Published in final edited form as:
Int J Cancer. 2007 June 1; 120(11): 2353–2358.

Inactivation of *LLC1* gene in nonsmall cell lung cancer

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Abstract

Serial analysis of gene expression studies led us to identify a previously unknown gene, c20orf85, that is present in the normal lung epithelium, but absent or downregulated in most primary non-small cell lung cancers and lung cancer cell lines. We named this gene *LLC1* for *L*ow in *L*ung *C*ancer 1. *LLC1* is located on chromosome 20q13.3 and has a 70% GC content in the promoter region. It has 4 exons and encodes a protein containing 137 amino acids. By *in situ* hybridization, we observed that *LLC1* message is localized in normal lung bronchial epithelial cells, but absent in 13 of 14 lung adenocarcinoma and 9 out of 10 lung squamous carcinoma samples. Methylation at CpG sites of the *LLC1* promoter was frequently observed in lung cancer cell lines and in a fraction of primary lung cancer tissues. Treatment with 5-aza deoxycytidine resulted in a reduced methylation of the *LLC1* promoter concomitant with the increase of *LLC1* expression. These results suggest that inactivation of *LLC1* by means of promoter methylation is a frequent event in nonsmall cell lung cancer and may play a role in lung tumorigenesis.

Keywords

nonsmall cell lung cancer; serial analysis of gene expression; promoter methylation

Lung cancer is the leading cause of cancer death, accounting for over 170,000 cancer deaths each year in the United States alone.[1] Nonsmall cell lung cancer (NSCLC) is the predominant form of lung cancer and consists of 2 major histological subtypes: squamous cell carcinoma and adenocarcinoma. Cytogenetic, chromosomal loss of heterozygosity and comparative genomic hybridization analyses have revealed multiple chromosomal as well as genetic changes in lung cancer.[2,3] In the past decade, gene expression-based studies, such as serial analysis of gene expression (SAGE) and cDNA microarray analyses, have revealed the gene expression signatures of lung cancer[4-11] and provided lung cancer subclassification[12] and prognosis.[10,13]

Using the SAGE, we have showed that 115 highly differentially expressed genes were able to distinguish the neoplastic state as well as the tissue type of the lung tumors.[4,5] We also identified PGP9.5 and TDE2 genes as candidate tumor markers commonly associated with

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Grant sponsor: Center for Cancer Research, National Cancer Institute; Grant sponsor: National Cancer Center; Grant number: 0510370-1; Grant sponsor: NIH; Grant numbers: R01-80127 and R01-84354.

lung cancers.[14,15] As a part of the effort to identify candidate genes that are expressed at significantly different levels between NSCLC and nontumor lung tissues, we used real-time PCR to screen additional genes based on SAGE analysis. Here, we report the identification of a previously uncharacterized gene, *LLC1*, whose expression was frequently lost in primary lung cancers and lung cancer cell lines.

Materials and methods

Tissues and SAGE libraries

Tissue samples were obtained and the SAGE libraries were generated, as previously described.[4,5] A total of 9 libraries were constructed, and they include 2 libraries of each primary culture of normal small or large airway epithelial cells (SAEC and NHBE, respectively), 2 of each primary squamous cell carcinoma tissue and adenocarcinoma tissue of the lung, and a library derived from the A549 lung adenocarcinoma cell line. A total of 374,643 tags were sequenced to include ~55,000 tags from squamous cell carcinoma, NHBE cells and A549 cell libraries, and 22,000 tags from each adenocarcinoma and SAEC libraries. The fresh-frozen lung adenocarcinoma and nontumor lung tissues used for real-time PCR and promoter methylation assay were obtained from the Mayo Clinic, using protocols approved by its institutional review board.[16] Normal human nonpulmonary tissue RNA samples were purchased from Stratagene (LaJolla, CA). All cell lines used were NSCLC cell lines and were obtained from American Tissue Culture Collection, Division of Cancer Treatment and Diagnosis Tumor Repository at NCI, and Korean Cell Line Bank.

Real-time RT-PCR for gene expression analyses

The paired nontumor tissues were stained with H&E to confirm the histology and tumor content. Approximately 10–15 sections (10 μm) were collected and placed in TRIzol (Invitrogen, Carlsbad, CA) for RNA extraction and 500 μg/mL of 1% SDS/proteinase K solution for DNA extractions, using standard procedures as described.[5,17] Samples were stored at −70°C until use. The quality of RNA was determined by formamide gel electrophoresis. Single-stranded cDNA was synthesized using 2 μg of the total RNA and the Superscript Kit (Invitrogen), and the cDNA was diluted at least 10 times for real-time quantitative PCR (Q-PCR) reactions.

