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Establishment and Validation of Quantitative Real-time PCR Assay for Aberrant Methylation of *14-3-3σ* Gene in Breast and Lung Carcinoma*

UBARADKA G. SATHYANARAYANA[1,2], MAKOTO SUZUKI[1], SHINICHI TOYOOKA[1], ASHA PADAR[1], KIYOMI O. TOYOOKA[1], ANDREA L. ZERN[1], KUNIHARU MIYAJIMA[1], TAKASHI TAKAHASHI[3], ELIZABETH BRAMBILLA[4] and ADI F. GAZDAR[1,2]

[1]*Hamon Center for Therapeutic Oncology Research and*
[2]*Departments of Pathology, University of Texas Southwestern Medical Center, Dallas, TX 75390, U.S.A.;*
[3]*Division of Molecular Oncology, Aichi Cancer Center, Nagoya 464-8681, Japan;*
[4]*Laboratoire de Pathologie Cellulaire, Centre Hospitalier Regional Universitaire, Grenoble 38043, France*

Abstract. *Background: 14-3-3σ gene has been shown to be responsible for G2 cell cycle checkpoint control by p53 in response to DNA damage in human cells. In order to increase the potential utility of 14-3-3σ gene as a molecular marker in tumor analysis and prognosis, we established and validated a quantitative real-time MSP assay and correlated our findings with the standard MSP assay. Materials and Methods: We examined the expression of 14-3-3 σ gene by reverse transcription PCR (RT-PCR) in breast and lung cancer cell lines and control non-malignant tissue samples. To elucidate the mechanism of gene silencing, we studied the methylation patterns in cell lines, tumors and non-malignant control tissues of breast and lung using previously reported MSP assay. For fluorescence based quantitative Real-Time PCR assay, we designed primers and probe specific to 14-3-3σ gene, validated the assay in cell lines and non-malignant control tissues of breast and lung and extended the study to primary tumors and corresponding non-malignant tissues. Results: The concordances between the standard MSP assay and the real-time assay were 95-100%. The overall concordances between standard MSP and real-time assay in 60 cell lines were 97%. By real-time assay, the differences in methylation frequencies between malignant and non-malignant breast and between malignant and non-malignant lung tissues; between NSCLC and SCLC cell lines; between MSP (-) and MSP (+) samples and between MSP (+) and MSP (++) samples were statistically significant. The mean real-time values for MSP (-), MSP (+) and MSP (++) samples were 2, 28 and 53 respectively. Conclusion: We conclude that promoter methylation is a valid pathway for silencing of 14-3-3σ gene in primary breast and lung carcinomas. The real-time assay to distinguish the extent and degree of methylation of 14-3-3σ gene among malignant and non-malignant tissues would potentially enhance the utility of this marker in breast and lung cancer analysis and prognosis.*

Introduction

Cells respond to DNA damage by stopping the cell cycle either at a G1 or G2 DNA-damage checkpoint. In contrast to the G1 checkpoint, the mechanisms controlling the G2 checkpoint have remained unclear until recently. A model now exists for epithelial cells in which distinct members of the 14-3-3 family target specific mitotic regulators to control various aspects of the G2 checkpoint (1-4). The 14-3-3 series of proteins comprise a family of eukaryotic regulatory molecules that are highly conserved among species. The *14-3-3σ* gene, one of the seven members of the 14-3-3 family, was originally characterized as an epithelial-specific marker, HME1 (5). The molecule was then detected as an abundant protein in stratified keratinizing epithelium and was termed stratifin (6). This gene has been

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Abbreviations: MSP, methylation specific PCR; TSG, tumor suppressor gene; S.E, standard error of the mean; RT, reverse transcription; 5-Aza-CdR, 5-aza-2'-deoxycytidine; NSCLC, Non-small cell lung cancer, SCLC, Small cell lung cancer.

Correspondence to: Dr. Adi F. Gazdar, Hamon Center for Therapeutic Oncology Research, University of Texas Southwestern Medical Center at Dallas, 6000 Harry Hines Boulevard, Dallas, Texas, 75390-8593, U.S.A. Tel: 214-648-4921, Fax: 214-648-4940, e-mail: adi.gazdar@utsouthwestern.edu

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shown to be responsible for G2 cell cycle checkpoint control by p53 in response to DNA damage in human cells (1). It has been suggested that 14-3-3σ normally sequesters cdc2-cyclin B1 complexes in the cytoplasm during G2 arrest, and that absence of 14-3-3σ eventually allows cdc2-cyclin B1 complexes to enter the nucleus, resulting in the mitotic catastrophe (3).

