Promoter Methylation of *DAL-1/4.1B* Predicts Poor Prognosis in Non–Small Cell Lung Cancer

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Abstract

Purpose: DAL-1/4.1B is an actin-binding protein originally identified as a molecule whose expression is down-regulated in lung adenocarcinoma. We have previously shown that a lung tumor suppressor, TSLC1, associates with DAL-1, suggesting that both proteins act in the same cascade. The purpose of this study is to understand the molecular mechanisms and clinical significance of DAL-1 inactivation in lung cancer.

Experimental Design: We studied aberration of the *DAL-1* in 103 primary non–small cell lung cancers (NSCLC) and 18 lung cancer cells. Expression and allelic and methylation status of DAL-1 was examined by reverse transcription-PCR, microsatellite analysis, and bisulfite sequencing or bisulfite single-strand conformational polymorphism, respectively.

Results: Loss of DAL-1 expression was strongly correlated with promoter methylation in lung cancer cells, whereas DAL-1 expression was restored by a demethylating agent, 5-aza-2′-deoxycytidine. The *DAL-1* promoter was methylated in 59 (57%) primary NSCLC tumors, 37% of which were associated with loss of heterozygosity around the *DAL-1* on chromosomal region 18p11.3. In squamous cell carcinomas, *DAL-1* methylation was observed in 9 of 10 tumors at stage I, whereas the incidence of methylation gradually increased in adenocarcinomas as they progressed [13 of 36 (36%), 4 of 12 (33%), 14 of 17 (82%), and 3 of 3 (100%) tumors at stages I, II, III, and IV, respectively; $P = 0.0026$]. Furthermore, in adenocarcinomas, disease-free survival and overall survival were significantly shorter in patients with tumors harboring the methylated *DAL-1* ($P = 0.0011$ and $P = 0.045$, respectively).

Conclusions: *DAL-1* methylation is involved in the development and progression of NSCLC and provides an indicator for poor prognosis.

Lung cancer is the most common cause of cancer death in developed countries, including the United States and Japan (1). Numerous studies have reported that genetic alterations, including mutations of the *K-ras* and *N-ras* genes, amplification of the *EGFR* and *CCND1* genes, and inactivation of the *TP53*, *RB1*, and *CDKN2A/p16* genes, as well as loss of heterozygosity (LOH) at chromosomal regions 3p, 11q, 13q, and 17p, were frequently observed in non–small cell lung cancer (NSCLC; ref. 2). Recently, epigenetic inactivation [e.g., the promoter methylation of various genes, including *p16* (3) and *RASSF1A* (4)] is also reported in NSCLC.

We have previously identified the tumor suppressor in lung cancer 1 (*TSLC1*) gene on chromosomal fragment 11q23.2 by functional complementation through the suppression of tumorigenicity in nude mice (5, 6). *TSLC1* encodes a membrane glycoprotein belonging to the immunoglubulin superfamily molecules (7) and participates in cell adhesion (8). Loss of TSLC1 expression was strongly correlated with the methylation of the gene promoter, and the hypermethylation of *TSLC1* was detected in 44% of primary NSCLC tumors, especially in those at advanced stages (9). We have recently shown that TSLC1 directly associates with DAL-1, a member of protein 4.1 family, and further interacts with the actin cytoskeleton at the cell-to-cell attachment site of epithelial cells (10).

The *DAL-1/4.1B* (*EPB41L3*) gene on 18p11.3 was initially isolated by differential-display PCR as a molecule whose expression was down-regulated in primary NSCLCs (11). DAL-1 belongs to the protein 4.1 family of molecules and is involved in the regulation of cytoskeleton through direct association with the actin filament. Reduced expression of DAL-1 was reported in ~50% of primary NSCLC tumors,

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whereas restoration of DAL-1 expression in NSCLC cell lines significantly suppressed cell growth *in vitro*. Furthermore, overexpression of DAL-1 impairs the motility of a rat schwannoma cell, RT4 (12). DAL-1 has also been shown to induce apoptosis and modulate cell attachment to a variety of extracellular matrices when introduced into a human breast cancer cell, MCF-7 (13). These findings suggest that *DAL-1* could be a candidate tumor suppressor in various cancers, especially NSCLC. However, the molecular mechanisms of *DAL-1* inactivation and its significance in primary NSCLC have not been elucidated.

In the present study, we investigated the expression, allelic status, and methylation status of the *DAL-1* gene in NSCLC cell lines and tumors and found that the *DAL-1* gene was methylated in 57% of primary NSCLC tumors.