SAGE tags with the highest differential expression between the control bronchial epithelial cells and the lung tumor tissues were searched against GenBank to identify the full length sequence, as previously described,[18] and then selected for analysis by Q-PCR. The Q-PCR primers for *LLC1* were LLC-F (CCT TGT GGG TCA GGA TGA GAT) and LLC-R (AAC TCC TCA AAA GGG GTT GTT A), and the reactions were carried out using the SYBR Green Core reagent (Applied Biosystems, Foster City, CA). For *PLUNC* and *XAGE1*, primers PLUNC-F (CTC ATT GTC TTC TAC GGG CTG TTA), PLUNC-R (GGC AGG GCT GGA TTC ACA TT), XAGE1-F (CGG CGT CAA GGT GAA GAT AA) and XAGE1-R (ACC AGC TTG CGT TGT TTC AG) were used for Q-PCR. Each Q-PCR reaction mixture contained 4 μL of diluted single-stranded cDNA, 25 μL of SYBR Green PCR master mix, 1.5 μL each of 10 μM of forward and reverse primers and water, to a final volume of 50 μL. The condition during the amplification was as follows: initial activation of 95°C for 10 min, 45 cycles of 95°C for 30 sec, 59°C for 30 sec and 60°C for 30 sec using the 7900HT sequence Detector System (Applied Biosystems). For normalization, *GUSB* (β-glucuronidase) level (Applied Biosystems) was determined for each cDNA samples. Four other genes [*eEF1γ* (BC024274); *RPS4* (BC071662); *RPL35* (BC000348); and *RPL41*(BC017820)] were also used as reference genes, based on their relatively constant tag levels in SAGE and as analyzed by Q-PCR. When analyzed using all 5 genes, the average ΔCt values between tumor and nontumor samples was similar to that of the differences obtained using *GUSB* alone. Therefore, *GUSB* expression

level was used to calculate the relative expression of candidate genes in paired tumor/nontumor samples.

For the analysis of Q-PCR, the comparative Ct method was used for comparing relative expression results, as suggested by the manufacturer (PE Applied Biosystems). Specifically, the relative ratio of expression = $2^{-[\Delta Ct(sample) - \Delta Ct(control)]} = 2^{-\Delta\Delta Ct}$, where ΔCt is the Ct difference between the Ct from *LLC1* and the Ct from a reference gene (*GUSB* in this study). Ct values were defined as the number of PCR amplification cycle, in which the SYBR Green signal from the amplified product is greater than the minimal detection level. In the analysis of the data from tumor and nontumor paired samples, ΔCt value from tumor was considered as ΔCt sample, and ΔCt for nontumor was the ΔCt control in the equation. In the analyses of cell lines or human normal tissues from different organs where the controls were not available, each ratio in cell lines or human normal tissues represents the relative *LLC1* expression level compared with *LLC1* expression level in nontumor lung tissues. When compared to *GUSB*, the relative median ΔCt value for *LLC1* was −3.4 for nontumor lung tissues.

The linearity of real-time PCR assay for *LLC1* according to the Ct value was assessed by spiking the *LLC1* plasmid DNA into 10 ng gDNA, and it was found that the amplification efficiency was 2.006 and there was a linear relationship between the log (input *LLC1*) and Ct values (data not shown).

In situ hybridization of LLC1 in normal and tumor lung tissues

The *in situ* hybridization was performed exactly as previously described,[15] or using a single-step amplification protocol with the GenPoint Fluorescein kit (Dako, Carpinteria, CA), and then visualized using the NovaRed HRP as a substrate (Vector Labs, Burlingame, CA). Specifically, the PCR product for *LLC1* genes was generated from nontumor cDNA using gene-specific primers: LLC-F (same 1 for real-time PCR) and IVT-LLC-R (GGA TCC TAA TAC GAC TCA CTA TAG GGA GAA CTC CGT CTG GAT TCA) with the same amplification condition as Q-PCR for *LLC1* gene. PCR product for the *vWF* gene was used as a control.[15] The quality of fresh-frozen tissue was evaluated based on the intensity and the specificity of *vWF* gene staining in the vascular endothelium.