Multiple mechanisms of gene silencing including loss of heterozygosity, point mutations, homozygous deletions and aberrant promoter methylation have been reported in tumors (7). Aberrant methylation of CpG rich sites (CpG islands) was identified as an epigenetic mechanism for the transcriptional silencing of tumor suppressor genes in many cancer types, and the number of methylated genes in individual cancers is estimated to be very high (8-10). Down regulation of *14-3-3σ* gene expression has been reported in v-Ha-ras-transformed mammary epithelial cells, mammary carcinoma cells (5), SV40-transformed human keratinocytes (11), head and neck squamous cell carcinoma lines (6), primary bladder tumors (12) and colonic polyp specimens (13). The implication of loss of 14-3-3σ function in tumorigenesis was further substantiated by results of a study showing hypermethylation of CpG islands and transcriptional silencing of the *14-3-3σ* gene in the majority of breast cancers (14) and in lung cancers (15). Furthermore, loss of *14-3-3σ* gene occurs early in neoplastic development in breast epithelium (16). Silencing of *14-3-3σ* gene expression has also been reported in 43% of primary gastric adenocarcinomas (17) and 89% of heaptocellular carcinomas (18).

Methylation of *14-3-3σ* gene in lymphocytes (5), non-malignant tissues and also considerable heterogeneity of methylation among malignant tissues makes it difficult to use as marker in tumors, especially when the standard non-quantitative MSP assay is used. In order to increase the potential utility of *14-3-3σ* gene as a molecular marker in tumor analysis and prognosis, in the present study, we established and validated a quantitative real-time MSP assay and correlated our findings with the standard MSP assay. We show here that real-time MSP assay distinguishes methylation in malignant and non-malignant tissues.

Materials and Methods

Cell lines. Human breast and lung cancer cell lines (20 breast, 20 NSCLC and 29 SCLC lines) were established by us (19, 20). Most breast and NSCLC lines were established from primary tumors, and most SCLC lines were established from metastases. Cell cultures were grown in RPMI 1640 (Life Technologies, Inc., Rockville, MD) supplemented with 5% fetal bovine serum and incubated in 5% CO_2 at 37˚C.

Clinical samples. Surgically resected specimens from 40 breast tumors, 25 corresponding non-malignant breast tissues and 5 unpaired non-malignant breast tissues from these patients were obtained from our Tumor and Tissue Repository. Tumor samples from 36 primary NSCLC (19 adenocarcinoma, 15 squamous cell, 1 large cell and 1 adenosquamous carcinomas) and 21 corresponding nonmalignant lung tissues were obtained from surgical resections performed at the UT Southwestern Medical Center and M.D. Anderson Cancer Center, Houston. Tumor samples from 26 primary SCLC and 24 bronchial carcinoids were obtained from surgical resections performed in the USA, France and Japan. For gene expression studies, 10 nonmalignant tissue samples (two breast epithelial cells, four bronchial brushes, one bronchial epithelium, one airway epithelial cell culture and two peripheral lung tissues) were obtained as far from the tumor tissue as possible. Epithelial cells from buccal swabs (n=15) of healthy nonsmoking volunteers and peripheral blood lymphocytes (n=14) from healthy volunteers were also obtained. Appropriate Institutional Review Board permission was obtained at participating centers, and written informed consent was obtained from all subjects. Tissues were stored at -80˚C prior to testing.

Expression of 14-3-3σ gene. Expression of *14-3-3σ* gene was analyzed by the RT-PCR technique. Total RNA was extracted from 20 breast and 49 lung cancer cell lines, 4 bronchial brushes, 1 bronchial epithelium, 1 airway epithelial cell culture and 2 peripheral lung tissues by using TRIzol (Life Technologies, Inc., Rockville, MD) reagent following the manufacturer's instructions. Two μg of total RNA treated with DNase I (1unit, Life Technologies, Inc., Rockville, MD) was reverse-transcribed into cDNA using SuperScript II First-Strand Synthesis System (Life Technologies, Inc., Rockville, MD) at 42˚C for 52 min. using oligo (dT) primer according to manufacturer's instructions. The resulting cDNA was subjected to PCR using primers and temperature conditions as described previously (14). The housekeeping gene β-actin was used as an internal control to confirm the success of the RT reaction (21). PCR products were analyzed on 2% agarose gels stained with ethidium bromide.