Materials and Methods

Tumor samples and cell lines. One hundred and three primary NSCLC tumors and corresponding noncancerous tissues from the same patients were surgically resected and histologically diagnosed at National Cancer Center Hospital, Japan. After surgical removal, all samples were immediately frozen and stored at −135°C. Clinicopathologic data were extracted from the medical records as well as pathology reports. Four NSCLC cell lines (RERF-LC-MS, RERF-LC-OK, VMRC-LCD, and A431) and six SCLC cell lines (Lu135, SBC1, SBC2, SBC3, Lu139, and SBC5) were obtained from the Human Science Research Resources Bank (Osaka, Japan). Five NSCLC cell lines (Calu-3, NCI-H441, NCI-H522, SK-LU-1, and NCI-H596) and a SCLC cell line (N417) were from the American Type Culture Collection (Rockville, MD). Two NSCLC cell lines (A549 and PC-14) were from the RIKEN Cell Bank (Ibaraki, Japan). The cells were cultured according to the suppliers' recommendations. The analyses of human samples were carried out in accordance with the institutional guidelines.

Reverse transcription-PCR. Human adult lung poly-A^+ RNA was obtained from Clontech (Palo Alto, CA). Poly-A^+ RNA and total cellular RNA were extracted and reverse transcription-PCR (RT-PCR) were carried out as previously described (9). A *DAL-1* fragment of 153 bp was amplified using 0.5 μmol/L primers 5′-GTAGTGGTCCATAAAGAGACAGAGA-3′ and 5′-GATACAAGTCAGTTGGGTTAGAAGA-3′, whereas a β-actin fragment of 646 bp was amplified using 0.1 μmol/L primers 5′-AAATCTGGCACCACACCTT-3′ and 5′-AGCACTGTGTTGGCGTACAG-3′.

Bisulfite sequencing. Bisulfite sequencing was carried out as previously described (9, 14). Modified DNA (100 ng) treated with sodium bisulfite was subjected to PCR using a pair of primers, DAL-1PR1F (5′-GGGTTAAAGTTATTGGTATTGGTAGTTG-3′) and DAL-1PR1R (5′-CCCCACTCCGAAAAACGAAAAAATTACC-3′), to amplify a 337 bp DNA fragment (−263 to +74 bp from the transcription initiation site), including the promoter sequence of the *DAL-1*. The PCR products were subcloned to confirm the sequences in at least four clones. The ratio of methylation was defined by calculating the bisulfite-resistant cytosine residues out of 38 CpG sites in all independent subclones examined. Samples with hypermethylation, partial methylation, and unmethylation are those with methylation ratios above 50%, between 10% and 50%, and below 10%, respectively.

Bisulfite single-strand conformational polymorphism analysis. For single-strand conformational polymorphism (SSCP) analysis, the 92 bp fragments (−64 to +28 bp from the transcription initiation site) were amplified by PCR using a pair of primers, DAL-1PR2F (5′-CGGAGTTTCGGTGTTTTTTGTAATAGG-3′) and DAL-1PR2R (5′-GCGCCGCGACGTAAAAACTAAAC-3′), the former of which was end-labeled with Texas Red. The PCR product was heat denatured and subjected to electrophoresis using SF5200 (Hitachi Electronics Engineering, Tokyo, Japan) as previously described (9). The criterion for hypermethylation was met when the ratio of the methylated fragments to the unmethylated fragments (methylation ratio) was >0.4. Hypermethylation and partial methylation were not discriminated in the bisulfite SSCP analysis.

Loss of heterozygosity analysis. Five polymorphic DNA fragments on 18p11.3, namely *IMS-JST119847*, *IMS-JST031621*, *IMS-JST143134*, *IMS-JST067229*, and *IMS-JST082513*, were amplified by PCR and examined for LOH as previously described (9). LOH was defined when the allelic ratio in a tumor was over or under the range of 3 × SD from the average of allelic ratio in noncancerous lung tissues.

Restoration of* DAL-1 *expression by 5-aza-2′-deoxycytidine. Cells (1×10^5) were seeded on day 0, treated with 5-aza-2′-deoxycytidine (10 μmol/L) for 24 hours on days 2 and 5, and collected on day 8, as previously reported (15). Amplification by RT-PCR was carried out as described above.

Statistical analysis. Statistical analysis was carried out using Fisher's exact tests or the χ^2 test. Disease-free survival and overall survival were calculated using Kaplan-Meier log-rank testing.

Results

Correlation of promoter methylation and loss of expression of the* DAL-1 *gene in human lung cancer cell lines. We initially examined the *DAL-1* gene expression in human lung cancer cell lines. RT-PCR analysis revealed that 7 of 11 (64%) NSCLC cell lines and 1 of 7 (14%) SCLC cell lines completely lost DAL-1 expression (Fig. 1A). To investigate the molecular mechanisms of gene silencing of the *DAL-1*, we analyzed the methylation status of the 5′ region of the gene. A fragment of ~330 bp, corresponding to the promoter and exon 1 of the *DAL-1* gene, contained 38 CpG sites with a GC content of 75%, matching the criteria of the CpG island (Fig. 2A; ref. 16). Therefore, we determined the methylation status of these CpG sites by bisulfite sequencing in 11 NSCLC cell lines. Of the seven cells lacking DAL-1 expression, three cells (NCI-H441, RELF-LC-OK, and A431) showed hypermethylation in almost all CpG sites, whereas the other four cells (NCI-H596, A549, Calu-3, and PC-14) showed methylation of portions of the CpG sites, especially in the middle portion of the fragment (CpG sites 19-32). In contrast, the remaining four cells expressing DAL-1 (RERF-LC-MS, NCI-H522, SK-LU-1, and VMRC-LCD) showed unmethylation in the majority of the CpG sites. Whereas the relatively upstream region of the fragment (CpG sites 1-9) showed low-grade methylation in these four cells, the middle portion of the fragment (CpG sites 19-32) showed predominant unmethylation. These results suggest that methylation of the 14 CpG sites in the middle portion of the fragments (CpG sites 19-32) strongly correlates with loss of DAL-1 expression. On the other hand, the allelic status of chromosomal region 18p11.3 around the *DAL-1* gene was not correlated with DAL-1 expression (Fig. 2B).

