# Activation of Placenta-Specific Transcription Factor *Distal-less* Homeobox 5 Predicts Clinical Outcome in Primary Lung Cancer Patients

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**Abstract**

**Purpose and Experimental Design:** To identify novel biomarkers and therapeutic targets for lung cancers, we screened for genes that were highly transactivated in lung cancers using a cDNA microarray representing 27,648 genes. *DLX5* gene, a member of the human *distal-less* homeobox transcriptional factor family that is expressed during early embryonic development, was found to be overexpressed in the great majority of lung cancers. Tissue microarray consisting of archival non–small cell lung cancer samples from 369 patients was applied to examine the clinicopathologic significance of DLX5 protein. A role of DLX5 in cancer cell growth and/or survival was investigated through small interfering RNA experiments.

**Results:** Northern blot and immunohistochemical analyses detected expression of DLX5 only in placenta among 23 normal tissues examined. Immunohistochemical analysis showed that positive immunostaining of DLX5 was correlated with tumor size (pT classification; $P = 0.0053$) and poorer prognosis of non–small cell lung cancer patients ($P = 0.0045$). It was also shown to be an independent prognostic factor ($P = 0.0415$). Treatment of lung cancer cells with small interfering RNAs for *DLX5* effectively knocked down its expression and suppressed cell growth.

**Conclusions:** These data implied that DLX5 is useful as a target for the development of anticancer drugs and cancer vaccines as well as for a prognostic biomarker in clinic.

Lung cancer is one of the most common causes of cancer death worldwide, and non–small cell lung cancer (NSCLC) accounts for nearly 80% of those cases (1). Many genetic alterations involved in development and progression of lung cancer have been reported, but the molecular mechanisms mostly remain unclear (2). In the last two decades, newly developed cytotoxic agents including paclitaxel, docetaxel, gemcitabine, and vinorelbine have emerged to offer multiple therapeutic choices for patients with advanced NSCLC. However, those regimens provide only limited survival benefits compared with cisplatin-based therapies (3, 4). Recently, new agents targeting the epidermal growth factor receptor pathway, erlotinib (Tarceva; OSI Pharmaceuticals) and gefitinib (Iressa; AstraZeneca), were shown to be very effective to a subset of NSCLC patients. However, even if all kinds of available treatments are applied, the proportion of patients showing good response is still very limited (5, 6). Hence, new therapeutic strategies are eagerly awaited.

Systematic analysis of expression levels of thousands of genes using cDNA microarray is an effective approach to identify molecules involved in carcinogenic pathways that can be candidates for development of novel therapeutics and diagnostics. We have been attempting to isolate potential molecular targets for diagnosis and/or treatment of lung cancer by analyzing genome-wide expression profiles of various types of lung cancer cells on a cDNA microarray containing 27,648 genes, using tumor-cell populations purified by laser microdissection (7–10). To verify the biological and clinicopathologic significance of the respective gene products, we did high-throughput screening of loss-of-function effects by means of the RNA interference technique as well as tumor tissue microarray analysis of clinical lung cancer materials (7–29). This systematic approach revealed that *distal-less* homeobox 5 (*DLX5*) was frequently overexpressed in the majority of primary lung cancers.

Homeobox genes are transcription factors of fundamental importance during development throughout evolutionarily diverse species. The redundant function of the *Dlx* genes was explained by their nearly identical homeodomains, whereas individual unique functions were supposed to be due to the divergence of their amino acid sequences in other domains (30). Inactivation of homeobox genes have been implicated in many congenital malformations as well as development of cancers (31). DLX5 is considered to be a master regulatory

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protein essential in initiation of the cascade involved in osteoblast differentiation and to play a critical role in regulation of mammalian limb development as shown by the evidences that the targeted disruption or ablation of *Dlx5* and *Dlx6* caused developmental abnormality of bone and inner ear, and craniofacial defects (32). However, the roles of DLX5 activation in carcinogenesis have not been clarified.

In this study, we describe that overexpression of DLX5 could contribute to the malignant nature of lung cancer cells. We suggest that targeting the DLX5 molecule might hold promise for development of a new diagnostic and therapeutic strategy in the clinical management of lung cancers.

## Materials and Methods

***Lung cancer cell lines and tissue samples.*** The human lung cancer cell lines used in this study were as follows: A427, A549, LC319, PC3, PC9, and NCI-H1373 (lung adenocarcinomas); NCI-H1781 (a bronchiolo alveolar carcinoma); RERF-LC-AI, SK-MES-1, EBC-1, LU61, NCI-H520, NCI-H1703, and NCI-H2170 (lung squamous cell carcinomas); NCI-H226 and NCI-H647 (lung adenosquamous carcinomas); LX1 (a lung large cell carcinoma); and DMS114, DMS273, SBC-3, and SBC-5 (small cell lung cancers). All cells were grown in monolayer in appropriate medium supplemented with 10% FCS and were maintained at 37°C in atmospheres of humidified air with 5% $CO_2$. Human small airway epithelial cells were grown in optimized medium (SAGM) purchased from Cambrex Bio Science, Inc. Fourteen primary NSCLCs (seven adenocarcinomas and seven squamous cell carcinoma) had been obtained from patients with written informed consent, as described previously (14). A total of 369 NSCLCs and adjacent normal lung tissue samples for immunostaining on tissue microarray were obtained from patients who underwent curative surgery at Saitama Cancer Center. This study and the use of all clinical materials were approved by the Institutional Research Ethics Committees.