5-Aza-CdR treatment. Three tumor cell lines with loss of expression for one or more of the three genes were treated with the demethylating agent 5-Aza-CdR (2 μg/ml) as described (22, 23).

DNA extraction. Genomic DNA was extracted from cell lines, primary tumors and nonmalignant cells by digestion with proteinase K (Life Technologies, Inc.) for 1 day at 50˚C, followed by two extractions with phenol: chloroform (1:1) (24).

Methylation-specific polymerase chain reaction (MSP). The MSP reaction employs an initial bisulfite reaction to modify the DNA (25). The primer sequences and temperature conditions for the MSP were done as described (14). *P16* unmethylated primer was used as control in MSP to check the integrity of tissue-extracted bisulfite treated DNA (25). DNA from peripheral blood lymphocytes (n=14) and buccal swabs (n=15) from healthy non-smoking subjects were used as negative controls for MSP assays. DNA from lymphocytes of healthy volunteers treated with Sss I methyltransferase (New England Biolabs, Beverly, MA) and subjected to bisulfite treatment was used as a positive control for methylated alleles. Water blanks and PCR cocktails (without template) were used as negative controls in each assay. PCR products were visualized on 2% agarose gels stained with ethidium bromide. Results were confirmed by repeating bisulfite treatment and MSP assays for all samples.

Semiquantitative real-time MSP assay. Sodium bisulfite treated genomic DNA was amplified by fluorescence based real-time MSP by using TaqMan technology (Perkin Elmer Corp., Foster City, CA) as described previously (26-28). We performed the MSP with the Gene Amp 5700 Sequence Detection System (Perkin Elmer Corp.). In brief, oligonucleotide primers were designed to specifically amplify bisulfite-converted DNA based on the previously reported methylation status of CpG rich regions of *14-3-3σ* gene (14, 16) and a probe was designed to anneal specifically within the amplicon during extension. For the internal reference gene, *MYOD1*, the primers and probe were designed to avoid CpG nucleotides. Thus, amplification of *MYOD1* occurs independent of its methylation status, whereas the amplification of *14-3-3σ* gene is proportional to the degree of cytosine methylation within the amplicon. The methylation ratio was defined as the ratio of the fluorescence emission intensity values for the *14-3-3σ* gene PCR products to those of the *MYOD1* PCR products, multiplied by 100. This ratio was used as a measure for the relative level of methylated *14-3-3σ* alleles in the particular sample. The sequences of the primers and probe used to amplify and detect methylated *14-3-3σ* gene were 5'-TTTAGGGCGTGTGCGATATC-3' (forward primer), 6FAM-5'-GAGGTCGGGGACGTCGAGAGTCG-3'-TAMRA (probe), and 5'-CGATAACCACCTCGACCAAATAAC-3' (reverse primer). The size of the amplicon was 132 bp. The sequences of the primers and probe used to amplify and detect *MYOD1* were 5'-CCAACTCCAAATCCCCTCTCTAT-3'(forward primer), 6FAM-5'-TCCCTTCCTATTCCTAAATCCAACCTAAA TACCTCC -3'-TAMRA (probe), and 5'-TGATTAATTTAGATT GGGTTTAGAGAAGGA -3' (reverse primer). Semiquantitative real-time MSP assays were performed in a reaction volume of 25 µl by using components supplied in a TaqMan PCR Core Reagent Kit (Perkin-Elmer Corp.). Separate amplification assays were performed for *14-3-3σ* gene and *MYOD1*; each assay was performed in duplicate. The final reaction mixtures contained the forward and reverse primers at 600 nmol/L each; the probe at 200 nmol/L; 200 µmol/L each of dNTPs; 5.5 mmol/L $MgCl_2$; 1X Hot Star enzyme buffer (Qiagen, Valencia, CA); 1 U of Hot Star enzyme and 2 µl of bisulfite modified genomic DNA. PCR was performed under following conditions: 95˚C for 12 minutes, followed by 45 cycles of 95˚C for 15 seconds and 60˚C for 1 minute. We used DNA from NCI-H1703, NCI-H2107 and HCC1569 cells in which *14-3-3σ* gene is methylated as positive controls and DNA from NCI-H2347 and NCI-H289 cells in which *14-3-3σ* gene is not methylated as negative controls, and two wells contained either water or cocktail without template as control for PCR specificity. We used serial dilutions of the positive control DNA to create a standard curve (1 to 1000 ng.).

