Homozygous deletion and reduced expression of the *DOCK8* gene in human lung cancer

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Abstract. A homozygous deletion of the DOCK8 (dedicator of cytokinesis 8) locus at chromosome 9p24 was found in a lung cancer cell line by array-CGH analysis. Cloning of the full-length *DOCK8* cDNA led us to define that the *DOCK8* gene encodes a protein consisting of 2,099 amino acids. *DOCK8* was expressed in a variety of human organs, including the lungs, and was also expressed in type II alveolar, bronchiolar epithelial and bronchial epithelial cells, which are considered as being progenitors for lung cancer cells. *DOCK8* expression was reduced in 62/71 (87%) primary lung cancers compared with normal lung tissue, and the reduction occurred irrespective of the histological type of lung cancer. 5-aza-2'-deoxy-cytidine and/or Trichostatin A treatments induced *DOCK8* expression in lung cancer cell lines with reduced *DOCK8* expression. Therefore, epigenetic mechanisms, including DNA methylation and histone deacetylation, were indicated to be involved in *DOCK8* down-regulation in lung cancer cells. Further screening revealed homozygous deletions of the *DOCK8* gene in a gastric and a breast cancer cell line. DOCK family proteins have been shown to play roles in regulation of migration, morphology, adhesion and growth of cells. Thus, the present results suggest that genetic and epigenetic inactivation of DOCK8 is involved in the development and/or progression of lung and other cancers by disturbing such regulations.

Introduction

It has been widely accepted that inactivation of tumor suppressor genes plays important roles in the development and/or progression of lung cancer (1,2). The *p53*, *RB* and *p16* tumor suppressor genes are frequently inactivated in lung cancer cells by chromosome deletions, mutations and/or epigenetic mechanisms. These alterations are thought to contribute to immortalization and autonomous growth of lung cancer cells by disturbing the signaling pathways regulating cell growth and death. In addition, inactivation of a few other (candidate) tumor suppressor genes, such as *PTEN*, *LKB1*, *RASSF1A* and *MYO18B*, was detected in a subset of lung cancer. The biological significance of these alterations has not been fully understood; however, it is considered that the disturbance of several signaling pathways by these alterations affects some biological properties of cancer cells, such as abilities of growth, survival and invasion (3-5). It is known that lung cancer cells carry deletions at a number of chromosomal loci in addition to the tumor suppressor gene loci described above (6). This suggests that these loci harbor genes whose inactivation contributes to lung carcinogenesis, and the identification of such genes is indispensable to the understanding of the molecular mechanisms of human lung carcinogenesis.

The microarray-based comparative genomic hybridization (array-CGH) method has been proved to be an effective tool to detect and map genomic regions deleted in lung cancer cells (7-10). We recently undertook a homozygous deletion search in 29 lung cancer cell lines by array-CGH analysis and found homozygous deletions at the *CBP* (cyclic AMP response element binding protein-binding protein) gene locus at 16p13 (11). Subsequent analyses revealed that *CBP* is deleted and mutated in a subset of lung cancers suggesting that *CBP* functions as a lung tumor suppressor gene (11). In the course of the array-CGH analysis on lung cancer cell lines, we detected a homozygous deletion at chromosome 9p24 in a lung cancer cell line. The deletion was discontinuous with the homozygous deletion at the *p16* locus at 9p21 in the cell line (Fig. 1), and removed the *DOCK8* gene encoding a member of DOCK (dedicator of cytokinesis) family proteins (12). Previous studies showed that hemizygous deletions at 9p regions distal to the p16 locus have frequently occurred in lung cancer cells (13,14). Several DOCK proteins, such as DOCK1, 2, 3, 4, 9 and 11, are shown to function as guanine