***Semiquantitative reverse transcription-PCR.*** Total RNA was extracted from cultured cells and clinical tissues using Trizol reagent (Life Technologies, Inc.) according to the manufacturer's protocol. Extracted RNAs and normal human tissue polyadenylate RNAs were treated with DNase I (Nippon Gene) and reversely transcribed using oligo (dT) primer and SuperScript II reverse transcriptase (Invitrogen). Semiquantitative reverse transcription-PCR (RT-PCR) experiments were carried out with the following *DLX5*-specific primers or with *ACTB*-specific primers as an internal control: *DLX5*, 5′-CTCGCTCAGCCACCACCCT-CAT-3′ and 5′-AGTTGAGGTCATAGATTTCAAGGCAC-3′; *ACTB*, 5′-GA-GGTGATAGCATTGCTTTCG-3′ and 5′-CAAGTCAGTGTACAGGTAA-GC-3′. PCR reactions were optimized for the number of cycles to ensure product intensity within the logarithmic phase of amplification.

***Northern blot analysis.*** Human multiple tissue blots (BD Biosciences Clontech) were hybridized with a $^{32}$P-labeled PCR product of *DLX5*. The cDNA probes of *DLX5* were prepared by RT-PCR using the primers described above. Prehybridization, hybridization, and washing were done according to the supplier's recommendations. The blots were autoradiographed at room temperature for 30 h with intensifying BAS screens (BIO-RAD).

***Anti-DLX5 antibodies.*** Plasmids expressing full-length fragments of DLX5 that contained His-tagged epitopes at their $NH_2$ terminals were prepared using pET28 vector (Novagen). The recombinant peptides were expressed in *Escherichia coli*, BL21 codon-plus strain (Stratagene), and purified using TALON resin (BD Bioscience) according to the supplier's protocol. The protein, extracted on an SDS-PAGE gel, was inoculated into rabbits; the immune sera were purified on affinity columns according to standard methodology. Affinity-purified anti-DLX5 antibodies were used for Western blotting as well as immunocytochemical and immunohistochemical studies. We confirmed that the antibody was specific to DLX5 on Western blots using lysates from cell lines that had been transfected with DLX5 expression vector and those from lung cancer cell lines, either of which expressed DLX5 endogenously or not, as well as by immunocytochemical staining of the cell lines.

***Western blot analysis.*** Cells were lysed in lysis buffer; 50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.5% NP40, 0.5% deoxycholate-Na, 0.1% SDS, plus protease inhibitor (Protease Inhibitor Cocktail Set III; Calbiochem Darmstadt). We used an enhanced chemiluminescence Western blotting analysis system (GE Healthcare Bio-sciences). SDS-PAGE was done in 12% polyacrylamide gels. PAGE-separated proteins were electroblotted onto nitrocellulose membranes (GE Healthcare Bio-sciences) and incubated with a rabbit polyclonal anti-human DLX5 antibody (generated to recombinant DLX5; please see above). A donkey anti-rabbit IgG-horseradish peroxidase antibody (GE Healthcare Bio-sciences) was served as the secondary antibodies.

***Immunocytochemistry.*** SBC-5 cells were seeded on coverslips, and cells were fixed in 4% formamide and permeabilized with cold methanol acetone (50:50) for 5 min at room temperature. After washing in PBS once, cells were incubated with the anti-DLX5 antibody for 1 h at room temperature, followed by incubation with Alexa488-conjugated goat anti-rabbit antibodies (Molecular Probes; 1:1,000 dilution) for 1 h in the dark. Images were captured on a confocal microscope (TCS SP2-AOBS; Leica Microsystems).

***Immunohistochemistry and tissue microarray analysis.*** To investigate the presence of DLX5 protein in clinical materials, we stained tissue sections using ENVISION+ kit/horseradish peroxidase (DakoCytomation). For antigen retrieval, slides were immersed in Target Retrieval Solution High pH (DakoCytomation) and boiled at 108°C for 15 min in an autoclave. Seven micrograms per milliliters of affinity-purified rabbit polyclonal anti-DLX5 antibodies (generated to recombinant DLX5; please see above) were added after blocking of endogenous peroxidase and proteins, and each section was incubated with horseradish peroxidase–labeled anti-rabbit IgG as the secondary antibody. Substrate chromogen was added and the specimens were counterstained with hematoxylin. Tumor tissue microarrays were constructed as published elsewhere, using formalin-fixed archived NSCLCs obtained by a single institutional group with an identical protocol to collect and fix the tissues after resection (13–28, 33–35). Considering the histologic heterogeneity of individual lung tumors, tissue areas for sampling were selected based on visual alignment with the corresponding HE-stained sections on slides. Three, four, or five tissue cores (diameter, 0.6 mm; height, 3-4 mm) taken from donor tumor blocks were placed into recipient paraffin blocks using a tissue microarrayer (Beecher Instruments). A core of normal tissue was punched from each case. Five-micrometer sections of the resulting microarray block were used for immunohistochemical analysis. Three independent investigators assessed the staining pattern of nuclear and cytoplasmic DLX5 (n-DLX5 and c-DLX5) individually, without prior knowledge of clinicopathologic data. Because the intensity of staining within each tumor tissue core was mostly homogenous, the intensity of DLX5 staining was semiquantitatively evaluated using following criteria: strong positive (2+), dark brown staining in >50% of tumor cells completely obscuring nucleus or cytoplasm; weak positive (1+), any lesser degree of brown staining appreciable in tumor cell nucleus or cytoplasm; and absent (scored as 0), no appreciable staining in tumor cell nucleus or cytoplasm. Lung cancers were scored as n-DLX5 or c-DLX5–strongly positive (2+) only if all reviewers defined them as such.