Detection of the methylation state of the* DAL-1 *promoter by bisulfite single-strand conformational polymorphism. Next, we focused on the methylation status of the 92-bp fragment containing the 14 CpG sites described above. Bisulfite sequencing of only a limited number of clones does not always reflect the quantitative state of methylation. To overcome this problem, we subjected the bisulfite-treated DNA to SSCP analysis after amplification by PCR. A clone with complete methylation and one with complete unmethylation showed distinct mobility in bisulfite SSCP analysis (Fig. 2C). The 36 fragments of known sequences with different methylation state examined also presented distinct patterns of

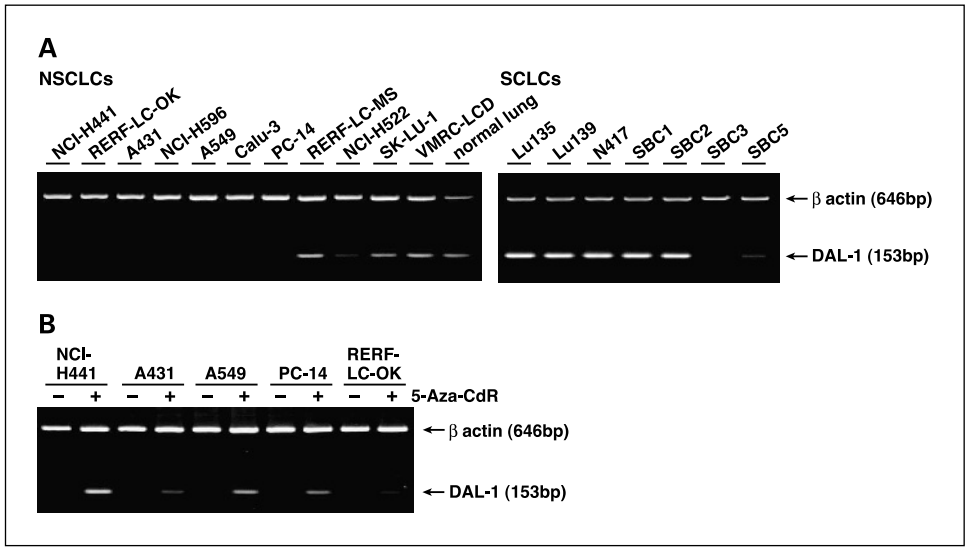


Fig. 1. Expression of the *DAL-1* gene in lung cancer cell lines. *A*, RT-PCR analysis of DAL-1 in NSCLC (*left*) and SCLC (*right*) cell lines. Poly-A$^+$ RNA from normal human lung tissues was also examined. *B*, restoration of DAL-1 expression by treatment with 5-aza-2′-deoxycytidine (*5-Aza-CdR*). cDNA fragments of DAL-1 and β-actin were amplified by the same RT-PCR reaction.

mobility from those of the completely methylated or unmethylated clones (Fig. 2C), although patterns of mobility were not always dependent on the number of methylated CpG (Fig. 2C, *clones II* and *III*). When we examined the DNA from NSCLC cells using this method, the results corresponded well to those obtained by bisulfute sequencing, where the cells with hypermethylation or partial methylation could be clearly distinguished from the cells with unmethylation (Fig. 2D). These results suggest that bisulfite SSCP provides a feasible and useful method to detect the status of methylation in the specific region of the *DAL-1* gene promoter. We subsequently analyzed seven SCLC cells and found that only SBC3 cells, which lack DAL-1 expression, showed hypermethylation. The causal involvement of the promoter methylation in the gene silencing of *DAL-1* was supported by the fact that DAL-1 expression was restored after treatment with a demethylating agent, 5-aza-2′-deoxycytidine, in five NSCLC cells lacking DAL-1 expression (Fig. 1B).