Promoter methylation of LLC1

The methylation status of *LLC1* was determined using methylation single base extension (MSBE) method as described.[19] Specifically, ~1 μg of genomic DNA was used for the sodium bisulfite treatment. Modified genomic DNA was amplified with *LLC1* gene-specific primers corresponding to the sodium bisulfite-treated sequences; c20mF (5′-GAG TAA ATG GGT TTA GAG GTG GAT AAA GG-3′) and c20mR (5′-CCC RCA AAC TCT AAC CCT AAA CTC AAC-3′, where R = A and G). PCR amplification was performed by initial incubation at 95°C for 10 min, then 35 cycles of 95°C for 30 sec, 56°C for 30 sec, 72°C for 1 min, with a final extension at 72°C for 7 min in a mixture containing 1× PCR Gold™ buffer (Roche, Indianapolis, IN), with 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 10 pmol of each primer and 50–100 ng of bisulfite-treated genomic DNA. The amplified products (20 μL) were then purified with a QIAquick™ PCR Purification Kit (Qiagen, Valencia, CA) and eluted in a final volume of 50 μL. For the SBE reaction, SNaPshot™ Ready Reaction Mix (Applied Biosystems) was used with 1 μL of purified PCR product and 2 pmole of SBE-primer mix of SBE-C (5′-TCA ACC CTA AAC TCA ACG TCT CGC-3′) and SBE-T (5′-CTC TCT CTC TCA ACC CTA AAC TCA ACT TCT CTC-3′). The SBE condition was 30 cycles of 96°C for 10 sec, 55°C for 5 sec and 60°C for 30 sec. The single base-extended samples were treated with 1 unit of shrimp alkaline phosphatase (Roche) at 37°C for 1 hr, followed by inactivation at 75°C for 15 min. The resulting 0.5 μL dephosphorylated samples were mixed with 9.4 μL of Hi-Di™ formamide and 0.1 μL of GeneScan 120 LIZ™ marker, and analyzed on an ABI Prism® (Applied

Biosystems) automatic sequencer, to determine the peak heights for the extended products corresponding to either methylated or unmethylated DNA.

For the comparison of MSBE and the standard methylation specific PCR (MSP) assay,[20] c20mF (the same primer for MSBE) was used as a common forward primer, and either c20Rt (CCC GCA AAC TCT AAC CCT AAA CTC AAT G) or c20Rc (CCA CAA ACTC TAA CCC TAA ACT CAA GA) was used for reverse primer specific for unmethylated CpG (c20Rt) or methylated CpG (c20Rc). The PCR reaction was carried out for 30 cycles at 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec, using the same sodium bisulfite converted template as those used for the MSBE assay. The degree of methylation was analyzed based on the ratio of the methylated and unmethylated (*M/U*) peaks between the paired tumor and normal tissues or the average of *M/U* ratios between the 2 groups using pair-wise *t*-test.

To determine whether promoter methylation played a role in *LLC1* expression, lung cancer cell lines (A549, H1299 and Hop92) were treated with culture medium containing a demethylating agent, 5-aza-deoxycytidine (final 2 μM; Sigma), for 4 days with a media change at day two. Cells were harvested by trypsinization and then placed in 1%SDS/proteinase K for DNA and TRI-zol (Gibco-BRL) for RNA isolations, respectively. The expression and DNA promoter methylation status of *LLC1* gene was determined by Q-PCR and MSBE, respectively, as described earlier.

Results

Identification of genes differentially expressed in NSCLC

We used SAGE libraries generated from lung cancers and normal bronchial epithelial cells to select genes most differentially expressed between the normal and the tumors. In total, 14 genes were selected for analyses, and they included *MUC5B*, *PLUNC*, *GAPDL4*, *AQP5*, *TSC*, *SERPIND1*, *XAGE1*, *AZGP1*, *WFDC2*, *VCC1*, *CAPS* and 3 hypothetical genes (c20orf85, or NM_178456, XM_498632, and XM_496360). In paired lung adenocarcinoma and nontumor tissues, the expression levels of *PLUNC* and *XAGE1* were increased (data not shown), and the expression of c20orf85 was decreased in a most consistent pattern in primary lung tumor samples. The expression levels of the other genes were not significantly different or were variably expressed among the tested tissues. Our observation of overexpressions of *PLUNC* and *XAGE1* was consistent with those previously reported for lung cancers.[21,22] We therefore focused on c20orf85 and designated it as *LLC1* for *L*ost in *L*ung *C*ancer 1.