***Statistical analysis.*** All analyses were done using statistical analysis software (StatView, version 5.0; SAS Institute, Inc.). We examined correlations between its expression levels and clinicopathologic variables such as age, gender, pathologic tumor-node-metastasis stage, and histologic type. Strong DLX5 immunoreactivity was assessed for association with clinicopathologic variables using the Fisher's exact test. Univariate and multivariate analyses were done with the Cox proportional hazard regression model to determine associations between clinicopathologic variables and cancer-related mortality. First,

we analyzed associations between death and possible prognostic factors including age, gender, histologic type, pT classification, and pN classification, taking into consideration one factor at a time. Second, multivariate Cox analysis was applied on backward (stepwise) procedures that always forced DLX5 expression into the model, along with any and all variables that satisfied an entry level of a $P$ value of <0.05. As the model continued to add factors, independent factors did not exceed an exit level of a $P$ value of <0.05.

***RNA interference assay.*** We had previously established a vector-based RNA interference system, psiH1BX3.0, which was designed to generate siRNAs in mammalian cells (12). Using 30 μL of Lipofectamine 2000 (Invitrogen), we transfected 10 μg of DLX5-specific siRNA expression vector into SBC-5 and NCI-H1781 cell lines that endogenously overexpressed DLX5. The transfected cells were cultured for 7 d in the presence of appropriate concentrations of geneticin (G418), and the numbers of colonies and viable cells were counted by Giemsa staining in triplicate 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. The target sequences of the synthetic oligonucleotides for RNA interference were as follows: control 1 [*enhanced green fluorescent protein gene* (*EGFP*), a mutant of *Aequorea victoria* GFP], 5′-GAAGCAGCACGACTTCTTC-3′; control 2 (Scramble, chloroplast *Euglena gracilis* gene coding for 5S and 16S rRNAs), 5′-GCGCGCTTTGTAGGATTCG-3′; siRNA-*DLX5*-#1, 5′-CCAGCCAGAGAAAGAAGTG-3′; and siRNA-*DLX5*-#2, 5′-GTGCAGCCAGCTCAATCAA-3′. To validate our RNA interference system, down-regulation of *DLX5* expression by functional siRNA, but not by controls or noneffective siRNA, was confirmed in the cell lines used for this assay.

## Results

***Expression of DLX5 gene in lung cancers and normal tissues.*** To identify target molecules for development of novel therapeutic agents and/or biomarkers for lung cancer, we first screened through a cDNA microarray for genes that showed 5-fold or higher expression in >50% of 86 NSCLCs or 15 small cell lung cancers analyzed (7–10). Among 27,648 genes screened, we identified the *DLX5* gene to be overexpressed in the majority of lung cancers, and confirmed its overexpression by semiquantitative RT-PCR experiments in 9 of 14 additional NSCLC cases (2 of 7 adenocarcinomas and all of 7 squamous cell carcinomas; Fig. 1A) as well as in 10 of 23 lung cancer cell lines, whereas its expression was hardly detectable in small airway epithelial cells derived from normal bronchial epithelium (Fig. 1B). We subsequently generated rabbit polyclonal antibody against human DLX5 and confirmed the expression of endogenous DLX5 protein in six lung cancer cell lines by Western blot analysis (three DLX5-positive and three DLX5-negative cell lines examined by RT-PCR; Fig. 1C). To determine the subcellular localization of endogenous DLX5 in lung cancer cells, we did immunofluorescence analysis using anti-DLX5 antibody and found its staining strongly in the nucleus and weakly in the cytoplasm of SBC-5 cells (Fig. 1D). DLX5 protein was diffusely detected in cells during the mitotic phase (Fig. 1D; *arrows*).

Northern blot analysis using *DLX5* cDNA as a probe identified a strong signal corresponding to a 1.8-kb transcript only in the placenta among 23 tissues examined (Fig. 2A). Furthermore, we compared DLX5 protein expressions in five normal tissues (heart, liver, kidney, lung, and placenta) with those in lung cancers using anti-DLX5 polyclonal antibodies by immunohistochemical analysis. In concordant with the result of northern analysis, DLX5 expression was observed in the placenta and lung cancers but was hardly detectable in the four other normal tissues (Fig. 2B). The positive signal by anti-DLX5 antibody obtained in lung cancer tissues was diminished by preincubation of the antibody with recombinant human DLX5, indicating its high specificity to DLX5 protein (Supplementary Fig. S1).