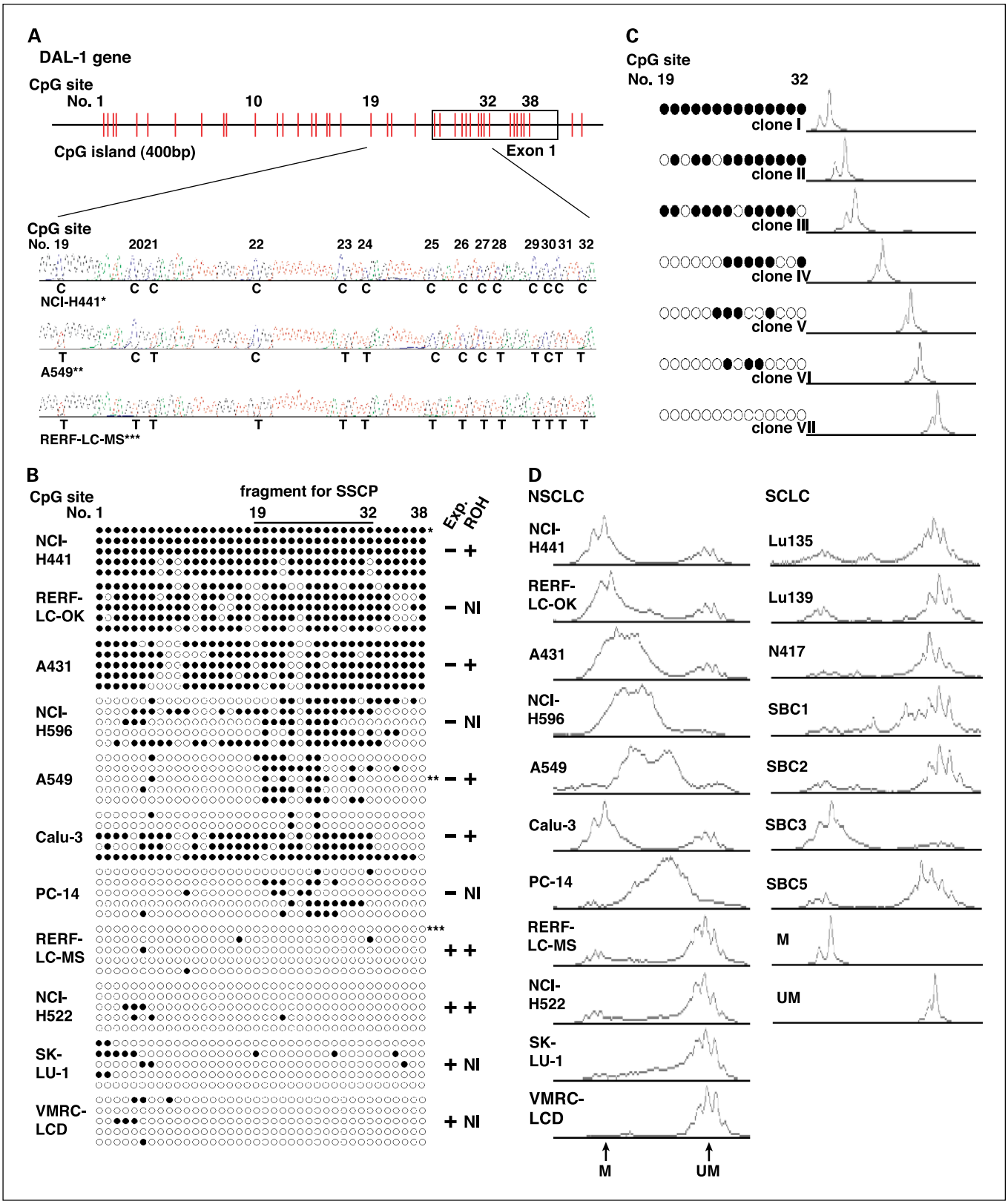
DAL-1 ***methylation in primary non–small cell lung cancer tumors.*** We then examined the methylation status of the 14 CpG sites of the *DAL-1* gene in 103 primary NSCLC tumors as well as noncancerous lung tissues from the same individuals by bisulfite SSCP. Representative results are shown in Fig. 3A, where signals defined as methylation were observed in L231C, L229C, and L292C, whereas only unmethylated signals were detected in L296C and L297C. The results of bisulfite sequencing of five independent clones in each sample, again, corresponded well to those of bisulfite SSCP. Similar analyses by bisulfite SSCP revealed that 59 of 103 tumors (57%) showed hypermethylation of the *DAL-1* gene. We also examined DAL-1 expression in a subset of samples by semiquantitative RT-PCR and found that all nine tumors with promoter methylation showed loss or marked reduction in the amount of DAL-1 expression, whereas five tumors without methylation expressed considerable amounts of DAL-1. Thus, promoter methylation seemed to be correlated with loss of DAL-1 expression in both cell lines and primary tumors of NSCLC (Fig. 3B).

LOH around the *DAL-1* locus on 18p11.3 was then analyzed using five polymorphic markers, and 39 of 100 (39%) informative cases of primary NSCLC showed LOH at least in one locus (Fig. 3C). Combinatorial analyses of LOH and promoter methylation showed that only 21 of 57 (37%) tumors with promoter methylation were associated with LOH on 18p11.3, whereas the remaining 36 (63%) tumors with methylation retained heterozygosity around the *DAL-1* locus, implying that the *DAL-1* gene could be inactivated by biallelic methylation.

