K-ras Gene Mutation Enhances Motility of Immortalized Airway Cells and Lung Adenocarcinoma Cells via Akt Activation

Possible Contribution to Non-Invasive Expansion of Lung Adenocarcinoma

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Point mutations of the *K-ras* gene, which are found in 10 to 30% of lung adenocarcinomas, are regarded as being an early event during the carcinogenesis. Autonomous vigorous motility of neoplastic cells, as well as growth and survival advantages, are considered to be necessary for cancer development and progression. The present study describes the contributions of the *K-ras* gene mutation and its downstream pathway via phosphatidylinositol 3-OH kinase (PI3K)-Akt to the cell motility in an immortalized human peripheral airway epithelial cell (HPL1D) and lung adenocarcinoma cells (A549, H820, TKB6, and TKB14). We have also evaluated the relationship between pathological events and the K-ras-Akt pathway using surgically resected lung tumors. The HPL1D cells transfected with the mutated *K-ras* gene (HPL-V12) showed a significant increase in cell motility compared to those transfected with empty vector (HPL-E) or wild-type *K-ras* gene (HPL-K). The enhanced motility in the HPL-V12 cells was markedly reduced by either treatment with inhibitors of ras, PI3K, and/or MEK, or by transfection with the dominant-negative mutant Akt (dnAkt). The lung adenocarcinoma cells bearing the *K-ras* gene mutation (A549 and H820) showed consistently higher levels of cell motilities than those without the mutation (TKB6 and TKB14), and the motility of A549 and H820 cells were significantly inhibited by dnAkt transfection.

These results suggest that the *K-ras* gene mutation could enhance the motility of neoplastic cells through a pathway involving PI3K-Akt. Actually, among the surgically resected lung tumors, the adenocarcinomas with the *K-ras* gene mutation tended to show a higher frequency and intensity of immunoreactivity for phosphorylated Akt (p-ser473Akt) than those without the mutation, supporting the *in vitro* observation that the mutated *K-ras* can activate the PI3K-Akt pathway. Immunoreactivity for p-ser473Akt was also seen in the pre-malignant and early lesions at a frequency similar to that in the advanced lung adenocarcinomas. No correlation was seen between p-ser473Akt immunoreactivity and lymphatic/organ metastasis or prognosis. These results taken together suggest that the K-ras-Akt pathway might facilitate the motility of neoplastic cells during the early period of carcinogenesis in lung adenocarcinomas, and may contribute to their non-invasive expansion along the alveolar septa, rather than invasion or metastasis. (*Am J Pathol* 2004, 164:91-100)

Malignant neoplasms are generally considered to develop through the accumulation of multiple genetic abnormalities. Point mutations of *ras* genes are among the most frequent events in human malignancies. The *ras* family is composed of three subtypes encoded by different genes, *H-*, *N-*, and *K-ras*. Point mutation of these genes is rather specific with regard to the organ or histological type of tumors. For example, mutations of *H-* or *N-ras* genes are seen in thyroid, breast, skin cancers, leukemia, etc.¹⁻³ On the other hand, mutations of the

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K-ras gene are seen in colorectal, pancreatic, endometrial, and lung cancers.^{2,4} In lung cancer, *K-ras* gene point mutations, which most frequently occur at codon 12, are restricted exclusively to adenocarcinoma, with a frequency of 10 to 30%. Since the *K-ras* gene mutation is observed not only in advanced adenocarcinomas, but also in pre-malignant and early lesions such as atypical adenomatous hyperplasia (AAH) and bronchioalveolar carcinoma (BAC) at a similar frequency,⁵⁻⁷ *K-ras* gene mutation has been considered to be an early event during tumorigenesis.⁵⁻¹¹ However, how this gene mutation mediates the development of lung adenocarcinoma has not been fully clarified.