Data analysis. The frequencies of loss of expression and methylation between two groups were compared using χ^2 test and Fisher's exact test with continuity correction. The quantitative ratios of different groups were compared using the Mann-Whitney U nonparametric test. For all of the tests, probability values $p<0.05$ were considered as statistically significant. All of the statistical tests were two-sided.

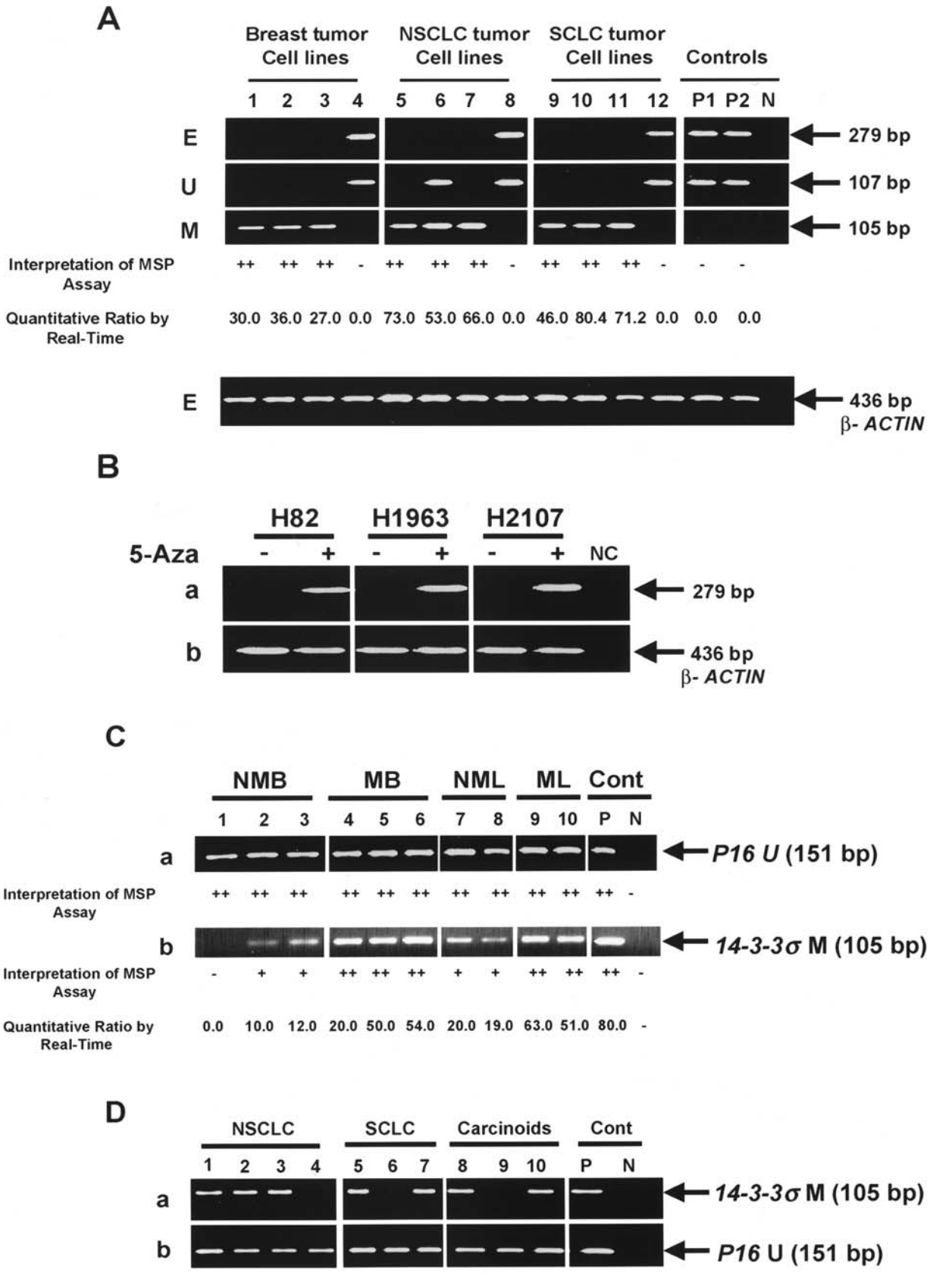
Results

Expression of 14-3-3σ gene in breast and lung cancer cell lines and non-malignant tissues. RT-PCR analysis revealed expression of *14-3-3σ* gene in all control tissues (samples of breast epithelial cells, bronchial brushes and epithelium, airway epithelial cells and peripheral lung tissues; 10 of 10, 100%). However, expression was lost in breast (3 of 20, 15%), NSCLC (5 of 20, 25%) and SCLC (17 of 29, 59%) cell lines. Representative examples are illustrated in Figure 1A. The loss of expression between NSCLC and SCLC cell lines were statistically significant ($P=0.02$). Treatment of expression negative cell lines- NCI-H82, H1963 and H2107 with demethylating agent restored expression in all of the cases (Figure 1B).

Standard MSP assay. Further, standard MSP assay was done in breast and lung cancer cell lines and in respective primary tumors and control tissues as mentioned in Materials and Methods. The results are detailed in Table I and representative examples are illustrated in Figure 1A, C and D. The methylation frequency of *14-3-3σ* gene in breast, NSCLC and SCLC cell lines were 3 of 20 (15%), 5 of 20 (25%) and 10 of 20 (50%) respectively. The concordances between loss of expression and methylation in cell lines for the three tumor types were 100% ($p<0.001$), 100% ($p<0.001$) and 80% ($p=0.0002$) respectively. The over all concordance between loss of expression and methylation in 60 cell lines were 93% ($p<0.0001$). As presented in the Table I and also in Figure 1C varying intensities of bands demonstrated heterogeneity, in primary breast tumors, 8 of 40 (20%) gave relatively weak (+) band and 27 of 40 (67%) gave