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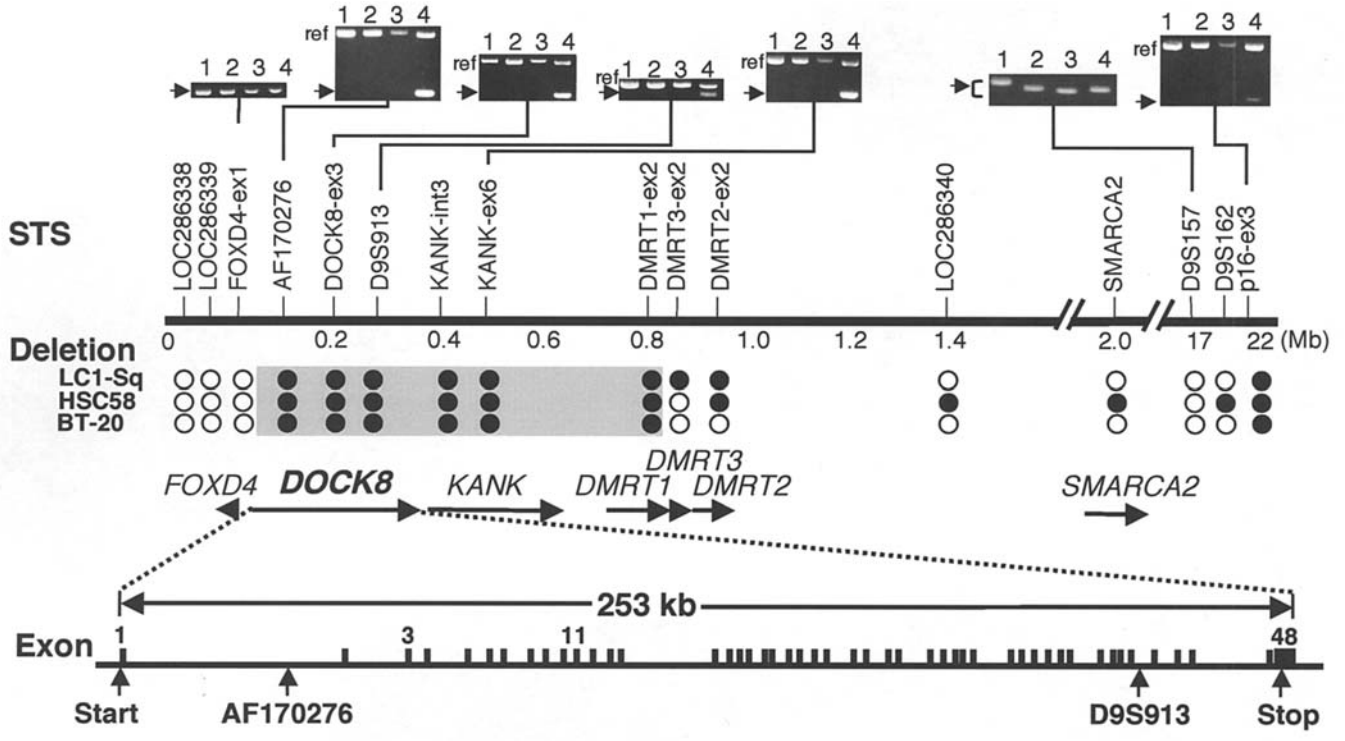

Figure 1. Homozygous 9p24 and 9p21 deletions in human lung and other cancer cell lines. STSs used for genomic PCR in this study are indicated on the map. Top, results of genomic PCR analysis. Lane 1, LC1-Sq (lung cancer); lane 2, HSC58 (gastric cancer); lane 3, BT-20 (breast cancer); lane 4, normal lung. ◦, retained; •, homozygously deleted. Gray region, a commonly deleted region at 9p24 in three cell lines.

nucleotide exchange factors (GEF) to regulate small GTPase proteins, such as Rac1, Cdc42, Rap1, and regulate migration, morphology, adhesion and growth of the cell (15-18). Recently, it was reported that *Dock4* was inactivated by homozygous deletion in a osteosarcoma generated in a *Tp53*+/- *Nf2*+/- mouse, and *DOCK4* was mutated in several human cancer cell lines (18). Restoration of *Dock4* in mouse osteosarcoma cells suppressed growth and invasion; therefore, it was indicated that *Dock4*/*DOCK4* functions as a tumor suppressor gene (18). Although the physiological and biological functions of *DOCK8* were unclear, the findings above prompted us to examine genetic alteration and the expression status of *DOCK8* in lung cancer to investigate the involvement of *DOCK8* alterations in human lung carcinogenesis.

Materials and methods

Samples. Sixty-one lung cancer cell lines [18 small cell lung carcinoma (SCC), 26 adenocarcinoma (ADC), 10 squamous cell carcinoma (SQC), 5 large cell carcinoma (LCC), and 2 adenosquamous cell carcinoma (ASC)], 18 gastric cancer, 14 breast cancer, 20 esophageal cancer, 12 colon cancer, 10 renal cell cancer, 9 glioma, and 2 pancreatic cancer cell lines were used in this study. Detailed information of cell lines used in this study can be obtained upon request. In six SCLCs and seven NSCLCs, corresponding lymphoblast cells were available. One hundred and seven macro-dissected primary lung tumors (15 SCCs, 56 ADCs, 30 SQCs, 4 LCCs, and 2 ASCs) and adjacent non-cancerous tissue pairs were obtained from lung cancer patients who were treated at the National Cancer Center Hospital, Tokyo. High-molecular-weight DNA was obtained from all cancer cell lines, and 95 of the 107 primary tumor and normal lung tissue pairs. PolyA RNA was obtained from all 61 lung cancer cell lines. Total RNA was obtained from 71 of the 107 primary tumors (46 ADCs, 21 SQCs, 3 LCCs and a SCC) and 7 normal lung tissues. Total RNA was also obtained from 3 samples of type II alveolar cell, bronchiolar epithelial cell and bronchial epithelial cell; and 5 samples of ADC and SQC, all of which were obtained by microdissection using the LM200 LCM System (Arcturus Engineering, Mountain View, CA), according to the protocol described (19). mRNA normal lung tissue was purchased from Clontech (Mountain View, CA) and used for *DOCK8* cDNA cloning and also as a control in the assessment of *DOCK8* expression as described below. Total RNA and mRNA were reverse-transcribed to cDNA as previously described (11,19).