Biochemical studies have revealed that *K-ras* gene point mutations cause constitutive activation of the protein product by reducing its intrinsic GTPase activity, resulting in excessive activation of its downstream factors.¹² Raf and phosphatidylinositol 3-OH kinase (PI3K) are among the best-studied downstream factors of *K-ras*.¹³ Raf transmits a signal via phosphorylation of MEK, which subsequently activates its downstream effector, mitogen-activated protein kinase (Erk1/2).¹⁴ Activated Erk1/2 binds to other kinases and translocates into the nucleus, and the complexes interact with various transcription factors to regulate the expression of genes facilitating cell cycle progression and inhibiting apoptosis.¹⁴⁻¹⁷ On the other hand, PI3K phosphorylates membrane phosphatidylinositide to recruit and activate many factors containing a pleckstrin homology (PH) domain, such as Akt and phosphatidylinositide-dependent kinase (PDK), which has also been shown to transmit signals mediating cell survival, cell cycle progression, and glucose metabolism.¹³ Thus, it is conceivable that the constitutive and excessive activation of the raf-Erk1/2 and/or PI3K-Akt pathways resulting from *K-ras* gene mutation promotes cancer development by providing survival and growth advantages for neoplastic cells. In addition, vigorous motility is also one of the essential properties of cancer cells, since malignant neoplasms are characterized by high invasive and metastatic potentials. Several recent studies have demonstrated that Erk1/2 and/or Akt could also transmit signals mediating the cellular motility, and have suggested their possible contribution to tumor expansion, invasion, or metastasis.^{18,19}

The purpose of the present study is to elucidate the cell biological significance of the *K-ras* gene mutations in the development of lung adenocarcinoma. In our preliminary study, we tried to generate *K-rasV12* gene transfectants of the primary human airway epithelial cells. However, we failed to obtain stable gene transfectants, as the *K-rasV12* induced a non-apoptotic type of cell death with vacuolar degeneration in these cells (unpublished observations). This observation suggested that a *K-ras* gene mutation alone is insufficient to induce the development of lung adenocarcinoma, and that additional genetic abnormalities are necessary. Although the abnormality participating in this process has not been strictly identified, it has been reported that inactivation of tumor suppressors such as p53 and retinoblastoma protein (Rb) could be observed along with *K-ras* gene mutations in the human

lung adenocarcinomas.⁸⁻¹⁰ The large T antigen of simian virus 40 (SV40) has the potential to disrupt the cell cycle regulation by binding to and inactivating p53 and Rb protein, and to immortalize various types of human cells.²⁰ We have therefore used a SV40-immortalized human peripheral airway epithelial cell line (HPL1D)²¹ to evaluate the significance of *K-ras* gene mutations in the lung adenocarcinoma. The present study focused on the role of the K-ras-PI3K-Akt pathway in cell motility, since vigorous autonomous motility is one of the important features of malignant neoplasms.

Materials and Methods

Cell Lines and Culture

The simian virus 40 (SV40)-immortalized human peripheral airway cell line (HPL1D) was established by the procedure described previously.²¹ The lung adenocarcinoma cell lines A549 and H820 were obtained from Riken Cell Bank (Tsukuba, Japan), and TKB6 and TKB14 were kind gifts from Dr. Kanma (Tsukuba University, Tsukuba, Japan). The A549 and H820 cells have a *K-ras* gene codon 12 point mutation, and the TKB6 and TKB14 cells do not. These cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 medium (GIBCO, Rockville, MD) with 1% fetal bovine serum (FBS, GIBCO). The cells in a semi-confluent condition were used for all of the following examinations.

Cell Migration Assay

Transwell chambers (6-well, 8- μ m pore size, Becton Dickinson, Franklin Lakes, NJ) were used, and the upper and lower chambers were filled with DMEM/F12 containing 1% FBS. The cells were harvested by trypsinization, and suspended in the medium. Five hundred thousand cells were seeded into the upper chambers, and hepatocyte growth factor (HGF) (final concentration 20 ng/ml, R&D systems, McKinley, NE) was added into the lower chambers of the transwell insert. For inhibitory experiments, inhibitors of ras (manumycin, 50 μ M, Calbiochem, Darmstadt, Germany), MEK (PD98059, 50 μ M, Cell Signaling Technology, Beverly, MA), and/or PI3K (LY29004, 50 μ M, Cell Signaling Technology) were added to the lower chambers. After 18 hours of incubation, the cells were fixed with 4% (w/v) paraformaldehyde, and stained with hematoxylin. Non-migrated cells on the upper side of the membranes were wiped away. The membranes were mounted on glass slides, and cells that had migrated through the membranes were counted in five random fields at a magnification of $\times 100$, under a light microscope (Olympus, Tokyo, Japan).