relatively strong (++) band. In non-malignant breast tissues, 5 of 30 (17%) gave relatively strong band (++) and 17 of 30 (57%) gave relatively weak band (+). In primary lung tumors, 10 of 36 (28%) gave relatively weak band (+) and 21 of 36 (58%) gave relatively strong band (++). Non-malignant lung tissues 16 of 21 (76%) gave relatively weak band (+) and 2 of 21 (10%) gave relatively strong band (++). Methylation frequencies and their band pattern in SCLC tumors, carcinoids, lymphocytes and buccal swabs are presented in Table I. The differences in methylation frequency between malignant and non-malignant tissues for both lung and breast were statistically significant ($p<0.0001$).

Real-time MSP assay. All the breast (n=20) and lung cancer cell lines (n=40), non-malignant breast and lung tissues, malignant breast and lung cancer tissues, lymphocytes and buccal swabs were analyzed by semi-quantitative real-time PCR. The real-time data for all the samples are presented by box plot in Figure 2.

Correlation between standard and real-time MSP assays. Correlations between the two assays are presented in Figure 2. Real-time values for both human bronchial epithelial cells and mammary epithelial cells were 0.00 indicating that *14-3-3σ* gene is not methylated in non-malignant epithelial cells.


A
Breast tumor Cell lines
NSCLC tumor Cell lines
SCLC tumor Cell lines
Controls
1 2 3 4 5 6 7 8 9 10 11 12 P1 P2 N
E
U
M
279 bp
107 bp
105 bp
Interpretation of MSP Assay
++ ++ ++ - ++ ++ ++ - ++ ++ ++ - - -
Quantitative Ratio by Real-Time
30.0 36.0 27.0 0.0 73.0 53.0 66.0 0.0 46.0 80.4 71.2 0.0 0.0 0.0
E
436 bp
β- ACTIN
B
H82 H1963 H2107
5-Aza
- + - + - + NC
a
b
279 bp
436 bp
β- ACTIN
C
NMB MB NML ML Cont
1 2 3 4 5 6 7 8 9 10 P N
a
P16 U (151 bp)
Interpretation of MSP Assay
++ ++ ++ ++ ++ ++ ++ ++ ++ ++ ++ -
b
14-3-3σ M (105 bp)
Interpretation of MSP Assay
- + + ++ ++ ++ + + ++ ++ ++ -
Quantitative Ratio by Real-Time
0.0 10.0 12.0 20.0 50.0 54.0 20.0 19.0 63.0 51.0 80.0 -
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1 2 3 4 5 6 7 8 9 10 P N
a
14-3-3σ M (105 bp)
b
P16 U (151 bp)