Array-CGH analysis. Genome copy number changes in lung cancer cells were assessed by a DNA array, Genosensor Array 300 (Visis, IL) covering 287 loci containing frequently amplified or deleted loci in a variety of cancers according to the procedure previously reported (11,20).

Homozygous deletion analysis. Homozygous deletions were examined by multiplex PCR using the *IRF1* locus on chromosome 5q as a reference as described previously (11). Primers and PCR conditions are available on request. If no PCR products were detected for the target locus, the locus was defined as being homozygously deleted.

Cloning of the full length DOCK8 cDNA. The first exon of the *DOCK8* gene was predicted based on the information of expressed sequence tags (ESTs) in the NCBI database and a CpG island detected using an algorithm at www.ebi.ac.uk/

emboss/cpgplot/. The full-length cDNA was amplified by PCR against normal lung cDNA. The obtained PCR product was directly sequenced in both directions using a Big Dye Terminator Sequencing kit and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). Transcription start sites were determined by 5'-RACE using a GeneRacer kit (Invitrogen, Carlsbad, CA). For 5'-RACE, a primer (5'-CCT GTTGATCTTGAGCGCGAACGC-3') and a nested primer (5'-TCTGCGCTCGGCAGAGTGGCCAT-3') were used. Obtained PCR products were subcloned into pGEM-T Easy Vector (Promega, San Luis Obispo, CA). Forty-eight clones were randomly selected and subjected to direct sequencing.

Subcellular localization of DOCK8 proteins. The coding region (nt 78-6413) of *DOCK8* cDNA was amplified by PCR from normal lung cDNA and was cloned into the *Not*I site of the pLPCX plasmid (Clontech). A Kozak rule-matched HA-tag sequence was fused to DOCK8 protein at the N-terminus. This construct was designated as pLPCX-HA-DOCK8. The sequence of the cDNA insert was confirmed by sequencing. NIH3T3 cells were cultured in the DMEM medium with heat-inactivated 10% fetal bovine serum, penicillin and streptomycin. pLPCX-HA-DOCK8 plasmid DNA was transfected into NIH3T3 cells using a Lipofectamine PLUS reagent (Invitrogen). After 24 h, the cells were fixed with 4% paraformaldehyde, permeabilized in 0.2% Triton X-100 in PBS, and incubated with the anti-HA 3F10 antibody (Roche Applied Science, Indianapolis, IN) for 90 min at room temperature. Cy3-conjugated goat anti-rat IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) and rhodamine-phalloidine were used for visualization of *DOCK8* and filamentous actin proteins, respectively, under a fluorescence confocal microscope, Radiance2000 (Bio-Rad, Hercules, CA).

Real-time quantitative PCR (RTQ-PCR) analysis. Expression of *DOCK8* was measured by RTQ-PCR using an ABI PRISM 7900HT (Applied Biosystems). Sequences of the Taqman probe and primer sets were as follows: 5'-AGAAAAACAAG CGTCTCATCACGGCAGA-3' (probe), 5'-GAGGTTATGC TTTAAGGAATTCATCA-3' (forward primer) and 5'-GATC ATTGGCCTGAGGTTCTC-3' (reverse primer). For macrodissected samples, the expression of *DOCK8* was normalized to RNA content for each sample by using *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) as an internal control. For microdissected samples, *ATP5F1* (ATP synthase, H^+ transporting, mitochondrial F0 complex, subunit b, isoform 1) was used as an internal control, since this gene showed less variable expression levels in microdissected lung epithelial cells (19). PCR was performed in a single tube in duplicate. Results were expressed as the average of these two independent tests. The expression levels of *DOCK8* relative to those of *GAPDH* or *ATP5F1* were shown after adjusting the level of *DOCK8* expression in normal lung tissue (values for the normal lung tissue mRNA purchased from Clontech) to 1.0.

Mutation analysis. Forty-seven coding exons of the *DOCK8* gene, i.e. exons 2 to 48, were amplified from 50 ng of genomic DNA from 61 lung cancer cell lines and 95 surgical specimens by PCR using 47 sets of primers. PCR products from cell lines were subjected to mutation search by direct sequencing using a Big Dye Terminator Sequencing kit and an ABI PRISM 3700 Genetic Analyser (Applied Biosystems). PCR products from surgical specimens were subjected to mutation search by WAVE analysis as described (11).

DNA methylation analysis. Genomic DNA was treated according to the urea/bisulfite method (21). PCR was performed with three sets of primers covering the *DOCK8* CpG island. Sequences of primer pairs used were as follows: 5'-GTA GAGTGGTTATGGTT-3' and 5'-CCCTTAATAACACCTA ACA-3'; 5'-GTTGAGGATATTTAGGAGTAT-3' and 5'-AAC CATAACCACTCTAC-3'; and 5'-GTGTTTTTGGATGTTT TTAGT-3' and 5'-ACCACCAAAAACCAAACTCTTTC-3'. PCR products were purified and subcloned into the pGEM-T easy vector (Promega). Six clones were randomly selected and subjected to direct sequencing.