Clinicopathologic features of tumors with a methylated **DAL-1** ***gene.*** The methylation status of the *DAL-1* gene was compared with the pathologic features of each NSCLC tumor (Table 1). *DAL-1* methylation was observed in all histologic subtypes of NSCLC, including 34 of 68 (50%) adenocarcinomas, 19 of 26 (73%) squamous cell carcinomas, 2 of 2 (100%) adenosquamous carcinomas, and 4 of 7 (57%) large cell carcinomas. The incidence of methylation tended to be higher in squamous cell carcinomas than in adenocarcinomas, although the difference was not statistically significant. A comparison with the pathologic stages of tumors revealed that *DAL-1* methylation seemed to be a relatively early event in squamous cell carcinomas because it was found in 9 of 10 tumors at stage I. In contrast, the incidence of *DAL-1* methylation in adenocarcinomas significantly increased as tumor stage advanced, and methylation was detected in 13 of 36 (36%), 4 of 12 (33%),

Fig. 2. Methylation status of the *DAL-1* gene promoter in lung cancer cell lines. *A*, schematic representation of the upstream region of the *DAL-1* gene is shown at the top. Vertical bars, CpG sites within the CpG island of the *DAL-1* gene. Open box, exon 1. The results of bisulfite sequencing in clones from three NSCLC cell lines are shown below. Sequence traces correspond to the 92 bp fragment containing the 14 CpG sites numbered 19 to 32. C and T indicate the nucleotides corresponding to the methylated and unmethylated cytosine residues at CpG sites, respectively. *B*, summary of the bisulfite sequencing in 11 NSCLC cell lines. The results of five independent clones in each cell line are shown. ●, methylated CpG sites; ○, unmethylated CpG sites. ***, clones whose sequences are presented in (*A*). Bold bar, 14 CpG sites that are further analyzed. The expression and allelic state of the *DAL-1* are summarized in the right. ROH, retention of heterozygosity; NI, not informative. *C*, SSCP analysis of the cloned DNA fragments of known sequences. Methylation status of the CpG sites, numbered 19 to 32, of each clone is shown on the left, whereas the signals in SSCP are shown on the right. *D*, bisulfite SSCP analysis of the *DAL-1* in NSCLC (*left*) and SCLC (*right*) cell lines. The results of completely methylated (*M*) and unmethylated (*UM*) fragments are shown at the bottom right side. Arrows, signals corresponding to completely methylated and unmethylated fragments.

14 of 17 (82%), and 3 of 3 (100%) tumors at stages I, II, III, and IV, respectively (P = 0.0026). Corresponding to these results, *DAL-1* methylation was preferentially observed in adenocarcinomas of pT_3 and pT_4 compared with those of pT_1 and pT_2 and in those of pN_1 to pN_3 compared with those of pN_0. These findings suggest that *DAL-1* inactivation could play distinct roles, at least in part, in the multistage tumorigenesis of squamous cell carcinomas and in that of adenocarcinomas.