***Association of strong DLX5 expression with poor prognosis for NSCLC patients.*** To verify the clinicopathologic significance of DLX5, we additionally examined the expression of DLX5 protein by means of tissue microarrays containing lung cancer tissues from 369 patients who underwent curative surgical resection. Positive staining was observed in nucleus and/or cytoplasm in NSCLC cells, but staining was negative in any of their adjacent normal lung cells or stromal cells surrounding tumor cells (Fig. 2C). We first classified a pattern of DLX5 expression on the tissue array ranging from absent/weak (scored as 0-1+) to strong (2+) staining in nucleus (n-DLX5) or cytoplasm (c-DLX5; Fig. 2C; please see the criteria in Materials and Methods). Of the 369 NSCLC cases examined, strong n-DLX5 staining was observed in 148 cases (40.1%; score 2+), weak staining in 148 cases (40.1%; score 1+), and no staining was in 73 cases (19.8%; score 0). Strong c-DLX5 staining was seen in 137 cases (37.1%; score 2+), weak staining in 153 cases (41.5%; score 1+), and no staining in 79 cases (21.4%; score 0; Supplementary Table S1). Because we found the strong n-DLX5 expression was significantly concordant with the strong c-DLX5 staining in these tumors ($\chi^2$ = 237; $P$ < 0.0001), we applied combined score for n-DLX5 and c-DLX5 for further clinicopathologic evaluation of DLX5; DLX5 was strongly stained in 160 cases (43.4%; score 2+) in nucleus and/or cytoplasm, weakly stained in 145 cases (39.3%; score 1+), and not stained in 64 cases (17.3%; score 0) in either nucleus or cytoplasm (Supplementary Table S1). We next examined a correlation of DLX5 expression (strong positive versus weak positive/absent in nucleus and/or cytoplasm) with various clinicopathologic variables and found its significant correlation with pT classification (higher in larger tumor; $P$ = 0.0053 by Fisher's exact test; Table 1). NSCLC patients whose tumors showed strong DLX5 expression (score 2+) in nucleus and/or cytoplasm revealed shorter tumor-specific survival periods compared with those with absent/weak DLX5 expression ($P$ = 0.0045 by the Log-rank test; Fig. 2D; Supplementary Fig. S2). To evaluate the importance of strong n-DLX5 and/or c-DLX5 expression, we further divided the 160 cases with DLX5-strong positive into three groups; 125 revealed strong DLX5 staining in both nucleus and the cytoplasm (group 1, n-DLX5++ and c-DLX5++), 23 revealed its strong staining in the nucleus with weak/no staining in the cytoplasm (group 2, n-DLX5++ and c-DLX5+/-), and 12 revealed its strong staining in the cytoplasm with weak/no staining in the nucleus (group3, n-DLX5+/- and c-DLX5++). Tumors with high levels of DLX5 staining (score 2+) in nucleus and/or cytoplasm (median survival days for the groups 1, 2, and 3, 1,626, 1,546, and 1,665, respectively) were likely to have worse tumor-specific survival compared with those without any strong DLX5 staining (n-DLX5+/- and c-DLX5+/-; 2,496 days; Supplementary Fig. S2). The difference in tumor-specific survival among the three groups of NSCLCs with strong DLX5 staining was not significant, as indicated by Kaplan-Meier analysis and Log-rank test (Supplementary Fig. S2). We also applied univariate analysis to evaluate associations between patient prognosis and other factors including age (<65 years