At the mRNA level, *LLC1* gene has a SAGE tag of GGATGTTGCA, which matched to a 788-bp cDNA sequence (accession no. BC035405) located on chromosome 20q13.32. The gene has 4 exons and encodes a 137 amino acids protein. Blast analysis revealed mouse (BC050790), bovine (BC109834) and canine (XM_849590). LLC1 amino acid sequences had about 70% homology to the human LLC1 sequence (NM178456) (Fig. 1a).

Expression of LLC1 in normal and tumor lung tissues

We performed real-time PCR using 16 paired lung adenocarcinoma and nontumor tissues, to determine the relative expression of the gene. As shown in Figure 1b, *LLC1* expression was reduced in 9 of 16 primary lung adenocarcinomas tested compared with the paired nontumor tissues. The expression of *LLC1* was also examined in 14 different human tissues by Q-PCR. We observed that *LLC1* expression was relatively high in ovary and fetal lung but low in liver and stomach (Fig. 1c). The *LLC1* gene message was minimal or not detectable in all other human tissues tested, including colon, heart, skeletal muscle, placenta, brain, spleen, bladder, skin, kidney and breast.

To determine the cellular localization of *LLC1* expression in normal and diseased lung, we performed *in situ* hybridization in 24 NSCLC tissues (14 adenocarcinomas and 10 squamous carcinomas) and 7 fresh-frozen adjacent nontumor lung tissues using a *LLC1* cRNA probe. Based on the staining pattern, *LLC1* expression was easily detectable and localized in bronchiolar epithelial cells of the airway (Figs. 2a and 2b). In contrast, there was no expression of *LLC1* in all but one lung adenocarcinoma tissue and 9 of 10 lung squamous carcinoma tissues (Fig. 2c). Two of the 24 primary tumor samples showed weak and diffused staining of *LLC1* (Fig. 2d). The expression of *LLC1* was also confirmed in normal appearing bronchiolar epithelial cells. The *vWF* message was localized in endothelial cells of blood vessels, and the pattern of expression was similar in both nontumor and tumor tissues, as shown earlier.[15]

Loss of LLC1 expression by promoter methylation in lung cancer cell lines

To investigate the mechanism of *LLC1* downregulation in lung cancer cell lines and primary NSCLC, we evaluated its promoter region and tested whether DNA methylation played a potential role. The GC content at the promoter region of *LLC1* was over 70% (Fig. 3a). When genomic DNA from lung cancer cell lines H1299, Hop92, H226 and H358 cells were examined by direct sequencing after sodium bisulfite treatment, all of the CpG sites within the analyzed promoter region were methylated in each of the 4 cell lines (data not shown). We therefore designed primers that could quantitatively evaluate the methylated and unmethylated alleles at the representative CpG sites of the *LLC1* promoter (boxed C in Fig. 3a), using a relatively quantitative MSBE assay.[19] The tested CpG site at the *LLC1* promoter sequence was methylated in a majority of the 9 cell lines tested (Figs. 3b and 3c). When real-time PCR assay was performed using cDNAs generated from 9 lung cancer cell lines, only 1 lung cancer cell line, H520, had a significant *LLC1* expression at the level of 2.4% compared with that of the average expression level observed in the normal lung tissues (Fig. 3c). The presence of the methylated allele in lung cancer cell lines was directly proportional to the presence of a methylated band when analyzed using the standard MSP method (Fig. 3d).

To investigate the causal relationship between the promoter methylation and the expression of *LLC1*, a demethylating agent, 5-aza-deoxycytosine, was used to treat lung cancer cell lines methylated at the *LLC1* promoter region. After treatment with 5-aza dC, the relative methylation (*M/U* ratio) decreased in all 3 tested cell lines (Fig. 4a), and this corresponded with an increase of LLC1 expression levels in the treated cells (Fig. 4b).

Methylation of the LLC1 promoter in primary lung cancers

To assess the mechanism of the frequent reduction of *LLC1* expression observed in primary NSCLC by quantitative RT-PCR and *in situ* hybridization, we assessed the extent of *LLC1* promoter methylation in primary tumors in a total of 67 primary nonsmall cell lung cancers and 29 paired nontumor lung tissues. We used MSBE assay to directly measure the presence of the methylated allele in the samples, based on the ratio of the detected methylated and unmethylated allele (*M/U* ratios, Fig. 3b). When considered individually, the presence of the methylated allele was higher in 10 tumors compared with the matched noninvolved lung tissues. When considered as a group, the average *M/U* ratio was significantly higher ($p = 0.02$) in tumors (*M/U* = 0.50) than in the nontumor lung (*M/U* = 0.34).