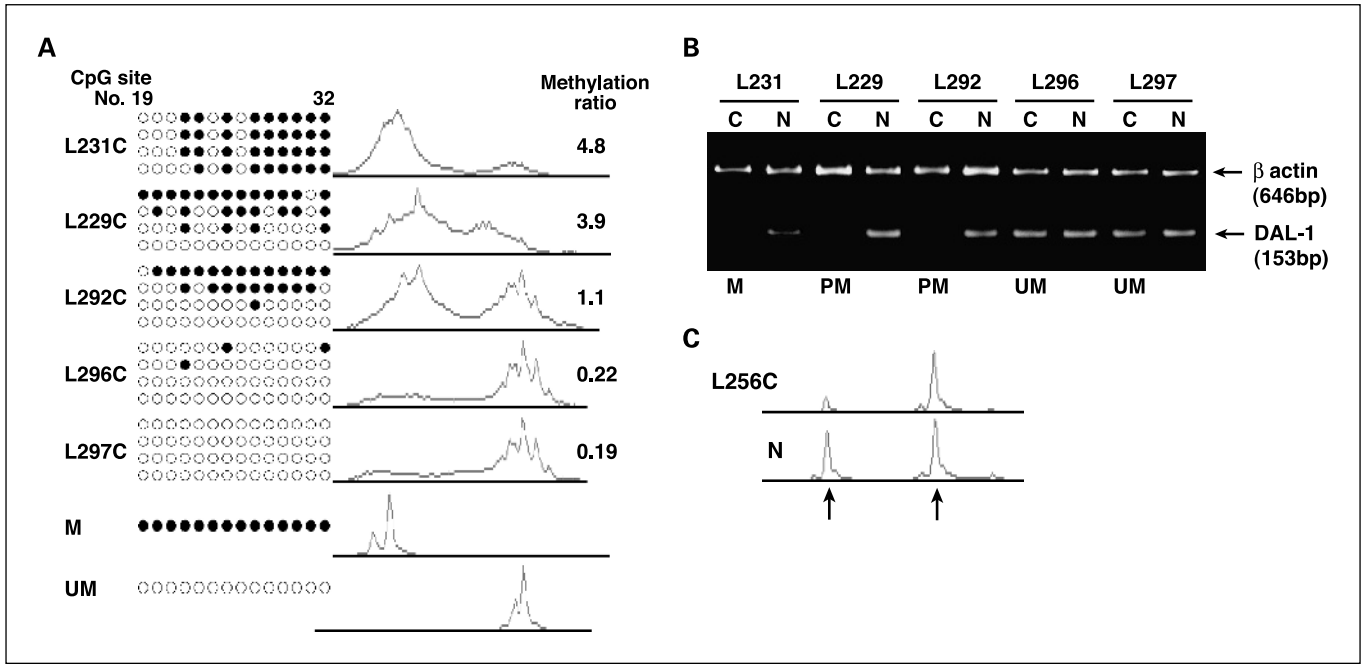


Fig. 3. Methylation, expression, and allelic state of the *DAL-1* promoter in primary NCSLC tumors. *A*, bisulfite SSCP analysis of the DNA fragments from primary NSCLC tumors. The sequences of four independent clones were determined in each sample and are schematically represented on the left. ●, methylated CpGs; ○, unmethylated CpGs. The results of completely methylated and unmethylated fragments are shown at the bottom. *B*, RT-PCR analysis of the *DAL-1* gene. C and N indicate DNA from cancerous and noncancerous portions of the lung, respectively. The methylation status in each tumor DNA is shown at the bottom. M, PM, and UM indicate hypermethylation, partial methylation, and unmethylation, respectively. *C*, LOH analysis at the polymorphic loci, *IMS-JST082513*, on 18p11.3. Arrows, signals corresponding to two polymorphic alleles determined by SSCP.

Furthermore, *DAL-1* methylation was correlated significantly with the degree of pleural involvement ($P = 0.045$). These results suggest that disruption of DAL-1 function would be implicated in tumor cell invasion. *DAL-1* methylation was also associated with the mitotic index ($P = 0.027$), one of the markers of cell proliferation, in adenocarcinomas. However, other clinical characteristics, including age, gender, and family history of the patients, were not correlated with *DAL-1* methylation (data not shown). In addition, no clinicopathologic parameters except for the mitotic index and histologic differentiation in adenocarcinomas were associated with LOH on 18p11.3 (Table 1).

Correlation with methylation and patient's prognosis. We finally investigated the correlation of *DAL-1* methylation in tumors with the disease-free survival of the patients using Kaplan-Meier analysis. When all subtypes of patients were examined, the methylation of the *DAL-1* gene was significantly associated with a shorter disease-free survival ($P = 0.045$). This association became apparent in adenocarcinomas ($P = 0.0011$), as shown in Fig. 4, but was not significant in squamous cell carcinomas ($P = 0.12$). *DAL-1* methylation also correlated with a shorter overall survival of the patients in adenocarcinomas ($P = 0.045$) but not correlated with that in squamous cell carcinomas ($P = 0.14$).

Discussion

A candidate lung tumor suppressor gene, *DAL-1*, on 18p11.3 was initially isolated by Tran et al. (11) using differential display analysis of primary adenocarcinoma of the lung. DAL-1 expression was observed in a variety of tissues, including lung, whereas its expression was greatly reduced in more than half of NSCLC tumors (11) or meningiomas (17). Although LOH on 18p11.3 was reported in ~40% of NSCLCs (18) as well as 60% to 70% of meningiomas (17) or breast cancers (19), a subsequent study showed that LOH in this region was not correlated with loss of DAL-1 expression (17). Moreover, mutational screening failed to identify inactivating mutations of the *DAL-1* gene (17, 20). Because many tumor suppressor genes carrying the CpG islands in their upstream regions are often silenced by epigenetic mechanisms, we examined the status of the promoter methylation of the *DAL-1* gene in the present study.

First, we determined the methylation status of 38 CpG sites around the promoter and exon 1 of the *DAL-1* gene in 11 NSCLC cells by bisulfite sequencing and found that the segmental methylation at the 14 CpG sites within the upstream region of the transcriptional start site and the beginning of exon 1 was strongly correlated with loss of expression. Complementary experiments using a methyltransferase inhibitor, 5-aza-2′-deoxycytidine, induced the reexpression of the *DAL-1* gene, providing supportive evidence that hypermethylation of this region of 92 bp is involved in gene silencing of *DAL-1*. In other words, the methylation state of the remaining regions at the 5′ and 3′ of the 14 CpG sites is not directly related to DAL-1 expression. These findings suggest that the relatively short fragment of 92 bp would play a critical role in silencing of the *DAL-1* gene, probably through the binding of some methyl-CpG–specific binding proteins instead of complexes of transcriptional factors. We checked the putative binding sites of transcription factors and found those of Sp1, although the

biological significance of these binding sites needs to be clarified in future studies.