5-aza-2'-deoxycytidine (5-aza-dC) and/or Trichostatin A (TSA) treatment. Lung cancer cell lines were cultured in the RPMI-1640 medium with heat-inactivated 10% fetal bovine serum, penicillin and streptomycin, and treated with 5-aza-dC and TSA as described (5). Briefly, for the first 48 h, cells were incubated with media containing or not containing 10 μM 5-aza-dC (Sigma) and then for another 24 h with or without 1.0 μM TSA (Wako, Tokyo). Total RNA was isolated using an RNeasy minikit (Qiagen) and was subjected to RTQ-PCR. A relative expression of >3 compared with basal levels was defined as being induced.

Results

Homozygous deletions at 9p24 in a human lung cancer cell line. Signal intensities at two markers, AF170276 and D9S913, at chromosome 9p24 were decreased (relative copy number <0.4) in a lung SQC cell line, LC1-Sq, in array-CGH analysis. Genomic PCR analysis revealed that the AF170276 and D9S913 loci were homozygously deleted in LC1-Sq cells (Fig. 1). Further genomic PCR analysis of several sequence tag sites (STSs) revealed that the deleted region in LC1-Sq is approximately 1-Mb in size and contained 5 genes, *DOCK8* (dedicator of cytokinesis 8), *KANK* (kidney ankyrin repeat protein) and *DMRT1-3* (doublesex and mab-3 related transcription factor 1-3). LC1-Sq cells also had a homozygous deletion at the *p16* locus at chromosome 9p21; however, the deletion was not contiguous with the one at 9p24. Homozygous deletions at these STSs were further searched for by genomic PCR analysis in 60 lung cancer cell lines other than LC1-Sq; however, additional homozygous deletions were not detected.

The structure of the DOCK8 gene. Two forms of *DOCK8* transcripts with differences in the N-terminal region, which encoded polypeptides of 2,031 and 1,701 amino acids, respectively, were cloned previously (12). However, the first exons (corresponding to exons 3 and 11 in Fig. 1) for these two forms were not located near the CpG island, and the nucleotide sequences surrounding the first ATG codons for the two forms did not meet the Kozak rule (22). Thus, it was possible that