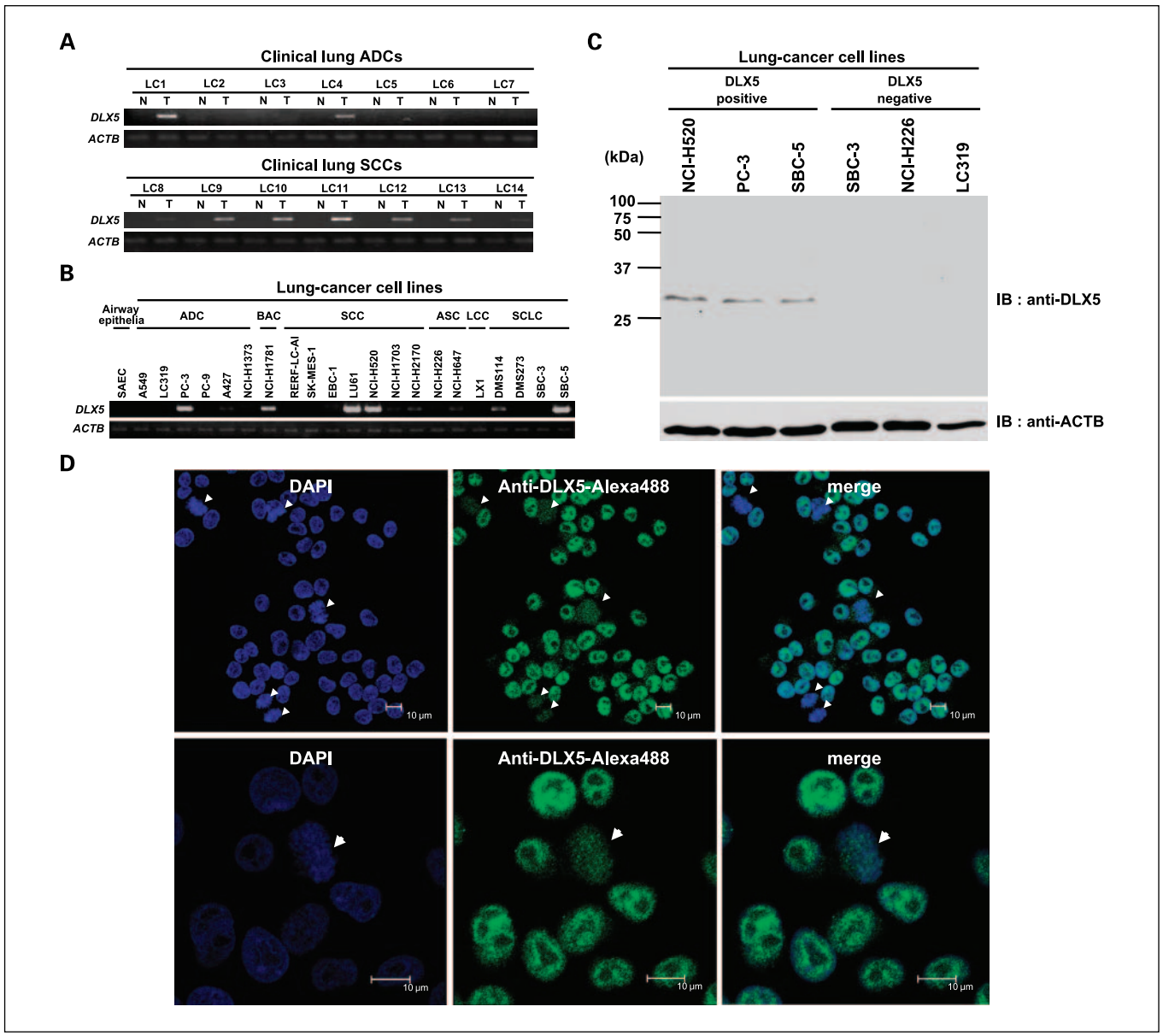


**Fig. 1.** Expression of DLX5 in lung tumors. *A,* expression of *DLX5* in clinical samples of NSCLC (adenocarcinoma and squamous cell carcinoma) and normal lung tissues, examined by semiquantitative RT-PCR. *B,* expression of *DLX5* in lung cancer cell lines, as revealed by semiquantitative RT-PCR. Expression of β-actin (*ACTB*) served as a quantity control. *C,* expression of DLX5 protein in lung cancer cell lines by Western blot analysis. Expression of ACTB served as a quantity control. *D,* subcellular distribution of the DLX5 proteins examined by confocal microscopy. *Arrows,* cells in the mitotic phase. IB, immunoblotting.

versus ≥65 years), gender (female versus male), histologic type (adenocarcinoma versus non-adenocarcinoma), pT classification ($T_1$ versus $T_2$, $T_3$, and $T_4$), pN classification ($N_0$ versus $N_1$ and $N_2$), and DLX5 status (0 and 1+ versus 2+ in nucleus and/or cytoplasm). Among those variables, strong DLX5 staining (2+ in nucleus and/or cytoplasm; $P = 0.0048$), elderly ($P = 0.0028$), male ($P = 0.001$), non-adenocarcinoma histologic classification ($P = 0.01$), advanced pT stage ($P < 0.0001$), and advanced pN stage ($P < 0.0001$) were significantly associated with poor prognosis (Table 2). In multivariate analysis of the prognostic factors, strong DLX5 expression (2+ in nucleus and/or cytoplasm), elderly, higher pT stage, and higher pN stage were indicated to be independent prognostic factors ($P = 0.0415$, 0.0007, 0.0004, and <0.0001, respectively; Table 2).

***Growth inhibition of NSCLC cells by specific siRNA against DLX5.*** To assess whether *DLX5* is essential for growth or survival of lung cancer cells, we constructed plasmids to express siRNAs against DLX5 (si-*DLX5-#1* and si-*DLX5-#2*) as well as two control plasmids (siRNAs for EGFP and Scramble), and transfected them into lung cancer cell lines, SBC-5 and NCI-H1781. The mRNA levels in cells transfected with si-*DLX5-#2* were significantly decreased in comparison with those transfected with either of the two control siRNAs or si-*DLX5-#1*. We observed significant decreases in the number of colonies and in the numbers of viable cells measured by MTT assay, suggesting that up-regulation of DLX5 is related to growth or survival of cancer cells (representative data of SBC-5 was shown in Fig. 3).