For the detection of the methylation status of the 14 CpG sites, we carried out bisulfite SSCP in addition to bisulfite sequencing. One of the advantages of the former method is its rapid and easy procedure, which is convenient for screening of a large number of samples. Another, more important, advantage is that bisulfite SSCP can detect the quantitative status of methylation based on its sensitive detection. It is, therefore, particularly useful when primary tumors are to be analyzed because aberrant methylation in tumor cells is often underestimated owing to inevitable contamination of the infiltrating leukocytes as well as the stromal cells. In fact, it is difficult to evaluate the low frequency of methylated clones by bisulfite sequencing of a limited number of the cloned DNA as previously discussed (9).

Using bisulfite SSCP, we detected a high incidence of methylation of the *DAL-1* promoter (59 of 103; 57%) in primary NSCLC tumors. *DAL-1* methylation was also found in a subset of SCLC cells. Therefore, the methylation and gene silencing of the *DAL-1* seem to be involved in all histologic subtypes of human lung cancer. However, the pathobiological significance of *DAL-1* methylation in multistage carcinogenesis might be different between squamous cell carcinomas and adenocarcinomas. *DAL-1* methylation seemed to be a relatively early event in squamous cell carcinomas because the incidence was 90% in tumors at stage I but did not essentially change in tumors of more advanced stages. In contrast, *DAL-1* methylation would be a late event in adenocarcinomas because the incidence increased significantly as tumor-stage advanced from stage I to stage IV ($P = 0.0026$). It is noteworthy that mutant p53 proteins were also reported to be an early event in squamous cell carcinomas but a late event in adenocarcinoma (21). Detailed analyses of DAL-1 expression by immunohistochemical studies in NSCLC tumors as well as its precursor lesions, such as atypical adenomatous hyperplasia, would be required to elucidate the pathologic function of DAL-1 in the multistage tumorigenesis of NSCLC. The incidence of *DAL-1* methylation as well as that of LOH on 18p11.3 also increased significantly in adenocarcinomas with higher mitotic indices, suggesting that DAL-1 could also be implicated in the suppression of cell proliferation. This would correspond well with previous reports of findings that the restoration of DAL-1 expression into its deficient cells from NSCLC, meningioma,

Table 1. Promoter methylation and allelic status of the *DAL-1* gene and clinicopathologic characteristics of primary NSCLCs

Characteristics	No. of tumors examined	No. of tumors with *DAL-1* methylation (%)		No. of tumors examined	No. of tumors with LOH on 18p11.3 (%)	
Total	103	59 (57)		100	39 (39)	
Age						
≤69	60	35 (58)		58	23 (40)	
≥70	43	24 (56)	NS	42	16 (38)	NS
Histological differentiation						
Adenocarcinoma	68	34 (50)		66	26 (39)	
Well differentiated	22	12 (55)		21	4 (19)	
Moderately differentiated	36	16 (44)	NS	35	16 (46)	$P = 0.050^*$
Poorly differentiated	10	6 (60)		10	6 (60)	
Squamous cell carcinoma	26	19 (73)	NS	25	8 (32)	NS
Well differentiated	6	5 (83)		6	4 (67)	
Moderately differentiated	17	11 (65)	NS	16	3 (19)	NS
Poorly differentiated	3	3 (100)		3	1 (33)	
Adenosquamous carcinoma	2	2 (100)		2	2 (100)	
Large cell carcinoma	7	4 (57)		7	3 (43)	
Adenocarcinoma						
pTNM classification						
pT1	26	9 (35)		25	10 (40)	
pT2	30	15 (50)		30	12 (40)	
pT3	3	3 (100)		3	1 (33)	
pT4	9	7 (78)	$P = 0.041^*$	8	3 (38)	NS
pN0	39	15 (38)		38	15 (39)	
pN1	13	5 (38)		13	5 (38)	
pN2	15	13 (87)		14	5 (36)	
pN3	1	1 (100)	$P = 0.0080^*$	1	1 (100)	NS
pM0	65	31 (48)		64	25 (39)	
pM1	3	3 (100)	NS	2	1 (50)	NS

Table 1. Promoter methylation and allelic status of the *DAL-1* gene and clinicopathologic characteristics of primary NSCLCs (Cont'd)