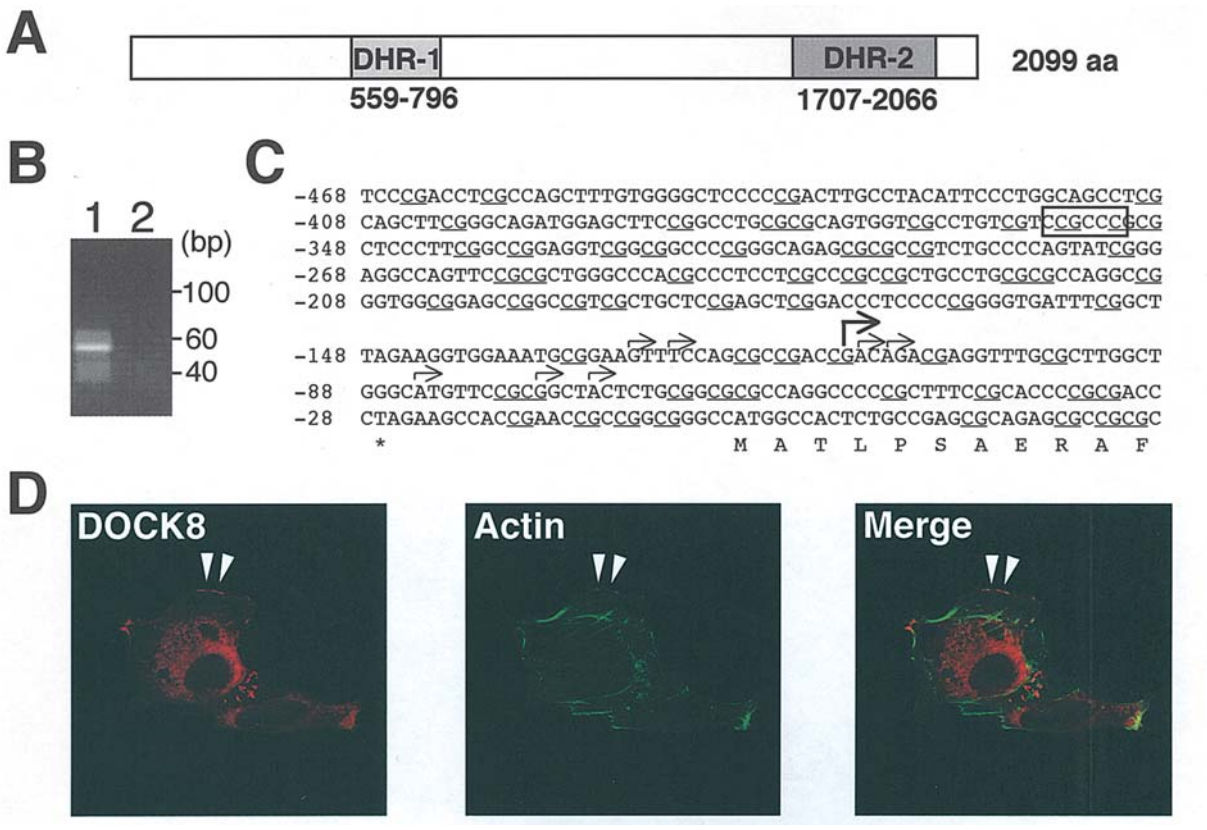

Figure 2. Cloning of the full-length *DOCK*8 cDNA. (A) The structure of DOCK8 protein. (B) An electrophoregram of the 5'-RACE products. Lane 1, normal lung cDNA; lane 2, no template. (C) Nucleotide sequence of the region containing transcription start sites of the *DOCK8* gene. CpG sites 1-62 are underlined. A thick arrow indicates the major transcription start site and other arrows indicate minor transcription start sites. A GC box in this region is indicated by an open box. Amino acids translated from the first ATG are shown below with an in-frame stop codon (asterisk) upstream of the ATG. (D) Subcellular localization of HA-tagged DOCK8 protein. DOCK8 protein was detected by an HA-specific mouse antibody followed by a FITC-labelled anti-mouse antibody, while filamentous actin was detected by rhodamine-phalloidine. Arrowheads mark lamellipodia containing HA-tagged DOCK8 protein.

these two forms were 3' portions of a longer *DOCK8* transcript. Analysis of the human genome sequence containing the *DOCK8* locus led us to identify a CpG island >60-kb upstream of the previously identified exons, and an exon was indicated to be present in the CpG island since five ESTs (Genbank accession no: BE326843, BP275915, BI560578, CF995096 and CB989814) were mapped in this region. RT-PCR against lung mRNA using a primer in the newly suggested exon and a primer in the last exon (exons 1 and 48, respectively, in Fig. 1) produced a single PCR product. Direct sequencing of the product revealed that this cDNA fragment was of 6,336 bp and contained an open reading frame of 6,297 bp encoding 2,099 amino acids (Fig. 2A). The deduced DOCK8 protein contained DHR-1 and DHR-2 (DOCK homology region-1 and -2, respectively) domains conserved in DOCK family proteins, while no other functional domains were found (Fig. 2A). The nucleotide sequence surrounding the first ATG codon matched the Kozak rule and an in-frame stop codon was present upstream of the ATG codon. Transcription start sites were determined by 5'-RACE against normal lung mRNA (Fig. 2B). Cloning and sequencing of the RACE product clones allowed us to define a major start site and seven minor start sites (Fig. 2C). Thus, the major *DOCK8* transcript (i.e., from the nucleotide at the major start site to the nucleotide before the polyA tract) was determined as 7,448 bp in size (deposited in Genbank with the accession no. AB191037). The two forms of the transcripts previously determined corresponded to the 3' portions of this newly identified form; thus, we concluded that the transcript identified in this study is the full-length one. A GC box sequence was present upstream of the transcription start sites, while TATA-box was absent (Fig. 2C). Northern blot analysis with the full-length cDNA probe detected a single band of 8 kb for mRNAs from a variety of adult and fetal organs (data not shown), as previously reported (12).

The full-length DOCK8 protein tagged with HA at the N-terminal was expressed in NIH3T3 cells, and the subcellular localization of the DOCK8 protein was examined by immunofluorescence analysis using an anti-HA antibody together with rhodamine-phalloidin to label filamentous actin (Fig. 2D). DOCK8 protein was detected in protrusions at cell edges, i.e. lamellipodia, and also in cytoplasm, and such localization was similar to that of DOCK8 protein lacking the N-terminal 68 amino acids produced from a shorter cDNA clone (12).

Mutation and expression of the DOCK8 gene in lung cancer cells. Ninety-five primary lung tumors and 60 lung cancer cell lines other than LC1-Sq were examined for mutations and homozygous deletions of *DOCK8* coding exons. Homozygous deletions were not observed in these samples. Somatic mutations were not detected in the 95 primary tumors, while five base substitutions associated with amino-acid substitutions were detected in five cell lines; Ala889Val (homozygous