## Discussion

Although advances have been made in development of molecular-targeting drugs for cancer therapy, the proportion of patients showing good response to available treatments is still very limited (36). Hence, it is urgent to develop new anticancer agents that will be highly specific to malignant cells, with minimal or no adverse reactions. Toward this direction, we have been taking a strategy to identify good molecular targets for drug development as follows: (*a*) screening for genes that

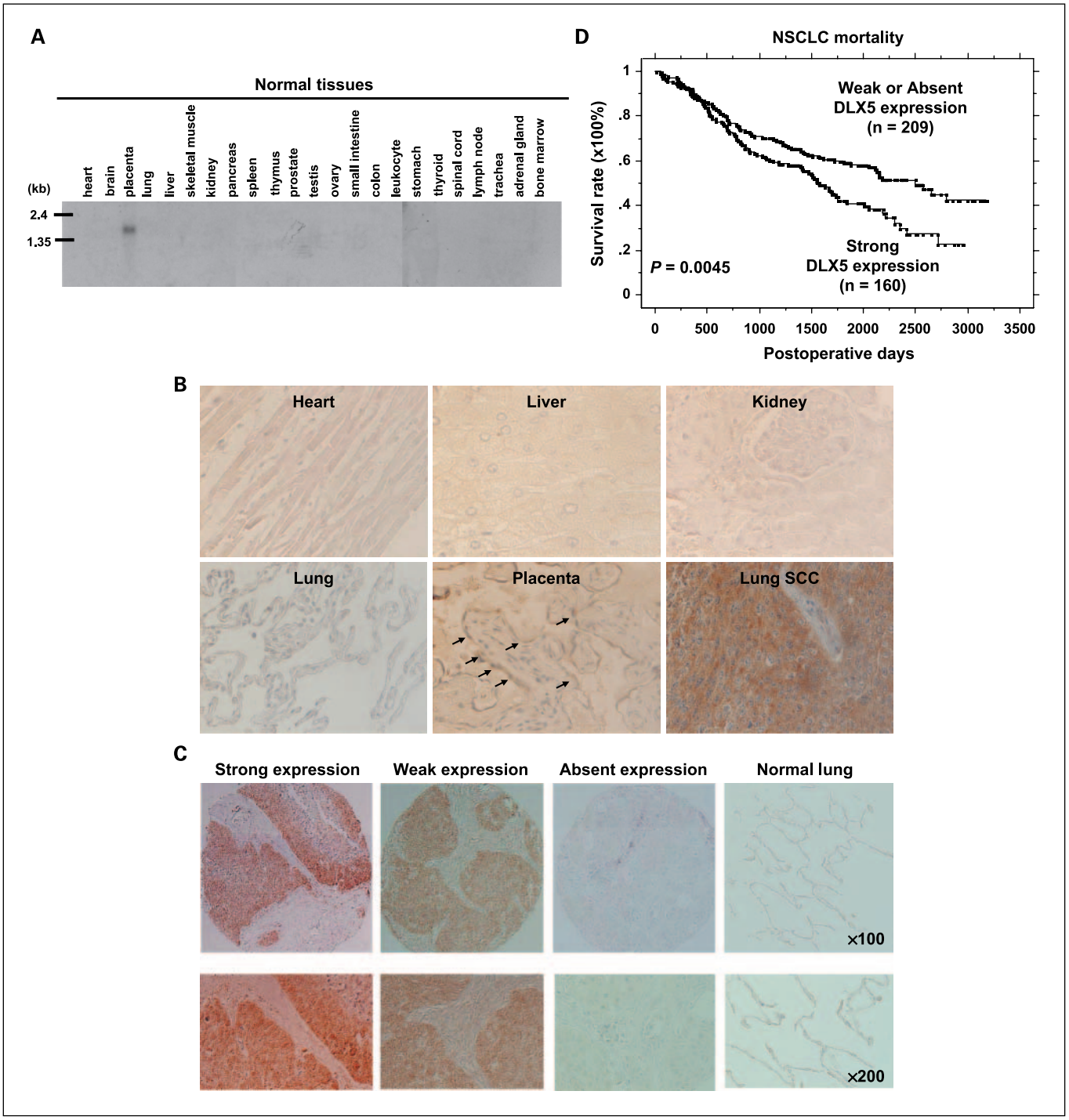


**Fig. 2.** Expression of DLX5 in normal tissues and association of DLX5 overexpression with poor prognosis for NSCLC patients. *A,* expression of *DLX5* in normal human tissues, detected by Northern blot analysis. *B,* expression of DLX5 in five normal human tissues as well as lung squamous cell carcinoma, detected by immunohistochemical staining using the rabbit polyclonal anti-DLX5 antibody; counterstaining with hematoxylin (×200). Positive staining appeared in the cytoplasm and/or nucleus of syncytiotrophoblasts in the placenta (*arrows*) and lung cancer cells. *C,* representative example of expression of DLX5 in lung cancer (squamous cell carcinomas, ×100) and normal lung (×100), and magnified view of squamous cell carcinoma – positive case (×200). *D,* Kaplan-Meier analysis of tumor-specific survival in NSCLC patients according to DLX5 expression level.