Characteristics	No. of tumors examined	No. of tumors with *DAL-1* methylation (%)		No. of tumors examined	No. of tumors with LOH on 18p11.3 (%)	
Stage						
Ia	18	4 (22)		17	7 (41)	
Ib	18	9 (50)		18	7 (39)	
IIa	3	1 (33)		3	1 (33)	
IIb	9	3 (33)		9	4 (44)	
IIIa	10	9 (90)		10	3 (30)	
IIIb	7	5 (71)		7	3 (43)	
IV	3	3 (100)	$P = 0.0074$ *,†	2	1 (50)	NS
Mitotic index						
1	31	10 (32)		31	7 (23)	
2	32	21 (66)		30	15 (50)	
3	5	3 (60)	$P = 0.027$*	5	4 (80)	$P = 0.014$*
Squamous cell carcinoma						
pTNM classification						
pT1	4	3 (75)		3	1 (33)	
pT2	19	13 (68)		19	7 (37)	
pT3	3	3 (100)	NS	3	0 (0)	NS
pT4	0	0		0	0	
pN0	12	11 (92)		11	4 (36)	
pN1	10	6 (60)		10	2 (20)	
pN2	4	2 (50)	NS	4	2 (50)	NS
pN3	0	0		0	0	
pM0	26	19 (73)		25	8 (32)	
pM1	0	0		0	0	
Stage						
Ia	4	3 (75)		3	1 (33)	
Ib	6	6 (100)		6	3 (50)	
IIa	0	0		0	0	
IIb	11	7 (64)		11	2 (18)	
IIIa	5	3 (60)	NS	5	2 (40)	NS
IIIb	0	0		0	0	
IV	0	0		0	0	
Pleural involvement of all NSCLC						
p0	48	22 (46)	$P = 0.045$ ‡	48	14 (29)	NS
p1	30	21 (70)		28	13 (46)	
p2	14	7 (50)		13	8 (62)	
p3	11	9 (82)		11	4 (36)	

Abbrevation: NS, not significant.
*P was calculated by χ^2 test.
†$P = 0.0026$ when tumors in stage I, II, III, and IV were analyzed.
‡P was calculated by Fisher's exact test.

or breast cancer significantly suppressed cell growth *in vitro* (11–13). In this connection, it is noteworthy that DAL-1 directly associates with 14-3-3 molecules both *in vivo* and *in vitro* although its significance in the regulation of cell growth is controversial (22, 23).

Preferential methylation of the *DAL-1* was also observed in lung adenocarcinomas with invasion to the surrounding tissues, metastasis to the lymph nodes, and involvement into the pleura in this study. We are particularly interested in these findings because they could suggest that the disruption of DAL-1 is involved in the invasion or metastasis of cancer cells. The principal role of DAL-1 seemed to be linking the actin cytoskeleton to the plasma membrane. Gutmann et al. (12) showed the expression of DAL-1 impaired cell motility

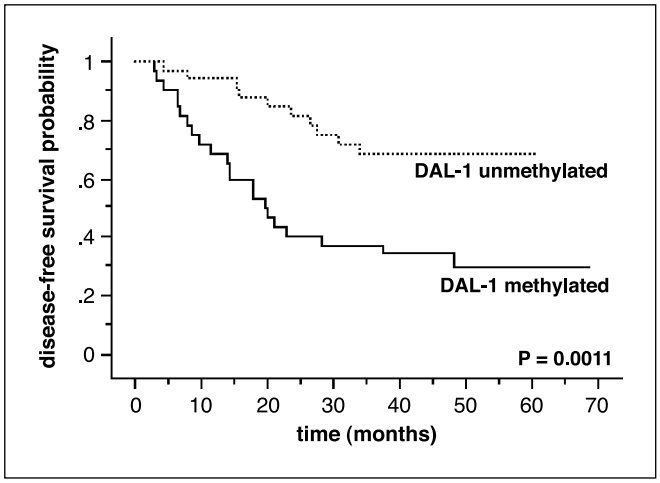


Fig. 4. Disease-free survival of 68 patients with adenocarcinoma of the lung. Kaplan-Meier log-rank test was used to examine the correlation of the *DAL-1* methylation in tumors with the disease-free survival of the patients.

by disrupting actin cytoskeleton in schwannoma cells. DAL-1 expression also enhanced the attachment of cells to the extracellular matrices in breast cancer cells (13). Furthermore, we have previously shown that DAL-1 directly interacts with another tumor suppressor, TSLC1, a cell-surface adhesion molecule (10) whose inactivation is observed in a variety of tumors, including NSCLC, breast cancer, and meningiomas, as they progress (9, 24, 25). Taken together, these results strongly suggest that DAL-1 is involved in cell-to-cell attachment and suppression of cell motility, whereas loss of its function could lead cancer cells to invasion or metastasis. In fact, the significant correlation of *DAL-1* methylation in tumors with a shorter disease-free survival ($P = 0.0011$) and a shorter overall survival ($P = 0.045$) was observed for the patients with lung adenocarcinoma. Thus, DAL-1 methylation would provide a potential biomarker of prognosis in patients with NSCLC, especially in those with lung adenocarcinoma.

Finally, comparison of the results of *DAL-1* methylation with those of *TSLC1* methylation in 48 primary NSCLC tumors examined in the previous study (9) revealed that 29 (60%) and 21 (44%) tumors showed hypermethylation of the *DAL-1* and *TSLC1*, respectively, and that total of 39 of 48 (81%) primary NSCLC tumors represented epigenetic inactivation of either the *TSLC1* or the *DAL-1* gene. Taking into consideration that DAL-1 and TSLC1 act in the same cascade of tumor suppression, these results strongly suggest that the aberration of the TSLC1 – DAL-1 cascade is deeply involved in the majority of primary NSCLC tumors.

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