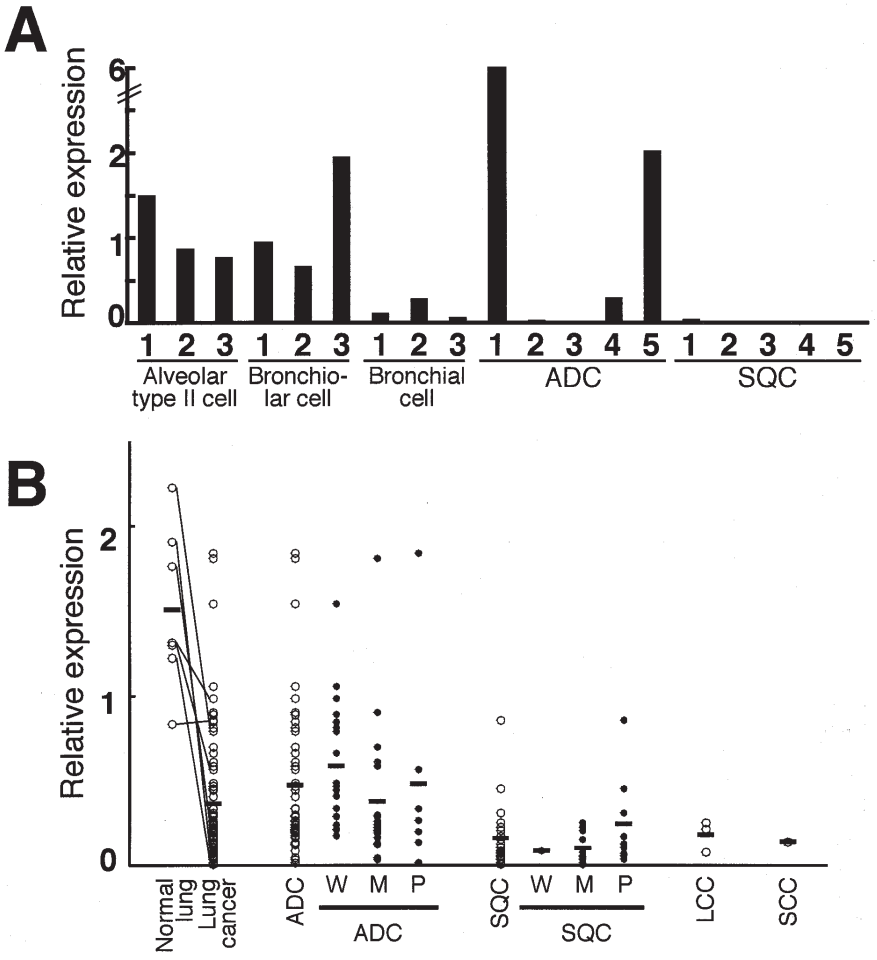

Figure 3. Expression of the *DOCK8* gene in cancerous and non-cancerous lung cells. (A) QRT-PCR analysis of microdissected lung cells. Three samples of type II alveolar cell, bronchiolar epithelial cell and bronchial epithelial cell; and five samples of ADC and SQC were analyzed. The expression levels of *DOCK8* are shown after adjusting the level in whole lung tissue to 1.0. (B) QRT-PCR analysis of macrodissected lung cells. The expression levels in 7 normal lung tissues, total 71 lung tumors, total 46 ADCs; 19 well (W), 19 moderately (M) and 7 poorly (P) differentiated ADCs; total 21 SQCs; 1 well (W), 11 moderately (M) and 9 poorly (P) differentiated SQCs; 3 LCCs, and 1 SCC are shown. The mean for *DOCK8* expression in each group is indicated by a thick bar. Paired cancerous and non-cancerous lung tissue is indicated by connecting with solid lines. The expression levels of *DOCK8* are shown after adjusting the level in normal lung tissue to 1.0.

substitution in PC3), Ile1221Met (homozygous substitution in H157), Tyr1340Cys (heterozygous substitution in HCC33), Met1379Thr (homozygous substitution in Ma29) and Asn1694Ser (homozygous substitution in Lu99). These substitutions were not deposited in the SNP database and were not observed in DNA from 95 normal lung tissues. Since corresponding normal DNA was not available for these 5 cell lines, it was not concluded whether these substitutions were somatic mutations or rare polymorphisms. It was likely that genetic *DOCK8* alterations are rare in lung cancer.

We then examined the expression status of the *DOCK8* gene in cancerous and non-cancerous lung cells. First, we examined the DOCK8 expression in type II alveolar cells, bronchiolar epithelial cells, and bronchial epithelial cells, which are considered as being progenitors for lung cancer cells, and also in ADC cells and SQC cells. These non-cancerous and cancerous lung cells were isolated by laser capture microdissection (19). RTQ-PCR analysis showed that *DOCK8* was expressed in lung epithelial cells (Fig. 3A). Two ADC cases (Cases 2 and 3 in Fig. 3A) and all five SQC cases showed less *DOCK8* expression than the normal lung epithelial cells examined, indicating that *DOCK8* expression is reduced in a fraction of lung cancer cells. We further examined the *DOCK8* expression in 71 macro-dissected primary lung tumors consisting of 46 ADCs, 21 SQCs, 3 LCCs and 1 SCC. For 7 of the 71 tumors (6 ADCs and 1 SQC), corresponding normal lung tissue was analyzed. Of these 7 tumors, 6 (86%, excluding 1 ADC) showed lower expression compared with the corresponding normal lung tissue (Fig. 3B). The expression levels of the *DOCK8* gene in 62 (87 %) of the 71 tumors [38 ADCs (83%), 20 SQCs (95%), 3 LCCs (100%) and 1 SCC (100%)] were lower than those in any of the 7 normal lung tissues examined (Fig. 3B). The expression levels in total lung tumor cases, ADCs, SQCs and LCCs were significantly lower than those in normal lung tissue by Mann-Whitney U test ($P<0.05$). The *DOCK8* expression levels were not significantly different by the differentiation grades in ADCs and SQCs (Mann-Whitney U test). The expression levels of *DOCK8* in 58 (95%) of 61 lung cancer cell lines were less than 1/10 of those in normal lung tissue. Thus, it was suggested that *DOCK8* gene expression was down-regulated in a majority of lung cancer cells irrespective of histological type.