Table I. *Promoter methylation frequencies of 14-3-3σ gene in breast and lung cancer cell lines, tumors and control tissues.*

Samples	No. tested	No. methylated (%)[a]
Breast carcinoma		
Breast cancer cell lines	20	3 (15)
Primary breast tumors	40	27 (++, 67); 8 (+, 20)
Total breast cancer samples	60	30 (++, 50); 8 (+, 13)
Lung carcinoma		
NSCLC		
Cell lines	20	5 (25)
Primary tumors		
Adenocarcinoma	19	15 (++, 79); 4 (+, 21)
Squamous cell carcinoma	15	5 (++, 33); 5 (+, 33)
Total tumors	36	21 (++, 58); 10 (+, 28)
Total NSCLC samples	56	26 (++, 46); 10 (+, 18)
SCLC		
Cell lines	20	10 (50)
Primary tumors	26	18 (++, 69); 8 (+, 31)
Total SCLC samples	46	28 (++, 61); 8 (+, 31)
Carcinoids	24	13 (++, 54); 9 (+, 37)
Total Lung cancer samples	126	67 (++, 53); 27 (+, 21)
Non-malignant tissues		
Peripheral blood lymphocytes [b]	14	13 (93)
Buccal swabs [b]	15	4 (+, 27)
Non-malignant breast [c]	30	5 (++, 17); 17 (+, 57)
Non-malignant peripheral lung [c]	21	2 (++, 10); 16 (+, 76)
Total non-malignant samples	80	20 (++, 25); 37 (+, 46)

[a] using standard MSP assay

[b] from healthy volunteers; [c] from resections for breast and lung cancers

+, relatively weak band; ++, relatively strong band

Based on our results, we selected a real-time value of 1.0 as the cut off between methylation-negative and methylation-positive samples. The concordances between the standard MSP assay and the real-time assay for breast, NSCLC and SCLC cell lines were 95% ($p<0.0001$), 100% ($p<0.0001$) and 95% ($p<0.0001$) respectively. The overall concordances between standard MSP and real-time assay in 60 cell lines were 97% ($p<0.0001$). By real-time assay, the differences in methylation frequencies between malignant and non-malignant breast ($p<0.0001$, Figure 2C) and lung ($p<0.0001$, Figure 2B) tissues; between NSCLC and SCLC cell lines ($p=0.04$, Figure 2B); between MSP (-) and MSP (+) samples ($p<0.0001$, Figure 2D) and between MSP (+) and MSP (++) samples ($p<0.0001$, Figure 2D) were statistically significant. The mean real-time values for non-malignant lung, malignant lung, non-malignant breast and malignant breast samples were 28±2, 58±6, 18±2 and 33±2 respectively. The mean real-time values for MSP (-), MSP (+) and MSP (++) samples were 2±0.7, 28±1 and 53±3 respectively.

Discussion

The class of molecular chaperones known as 14-3-3 is involved in the control of cellular growth by virtue of its apparent regulation of various signaling pathways (29). The only p53 target gene down-regulated with the loss of BRCA1 was *14-3-3σ*, a major G2/M checkpoint control gene (30). Several lines of evidence suggest a link between loss of *14-3-3σ* function and cell transformation and also it is strongly postulated that the inactivation of *14-3-3σ* might play an important role in tumor progression. Therefore, in terms of the regulatory role in G2/M checkpoint and suggestive association with the carcinogenesis of a large number of tumor types (14, 16, 29, 31), *14-3-3σ* gene has received a considerable research interest.

Epigenetic inactivation of *14-3-3σ* gene has been reported in several human cancers (14, 16, 29, 31). It has been reported that hypermethylation of *14-3-3σ* locus is detectable with