**Table 1.** Association between DLX5-positivity in NSCLC and patients' characteristics ($n$ = 369)

| | Total | DLX5 strong positive (score 2+)* | DLX5 weak positive (score 1+) † | DLX5 absent (score 0) ‡ | $P$ strong vs weak/absent |
|---|---|---|---|---|---|
| | $n$ = 369 | $n$ = 160 | $n$ = 145 | $n$ = 64 | |
| Gender | | | | | |
| Male | 255 | 109 | 99 | 47 | NS |
| Female | 114 | 51 | 46 | 17 | |
| Age (y) | | | | | |
| <65 | 189 | 90 | 64 | 35 | NS |
| ≥65 | 180 | 70 | 81 | 29 | |
| Histologic type | | | | | |
| ADC | 234 | 96 | 95 | 43 | NS§ |
| SCC | 95 | 44 | 36 | 15 | |
| Others‖ | 40 | 20 | 14 | 6 | |
| pT factor | | | | | |
| $T_1$ | 121 | 40 | 59 | 22 | 0.0053¶ |
| $T_2$-$T_4$ | 248 | 120 | 86 | 42 | |
| pN factor | | | | | |
| $N_0$ | 226 | 90 | 97 | 39 | NS |
| $N_1$ + $N_2$ | 143 | 70 | 48 | 25 | |

NOTE: Combined score for both n- and c-DLX5 staining (see the criteria below).
Abbreviations: ADC, adenocarcinoma; SCC, squamous cell carcinoma; LCC, large cell carcinoma; ASC adenosquamous carcinoma; NS, no significance.
*NSCLC cases with strong DLX5 staining in nucleus and/or cytoplasm.
†NSCLC cases with weak DLX5 staining.
‡NSCLC cases without DLX5 staining in either nucleus or cytoplasm.
§Adenocarcinoma versus non-adenocarcinoma.
‖Others, large cell carcinoma plus adenosquamous carcinoma.
¶$P$ < 0.05 (Fisher's exact test).

were up-regulated in cancer cells, but not expressed in normal organs, on the basis of cDNA microarray analysis; (*b*) investigating loss-of-function phenotypes using RNA interference systems and defining biological functions of the proteins; and (*c*) systematic analysis of protein expression among hundreds of clinical samples on tissue microarrays. Taking this approach, we have shown here that *DLX5*, a member of *distal-less* homeobox protein family, was frequently overexpressed in the great majority of clinical lung cancer samples and cell lines, and that the gene product is necessary for survival/growth of lung cancer cells.

The vertebrate *Dlx* genes, which encode a family of homeobox-containing transcription factors related in sequence to the *Drosophila Distal-less* (*Dll*) gene product, constitute one example of functional diversification of paralogs. All vertebrates investigated thus far have at least six *Dlx* genes that are

**Table 2.** Cox's proportional hazards model analysis of prognostic factors in patients with NSCLCs

| Variables | Hazards ratio (95% CI) | Unfavorable/favorable | $P$ |
|---|---|---|---|
| Univariate analysis | | | |
| DLX5* | 1.517 (1.136-2.026) | Strong (+)/weak (+) or (-) | 0.0048 † |
| Age (y) | 1.665 (1.192-2.324) | ≥65/<65 | 0.0028 † |
| Gender | 1.620 (1.157-2.269) | Male/female | 0.001 † |
| Histologic type | 1.466 (1.096-1.963) | non-ADC/ADC | 0.01 † |
| pT factor | 2.699 (1.867-3.902) | $T_2$ + $T_3$ + $T_4$/$T_1$ | <0.0001 † |
| pN factor | 2.674 (1.999-3.576) | $N_1$ + $N_2$/$N_0$ | <0.0001 † |
| Multivariate analysis | | | |
| DLX5* | 1.354 (1.012-1.811) | Strong (+)/weak (+) or (-) | 0.0415 † |
| Age (y) | 1.674 (1.244-2.254) | ≥65/<65 | 0.0007 † |
| Gender | 1.387 (0.960-2.004) | Male/female | NS |
| Histologic type | 1.099 (0.799-1.512) | non-ADC / ADC | NS |
| pT factor | 2.206 (1.357-2.912) | $T_2$ + $T_3$ + $T_4$/$T_1$ | 0.0004 † |
| pN factor | 2.536 (1.879-3.421) | $N_1$ + $N_2$/$N_0$ | <0.0001 † |

Abbreviations: 95% CI, 95% confidence interval; NS, no significance.
*Combined score for both n- and c-DLX5 staining.
†$P$ < 0.05.