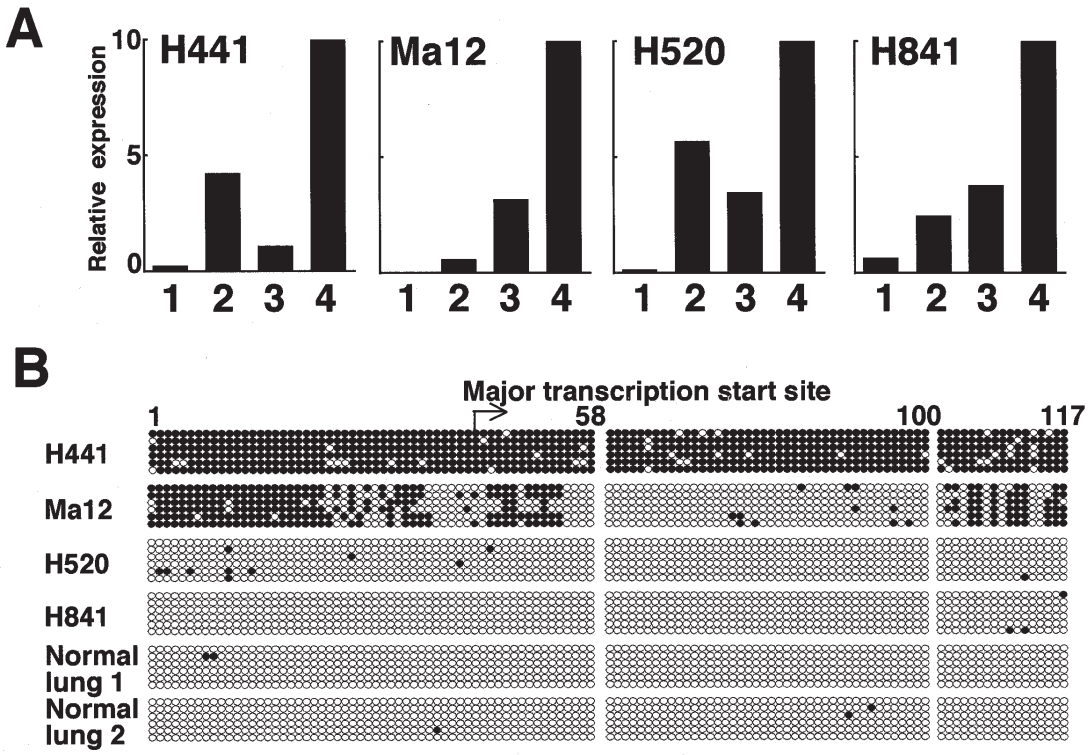

Figure 4. Epigenetic *DOCK*8 alterations in human lung cancer cells. (A) Induction of *DOCK8* expression by treatment with 5-aza-dC and/or TSA in lung cancer cell lines. 1, no treatment; 2, 10 μM 5-aza-dC treatment for 72 h; 3, 1.0 μM TSA treatment for 24 h; 4, 10 μM 5-aza-dC and 1.0 μM TSA treatment for 72 and 24 h, respectively. The expression levels of *DOCK8* are shown after adjusting the level of condition 4 in each cell line to 10.0. (B) Bisulfite sequencing of the *DOCK8* CpG island in lung cancer cell lines and normal lung tissue. Each circle indicates a CpG site in the primary DNA sequence, and each line of circles represents analysis of a single cloned allele. The numbers of the CpG sites are indicated at the top. ◦, unmethylated CpG sites; •, methylated CpG sites; arrow, the major transcription start site.

Epigenetic mechanisms for reduced DOCK8 expression in lung cancer cell lines. To examine the mechanism for reduced *DOCK8* expression in lung cancer cells, four lung cancer cell lines, H441, Ma12, H520 and H841, which showed reduced *DOCK8* expression by RTQ-PCR analysis as described above, were treated with 5-aza-dC and/or TSA (Fig. 4A). *DOCK8* expression was induced in all four cell lines by the 5-aza-dC, TSA and 5-aza-dC plus TSA treatments. Thus, DNA hypermethylation and/or histone deacetylation were likely to be involved in the reduced *DOCK8* expression in lung cancer cells.

To further investigate the involvement of DNA hypermethylation in reduced *DOCK8* expression, we examined the methylation status of 117 CpG sites in the CpG island of the *DOCK8* gene in two normal lung tissues and the four lung cancer cell lines described above (Fig. 4B). In the two normal lung tissues, the CpG island was free of methylation. The CpG island was fully methylated in H441 cells, while it was partially methylated in Ma12 cells. However, the CpG island was free of methylation in H520 and H841 cells. We then examined the methylation status in 6 primary lung tumors (2 ADCs, 3 SQCs and 1 LCC), which showed reduced *DOCK8* expression. The CpG sites, 1-58 and 101-117, which were commonly methylated in H441 and Ma12 cells, were examined; however, methylation of these CpG sites was not observed in the six tumors (data not shown). Thus, the methylation sites responsible for the reduced *DOCK8* expression remained obscure.