Discussion

Our work and that of others showed that SAGE is a powerful tool to identify highly differential expressed genes.[23,24] However, the actual expression of individual genes will need to be further examined using primary tissues, because SAGE usually relies on the analyses of only a few tissues and controls.[13] Taking this into consideration, we first selected candidate genes based on SAGE data and then compared the expression profiles of the candidate genes in paired

primary tumor and nontumor lung tissues, using independent methods such as Q-PCR and *in situ* hybridization. The comparison of expression patterns in paired tissues excluded the expression variation among individuals.

Using this approach, we identified *LLC1* as one of the most consistently downregulated genes in all lung cancer cell lines and primary lung adenocarcinomas tested. Since primary tumor tissues often contain normal lung epithelial components that express *LLC1*, the true extent of *LLC1* promoter methylation as well as downregulation in primary lung tumor tissues is likely to be higher than that observed in this study. When the extent of *LLC1* downregulation in lung cancers was directly assessed by *in situ* hybridization, *LLC1* message was readily detectable in the normal lung bronchial epithelial cells, but absent in most of the NSCLC tumors examined (Fig. 2).

Genetically, *LLC1* is localized on chromosome 20q13.32. The amplification of chromosome 20q is a frequent event in breast cancers.[25] In our study, we have no evidence that directly address the genetic status of *LLC1* gene. However, the promoter site of *LLC1* has a GC content of over 70%, and the CpG island of *LLC1* promoter was highly methylated in most of the lung cancer cell lines tested and in at least a portion of the primary lung tumors. Experiments using a demethylating agent, 5-aza dC, showed that promoter methylation play a role in the transcriptional control of *LLC1* gene expression (Fig. 3). Therefore, our results suggest that the hypermethylation of *LLC1* promoter is a primary mechanism contributing to the loss of *LLC1* expression in NSCLC, and that downregulation of the *LLC1* gene is a frequent event in lung cancers.

However, it is likely that *LLC1* expression also subjects to other mechanisms of transcriptional regulation, and that *LLC1* promoter methylation may not be restricted only to lung cancers. As shown in Figure 4c, most noninvolved lung tissues were unmethylated, but some also demonstrated methylation at the *LLC1* promoter. This result suggests that *LLC1* methylation might be an early or premalignant alteration as in the case of *RASSF1A* promoter methylation[26] or p16 methylation in histologically normal human mammary epithelia[27] or in premalignant lung endobronchial lesions.[28]

DNA methylation at the promoter has been shown to be a common mechanism of gene inactivation in human cancer.[29] Particularly for lung cancer, the CDK4 inhibitor *p16* was the first to be identified as a gene inactivated by either deletion or inactivation *via* promoter methylation.[30] Lung tumor suppressor genes on chromosome 3p, such as *RASFF1A* and *BLU*, are examples of genes whose inactivation is primarily caused by promoter methylation.[31,32] Our identification and analysis of *LLC1* adds to a growing list of candidate tumor genes that are potentially involved in lung tumorigenesis and frequently inactivated in lung cancers *via* promoter DNA methylation. The precise role of *LLC1* in lung cancer development will await further biochemical and functional investigations.

Acknowledgements

This work was supported by the Intramural Research Funding from the Center for Cancer Research at the National Cancer Institute of US and supported in part by a research grant from National Cancer Center (no. 0510370-1, Korea) to K. Hong, and NIH Grants R01-80127 and R01-84354 to P. Yang. We thank Dr. Marie-Christine Aubrey and Dr. Abbas Shakoori for assistance with the pathological diagnosis of the tumors used in this study.

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Human LLC1 MAQKPLSTAAAERMNLVGQDEIWKYRLKAESEARQNWPQNWGFLTTPFEE 50
Mouse LLC1 MAQKPLSTTAAERMNLVAQDEIWKYRLRAESEARQNWPAKWGYLTTSMKE 50
BovineLLC1 MAQKPISTAAAERMNLVAQDEIWKYRLKAENEARQNWAQNWGFLSTPFEE 50
Canis LLC1 MAQKPLSTAAAQRMNLVAQDEIWKYRLKAEGEARQNWAQNWGFLTTPVEE 50

Human LLC1 LIKCEEDLPTPKPKIELPERFRIRPVTPVEKYIKVFPSPPVPQTTQGFIG 100
Mouse LLC1 LLEGEEEPQTPKPKPELPSHFYVRPVSPMDKHIKILPSPPVPKTTQGFIG 100
BovineLLC1 LIKCEEEPATPKPKIELPERFRIRPVTPVEKYIKVLPSPPVPKTTQGFIG 100
Canis LLC1 LLKGEEEPPTLKPKIELPQRFRIRPVTPVEKYIKVLASPPVPKTTQGFIG 100