Figure 1. *Expression and methylation specific PCR (MSP) analysis of 14-3-3 σ gene in representative breast and lung cancer cell lines. Lanes 1-4, breast cancer cell lines - HCC1569, HCC1187, HCC1428 and HCC1143; Lanes 5-8, NSCLC cell lines – H1299, H1155, H522 and H2347; Lanes 9-12, SCLC cell lines – H524, H2107, H711 and H211. P1 and P2 are bronchial epithelial and breast epithelial samples respectively. E, expression by RT-PCR; U, Unmethylated form; M, Methylated form. Positive control (P), DNA from bronchial brushes or airway epithelial cells (for unmethylated form) or known cell line DNA in which 14-3-3 σ gene is not methylated treated with Sss I methyl transferase (for methylated form); N, negative control is water blank or PCR cocktail. A, agarose gel showing RT-PCR products (279 bp) and MSP products (unmethylated form 107 bp and methylated form 105bp) for 14-3-3 σ. Interpretation of MSP assay for methylated form is shown as -, band is absent; ++, strongly positive band. For the methylated form, the corresponding results of the semi-quantitative ratio of the real-time MSP assay are provided. E, Expression of house keeping gene β-actin (436 bp) by RT-PCR as an internal control for RNA integrity. B, Lack of expression of transcripts by RT-PCR for 14-3-3 σ in methylated cell lines in untreated state and restoration of expression after 5 – Aza – CdR (drug for demethylation). -, And +, indicate before and after 5 – Aza – CdR treatment respectively. NC, negative control is water blank or PCR cocktail. Panel a, demonstrates loss of expression and restoration of expression before and after drug treatment by RT-PCR respectively for the same cell line. Panel b, expression of β-actin transcript (436 bp) by RT-PCR as an internal control for RNA integrity. C, Representative examples of MSP assay in primary breast and lung carcinoma and corresponding non-malignant tissues. NMB, non-malignant breast; NML, non-malignant lung; MB, Malignant breast; ML, Malignant lung; Cont, controls. Results of testing for the unmethylated (P16 U) and methylated forms (14-3-3 σ M) are illustrated. The unmethylated form of P16U was run as a control for DNA integrity, and all samples yielded a strongly positive (++) band. The methylated bands demonstrated heterogeneity, with some being absent (-), weakly positive (+), or strongly positive (++). For the methylated form, the corresponding results of the semi-quantitative ratio of the real-time MSP assay are provided D, Illustration of MSP for 14-3-3 σ gene in lung tumor samples. Lanes 1-4, non-small cell lung cancer (NSCLC) tumors; lanes 5-7, small cell lung cancer (SCLC) tumors; lanes 8-10, carcinoids; P, positive control; N, negative control is water blank or PCR cocktail. U, unmethylated form; M, methylated form.*