9p24 homozygous deletion in human cancers other than lung cancer. To examine the prevalence of 9p24 homozygous deletions in human cancers, genomic PCR analysis of STSs mapped in the LC1-Sq deletion region was performed in 85 cancer cell lines consisting of 20 esophageal, 18 gastric, 12 colon, 10 renal, 14 breast, two pancreas and nine brain cancers. A gastric cancer cell line (HSC58) and a breast cancer cell line (BT20) showed homozygous deletions (Fig. 1). The deleted region contained the *DOCK8*, *KANK*, and *DMRT1* genes. These two cell lines also carried *p16* homozygous deletions, and the deletions were not contiguous with deletions at 9p24.

Discussion

In this study, a homozygous deletion including the *DOCK8* locus at chromosome 9p24 was identified in a lung SQC cell line. Findings to date on biological roles of DOCK family proteins, i.e. regulators of cell migration, morphology, adhesion and growth, as well as frequent allelic loss at 9p24 in human lung cancers prompted us to further investigate *DOCK8* alterations in lung cancer. We determined the structure of the *DOCK8* gene and performed a comprehensive analysis of genetic alterations and expression of the *DOCK8* gene in human lung cancer. Since *DOCK8* was expressed in lung epithelial cells, DOCK8 protein was suggested to play some roles in the maintenance and/or function of lung epithelial cells. Although genetic *DOCK8* alterations were rare, its expression was reduced in a majority of lung cancer

cells. The reduction occurred irrespective of histological type. Analysis of lung cancer cell lines indicated that the reduced *DOCK8* expression is caused by DNA methylation and/or histone deacetylation, as with other (candidate) tumor suppressor genes, such as *p16*, *RASSF1A* and *MYO18B* (1,2,5,23,24). Thus, it was suggested that down-regulation of *DOCK8* by epigenetic mechanisms is involved in the development and/or progression of lung cancer.

The deduced DOCK8 protein contained DHR-1 and DHR-2 domains, which were conserved among DOCK family proteins and are critical for GEF enzyme activity (12,15-18). In a recent study, DOCK8 did not show GEF activity against small GTPases, Cdc42, Rac1 and RhoA; therefore, it remains unclear whether DOCK8 functions as a GEF (12). Functional domains other than the DHR-1 and DHR-2 domains were not detected (Fig. 2A). Thus, at present, the physiological and biological functions of DOCK8 protein remain unknown. Localization of DOCK8 protein in lamellipodia suggests that DOCK8 functions as a regulator of cell migration, morphology, adhesion and growth, as do a few other DOCK proteins. In preliminary studies, we exogenously expressed *DOCK8* protein in lung cancer cells without *DOCK8* expression; however, changes in the growth and motility of the cells were not evident under standard culture conditions (data not shown). Further functional analysis of DOCK8 protein is needed to elucidate the biological and pathogenic significance of *DOCK8* alterations in lung cancer.

Previous studies on allelic 9p losses in human lung cancer suggested that chromosome arm 9p contains multiple target regions for deletions (13,14,23,25,26). In this study, homozygous deletion at the 9p24 region was detected in a breast and a gastric cancer cell line in addition to the LC1-Sq lung cancer cell line. All three cell lines carried a homozygous deletion at the *p16* locus at chromosome 9p21, but the 9p21 deletions were not contiguous with the 9p24 deletions. In a recent report, a pancreatic carcinoma cell line was also shown to have a homozygous deletion of this region (27). Thus, 9p24 can be defined as an independent target region for chromosome 9p deletions in several human cancers. The common region of the homozygous deletions in the three cell lines included three genes, *DOCK8*, *KANK* and *DMRT1* (Fig. 1). The *DMRT* gene encodes transcription factors specifically expressed in testis tissue. Therefore, it is unlikely that alterations of *DMRT1* contribute to the development of lung and other carcinomas. Interestingly, the *KANK* gene has been reported as being inactivated by promoter methylation in human renal cell carcinoma (28). Thus, we examined the methylation status of the promoter region of *KANK* in cancerous and non-cancerous lung cells. However, the *KANK* promoter was methylated both in normal lung tissue and lung cancer cells. Therefore, methylation of the *KANK* promoter would not be an epigenetic alteration specific to lung cancer cells. Thus, *DOCK8* seems to be the most likely target of 9p24 homozygous deletion, at least in lung cancer.

Lastly, the present study indicated that down-regulation of *DOCK8* is involved in human lung carcinogenesis. *DOCK8* is the second *DOCK* gene whose implication in carcinogenesis was suggested, following *DOCK4* (18). DOCK family proteins play roles in the regulation of physiological activities of epithelial and non-epithelial cells. Thus, the present study also proposes the necessity of analysing *DOCK* family gene alterations in a variety of human cancers for further understanding of the mechanisms of human carcinogenesis.

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