Human LLC1 WRSAVPGLNKCLELDDAIRSCKGAFARELCWPKQGVH 137
Mouse LLC1 WRSGKPAL-YCLEKYSEVCSCKGAYARELCWPEQGVH 136
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Canis LLC1 WRSGVPNLNKCLEHDYEIRSCKGAYAKELGWPKQGIH 137
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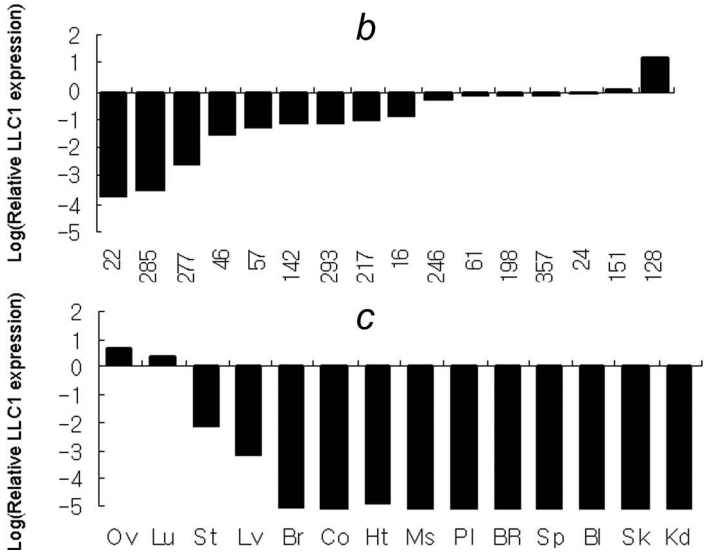

Figure 1.

LLC1 gene and its expression in lung tumor and normal tissues. (*a*) LLC1 protein sequence and its homologues. Mouse (BC050790), bovine (BC109834) and canine (XM_849590). LLC1 amino acid sequences had about 70% homology to the human sequence (NM178456). The bold characters represent identical amino acids. (*b*) The level of *LLC1* expression was determined by real-time RT-PCR. The expression of *LLC1* was decreased in 9 out of 16 adenocarcinoma tissues. (*c*) In human tissues, *LLC1* expression was determined in ovary (Ov), fetal lung (Lu), stomach (St), liver (Lv), breast (Br), colon (Co), heart (Ht), skeletal muscle (Ms), placenta (Pl), brain (BR), spleen (Sp), bladder (Bl), skin (Sk) and kidney (Kd). *Y*-axis is the relative level of *LLC1* expression (on a log scale) in tumor samples compared with paired non-tumor tissues (*b*) or the median Ct difference of *LLC1* and *GUSB* (−3.4) for nontumor lung tissues (*c*).