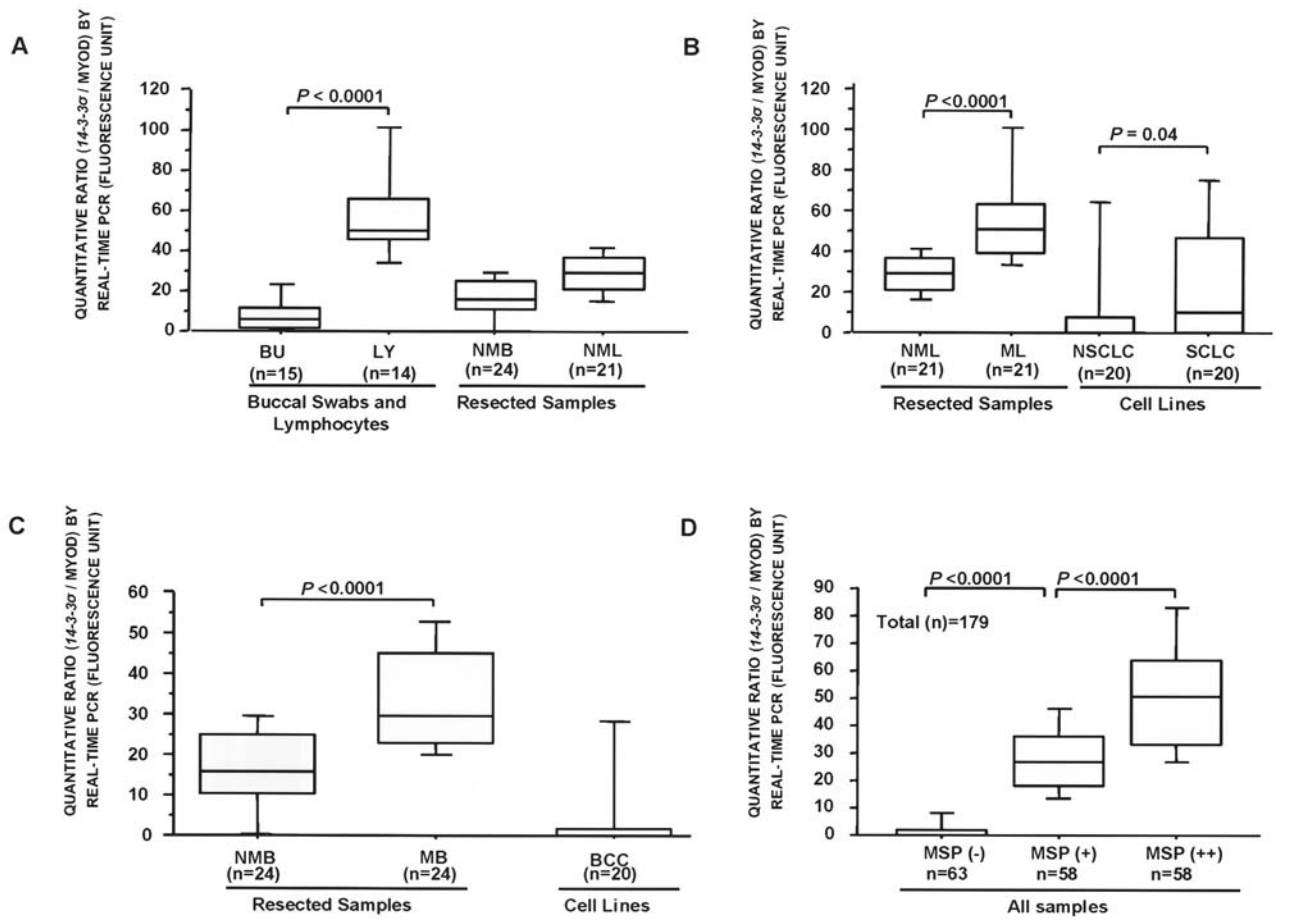

Figure 2. *Quantitative ratio by semi-quantitative real-time MSP assay for different samples shown by Box Plot analysis. Each box plot is composed of five horizontal lines that display 10th, 25th, 50th, 75th and 90th percentiles of a variable. For comparing different categories of samples different illustrations have been made (A-D). BU, buccal swabs; LY, lymphocytes; NMB, non-malignant breast; NML, non-malignant lung; MB, malignant breast; ML, malignant lung; NSCLC, non-small cell lung cancer cell lines; SCLC, small cell lung cancer cell lines; BCC, breast cancer cell lines; n=sample number. D, MSP results were classified into three groups based on the intensity of MSP product; negative (-), relatively weak positive (+) and relatively strong positive (++).*

increasing frequency as the breast lesions progress from normal to atypical hyperplasia, to ductal carcinoma *in situ*, and finally to invasive carcinoma (16). Others have reported and we have confirmed that lymphocytes do not express *14-3-3σ* gene and also their promoter region is methylated (5, 16). We confirm methylation as the mechanism of silencing of *14-3-3σ* gene in breast cancer, NSCLC and SCLC cell lines and tumors and extend these findings and demonstrate in increasing the utility of *14-3-3σ* as a molecular marker in tumor analysis and prognoses by employing real-time MSP assay. *14-3-3σ* gene is expressed uniformly in bronchial epithelial cells, mammary epithelial cells and in non-malignant lung and breast tissues whereas lung and breast cancer cell lines had varying frequencies of losses of *14-3-3σ* gene. Treatment with 5-Aza-CdR restored the expression of *14-3-3σ* gene in all the 3 expression negative cell lines tested indicating methylation as a mechanism of transcriptional silencing of *14-3-3σ* gene. There were significant differences in methylation frequencies between NSCLC and SCLC cell lines and tumors. SCLC is perhaps the most invasive/metastatic of all human cancers, and differences in methylation frequencies between SCLC and NSCLC have been described for other genes including *CDH1*(32), *CDH13*(33) and *CASP8*(34). Differences in methylation between NSCLC and SCLC also suggest that the two major forms of lung cancer arise via different pathogenic pathways.

In order to increase the potential utility of *14-3-3σ* gene as a molecular marker in tumor analysis and prognosis, we established and validated a quantitative real-time MSP assay and correlated our findings with the standard MSP assay. There was excellent concordance between standard MSP

assay and semiquantitative real-time PCR assay and also there was statistically significant differences in methylation by real-time assay between malignant breast / lung and corresponding non-malignant tissues. Real-time assay could clearly distinguish methylation negative (-), relatively weak (+) and relatively strong bands (++), thereby making the tumor analysis more rigorous, which might help in prognosis. Our semi-quantitative real-time MSP may help to interpret the dynamic nature of the promoter methylation.

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