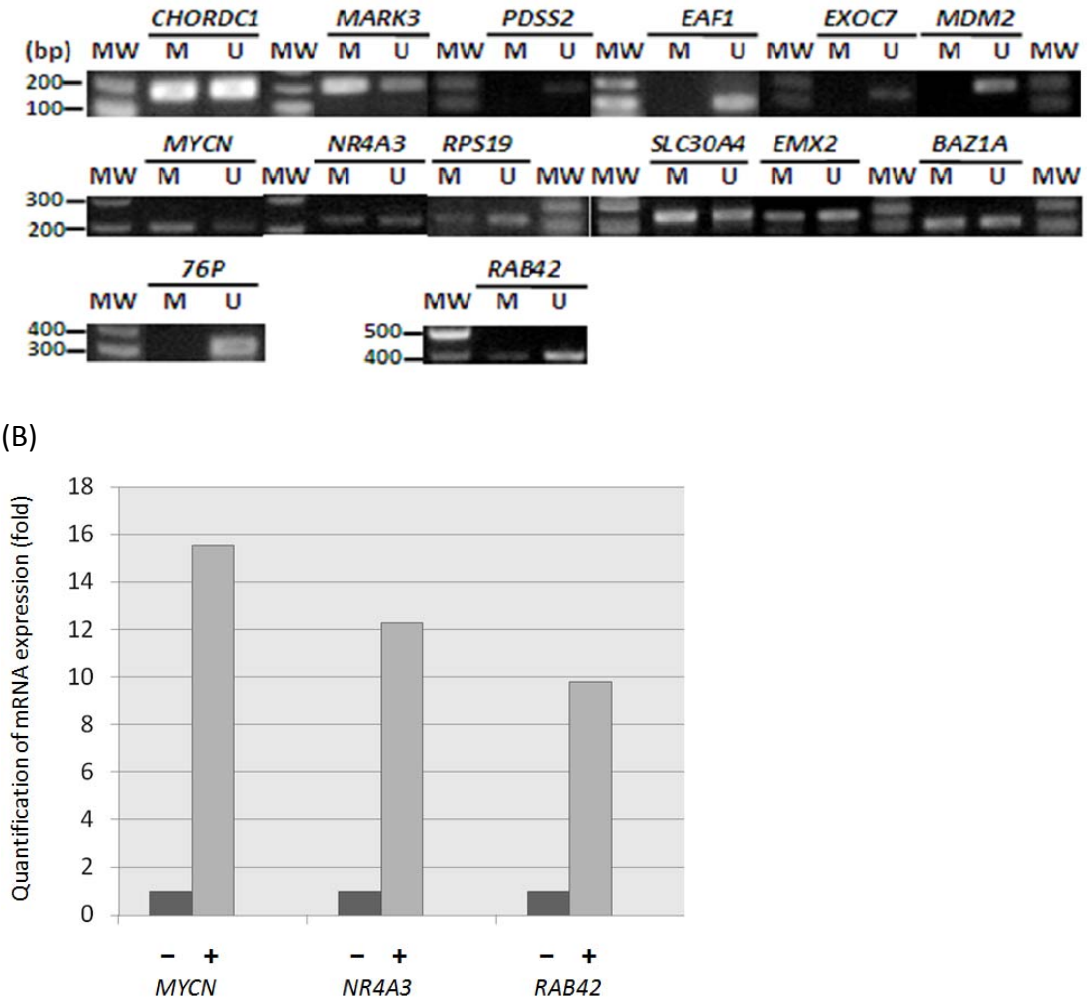

Fig. 1. (A) Shows the MSP result. U, primers specific for unmethylated DNA. M, primers specific for methylated DNA. (**B**) Quantification of *MYCN*, *NR4A3* and *RAB42* mRNA re-expression in 5-Aza-dC-treated CL1-5 cells.

Reference

Cameron EE, Bachman KE, Myohanen S, Herman JG, Baylin SB. (1999). Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. *Nat Genet* **21**: 103–107.

Chu YW, Yang PC, Yang SC, Shyu YC, Hendrix MJ, Wu CW *et al*. (1997). Selection of invasive and metastatic subpopulations from a human lung adenocarcinoma cell line. *Am J Resp Cell Mol Biol* **17**: 353–360.

PANC YANG, KTAY LUH, R WU, CWEN WU (1992). Characterization of the mucin differentiation in human lung adenocarcinoma cell lines. *Am J Resp Cell Mol Biol* **7(2)**: p.161–171.