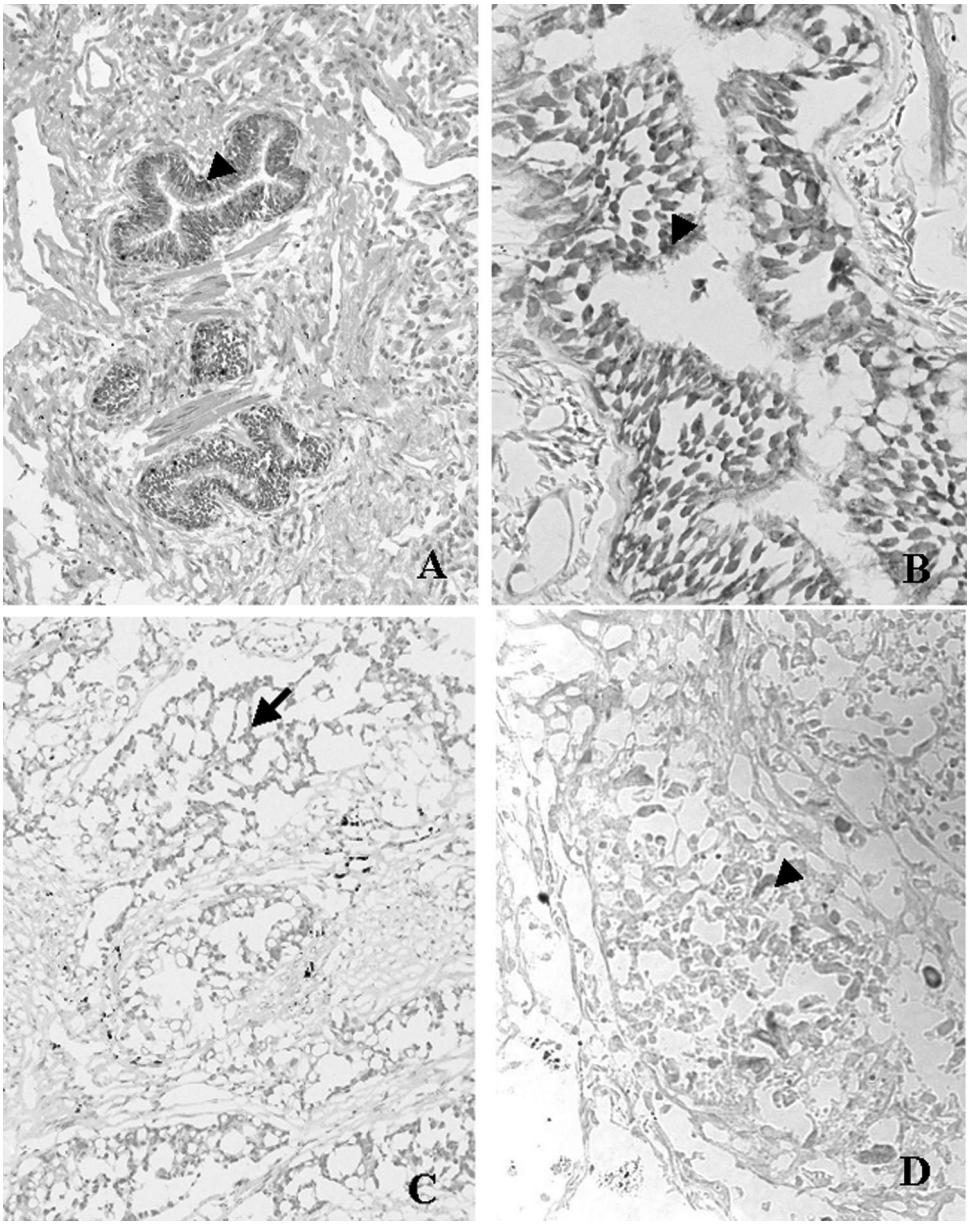

Figure 2.

The expression of *LLC1* in lung tissue and lung adenocarcinoma by RNA *in situ* hybridization. The *LLC1* was expressed in lung epithelial cells (*a* and *b*). (*c*) Lack of *LLC1* expression in primary lung cancers. (*d*) Lung cancer with a weak LLC1 expression. Tissue samples were counterstained with hematoxylin, and photographed under ×100 (*a* and *c*) or ×400 (*b* and *d*) magnification. Arrow heads (▲) indicate tissue areas of *LLC1* expression. Arrow (→) indicates tumor regions without *LLC1* expression.