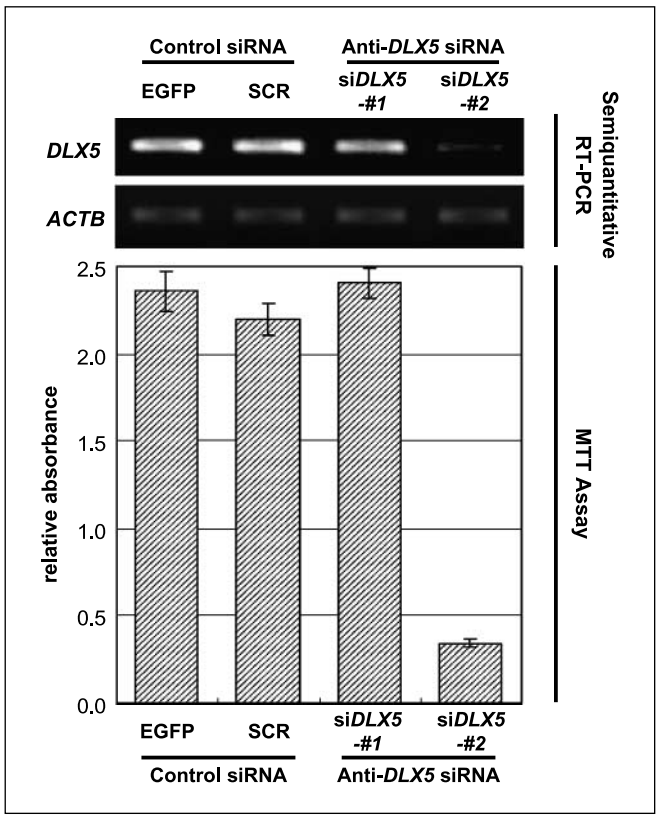


**Fig. 3.** Inhibition of growth by siRNA against *DLX5* in SBC-5 cancer cells. *Top,* the level of *DLX5* expression detected by semiquantitative RT-PCR in SBC-5 cells treated with either control siRNAs (si-EGFP or si-Scramble/SCR) or si-*DLX5*. *Bottom,* the effect of siRNA against *DLX5* on cell viability, detected by MTT assays.

generally arranged as three bigene clusters: *Dlx1*/*Dlx2*, *Dlx5*/*Dlx6*, and *Dlx3*/*Dlx4* (*Dlx7*; refs. 30, 37–39). The Dlx5 protein is first expressed in the anterior region of mouse embryos during early embryonic development (37). It has been reported that homozygous Dlx5/Dlx6 double-knockout mice exhibit split hand/foot malformation phenotypes, a heterogeneous limb disorder characterized by missing central digits and claw-like distal extremities, suggesting that *DLX5* gene is one of critical regulators for mammalian limb development (40). In fact, DLX5 was indicated to be a master regulatory transcriptional factor essential for initiating the cascade involved in osteoblast differentiation in mammals (41, 42).

In the present study, we showed that *DLX5* gene was frequently overexpressed in lung cancer and might play an important role in the development/progression of lung cancers. In this study, knockdown of *DLX5* expression by siRNA in lung cancer cells resulted in suppression of cell growth. Moreover, clinicopathologic evidence obtained through our tissue microarray experiments indicated that NSCLC patients with DLX5-strong positive tumors had shorter cancer-specific survival periods than those with DLX5-weak positive/negative tumors. The results obtained by *in vitro* and *in vivo* assays strongly suggested that DLX5 is likely to be an important growth factor and be associated with a more malignant phenotype of lung cancer cells. Because the DLX5 protein is present mainly in the nucleus and includes a homeodomain, it should play an important role in the transcriptional regulation and directly or indirectly transactivate various downstream genes in lung cancer cells. Interestingly, we also found 12 NSCLC cases with strong DLX5 staining in the cytoplasm of tumors but with weak/no staining in the nucleus (group3, n-DLX5+/-, and c-DLX5++), and their shorter tumor-specific survival (Supplementary Fig. S2). Some homeobox transcriptional factors are localized not only in the nucleus but also more predominantly in the cytoplasm (43, 44). *HOXA7*, a member of homeobox genes, changes its subcellular localization from the nucleus to the cytoplasm according to follicle maturation during ovarian folliculogenesis (43). Cell type- and stage-specific *HOXA7* localization is likely to regulate granulosa cell proliferation, and granulosa cell tumors also express cytoplasmic *HOXA7* (43). Other homeodomain-containing transcription factors, pre–B-Cell leukemia transcription factor families, are reported to be localized in the cytoplasm of the developing vertebrate embryo cells (44). Cytoplasmic localization of pre–B-Cell leukemia transcription factor is due to the modulation of nuclear localization signals, nuclear export sequences, and interaction with a cytoplasmic anchoring factor of nonmuscle myosin heavy chain II, whereas cytoplasmic distribution of pre–B-Cell leukemia transcription factor/knotted 1 homeobox 2 (PKNOX2 alias PREP2) is due to the concerted action of nuclear export and cytoplasmic retention by the actin and microtubule cytoskeletons (44). The precise molecular mechanism of DLX5 transport between the nucleus and cytoplasm, and whether c-DLX5 has an additional cytoplasm-specific function are not clear, but our data raise a possibility that c-DLX5 as well as n-DLX5 could contribute to the highly malignant phenotype of lung cancer cells by activating some unidentified signaling pathway(s). Further investigations of new pathway(s) involving c- and n-DLX5 could lead to a better understanding of the mechanisms of oncogene activation in pulmonary carcinogenesis. Because DLX5 is not expressed in any of normal adult tissues except the placenta, selective inhibition of DLX5 activity could be a promising therapeutic strategy that is expected to have a powerful biological activity against cancer with a minimal risk of adverse events.

In summary, *DLX5* gene might play an important role in the growth/progression of lung cancers. DLX5 overexpression in resected specimens may be a useful index for application of adjuvant therapy to the patients who are likely to have poor prognosis. In addition, the data strongly imply the possibility of designing new anticancer drugs and cancer vaccines to specifically target the DLX5 for human cancer treatment.

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