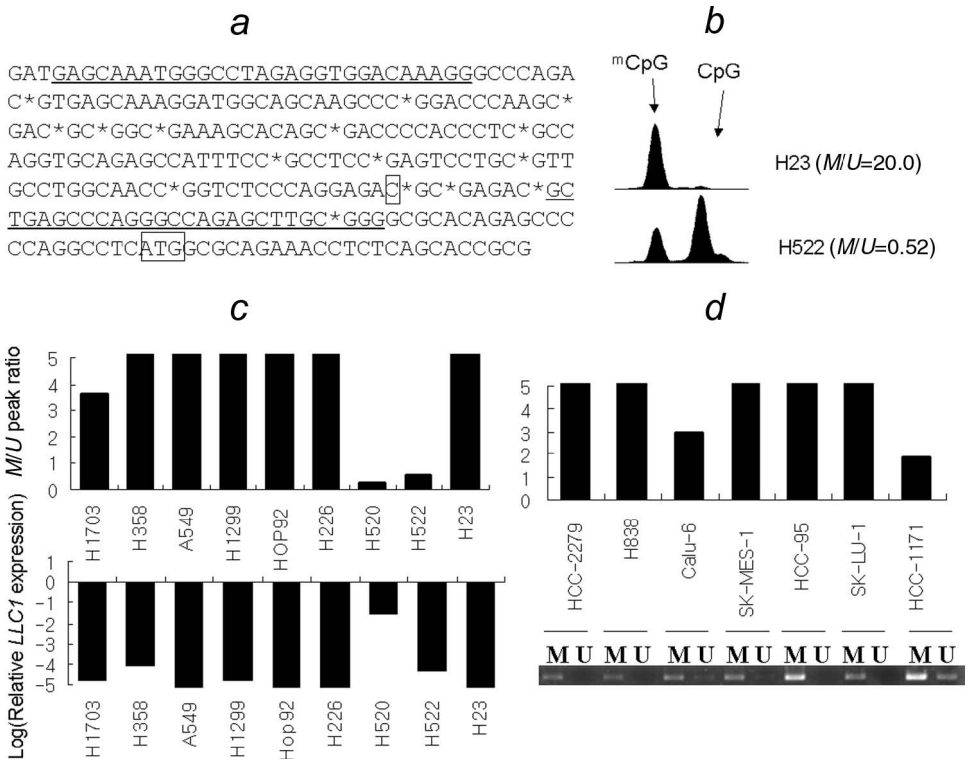

Figure 3.

The expression by real-time RT-PCR and the promoter methylation status of *LLC1* in NSCLC cell lines. (*a*) The promoter region of *LLC1* is shown with the CpG islands (*); and the PCR primer sites for the MSBE assay (underlined). The ATG start site of *LLC1* is boxed, as well as the selected CpG site for the MSBE assay. (*b*) Typical results from MSBE for the methylation of *LLC1* promoter are shown for cell lines, H23 and H522. The relative peak heights (*M/U* ratios) are indicated. (*c*) The increased methylation (upper panel) is correlated with the decreased *LLC1* expression in most lung cancer cell lines (lower panel). (*d*) The methylation status, as assessed by MSBE method (upper panel), directly correlates with those observed by MSP (lower panel). Cell lines are indicated on the *X*-axis and *Y*-axis as the methylation/unmethylation peak ratio (*M/U*) from the MSBE assay or the relative Ct value in Q-PCR analysis as described in the Material and Method.

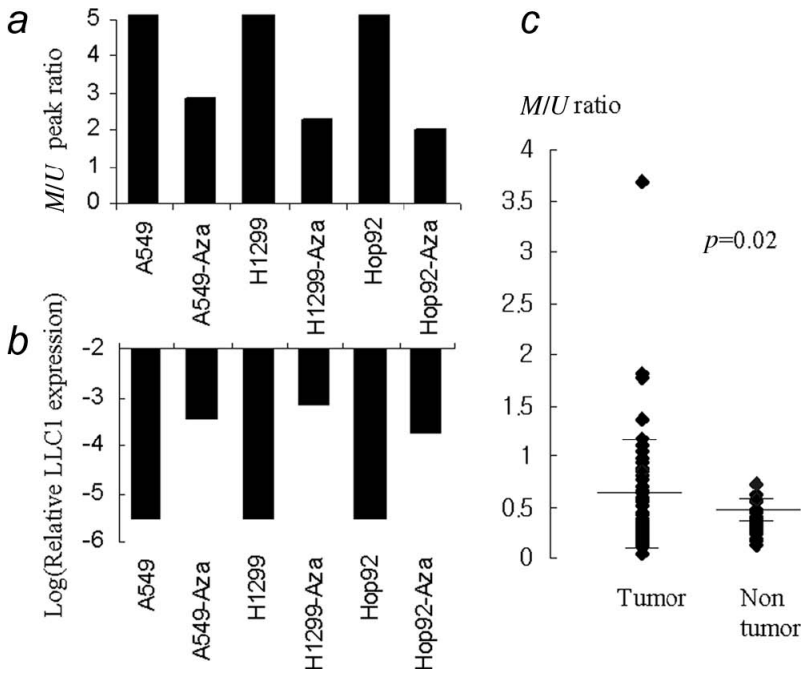

Figure 4.

The restoration of *LLC1* expression in NSCLC cell lines and methylation of *LLC1* promoter in primary lung cancers. (*a*) Promoter methylation status in A549, H1299 and HOP92 cells before and after treatment with 5-aza dC. (*b*) The expression of *LLC1* in indicated cell lines before and after 5-aza dC treatment. (*c*) The methylation of *LLC1* promoter in 67 primary NSCLC and 29 nontumor lung samples. *Y*-axis is the methylation/unmethylation peak ratio (*M/U*) from MSBE assay. The average *M/U* ratios for tumor and nontumor groups are indicated by the horizontal lines along with standard deviations. *p* value was determined by pair-wise *t* test.