Cell Growth and Cell Death Assay

Five hundred thousand cells were seeded into 18-well culture plates (which have the same diameter as the upper chamber of the transwells used in the migration assays). If needed, the inhibitors described above were

added to the medium at the same concentration. After 18 hours, the cells were harvested and stained with trypan blue. The numbers of living cells were determined as cell growth values, and the percentages of dead cells were determined as the cell death index.

Plasmid Vector Construct and Gene Transfection

K-ras cDNA with a codon 12 point mutation (GGT to GTT; glycine to valine, pSw11-1) was purchased from Riken Gene Bank. Using pSw11-1 as a template, mutated *K-ras* cDNA was amplified with primers containing restriction enzyme recognition sites, GACGGCCGAAGCTTGCTGAAAATGACTGAATATAAACTTG (underline shows the *Hind*III site) and CAGGATCCTCATTACATAATTACACACTTTG (underline shows the *Bam*HI site). The wild-type construct was also generated by PCR using pSw11-1 as a template with primers 5'-GACGGCCGAACGTTATGACTGAATATAAACTTGTGGTAGTTGGAGC-TGGTGGCGT (double underline shows codon 12) and 5'-CAGGATCCTCATTACATAATTACACACTTTG. The resultant PCR products were inserted into the mammalian expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA) at the position between the *Hind*III and *Bam*HI sites.

Akt1 cDNA was amplified by RT-PCR (RNA LA PCR kit, Takara, Kyoto, Japan) with primers containing restriction enzyme recognition sites 5'-GAGCCTCGGGCATCAT-GAGCGAC (underline shows *Bsp*HI site) and 5'-TAAAG-GCCGTGCTGCTGGCCGAGTAG. Template RNA prepared from HPL1D cells using an Isogen RNA Extraction Kit (Nippon Gene, Tokyo, Japan). The cDNA was inserted into pT7blue vector (Takara). A dominant-negative mutant of Akt1 (dnAkt; K179 mol/L) was generated from the Akt1 cDNA using an In Vitro Mutagenesis Kit (Takara) with a mismatch primer, 5'-CTACGCCATGATGATCCTCAA (underline shows codon 179). Then, the Akt or dnAkt cDNA was inserted into the pTriEx Vector (Takara) at the position between the *Nco*I and *Bam*HI sites to be tagged with polyhistidine. The Akt-His or dnAkt-His fusion construct was amplified by PCR with primers containing restriction enzyme recognition sites, 5'-AATCAAAG-GAGAGCTAGCATGAGCGACG (underline shows the *Nhe*I site) and 5'-TAGGCAGCCTGCACTTAAGGTTAATCAC (underlines shows the *Afl*II site), and was inserted into the expression vector pZeoSv(-) (Invitrogen) or pIRES-EGFP2 (Clontech, Palo Alto, CA). The constructions of the designed vectors were confirmed by DNA sequencing using a Dye-deoxy sequencing kit (Amersham Life Science, Piscataway, NJ). The genes were transfected into cells using Lipofectamine Plus reagents (GIBCO). To obtain the stable gene transfectants, the vectors pcDNA 3.1(K-ras/K-rasV12) and pZeo SV(-) (Akt-His/dnAkt-His) were linearized by *Ssp*I-digestion before use. The transfectants were selected with 1 mg/ml Neomycin (G418, Invitrogen) or Zeocin (Invitrogen). The expression of the desired genes was confirmed by Western blotting.

Western Blotting

The cultured cells were washed twice with cold 0.1 mol/L phosphate-buffered solution (pH 7.5), and were mixed with an appropriate volume of extraction buffer containing 10 mmol/L HEPES (pH 7.5), 0.1% Nonidet P-40 (NP-40), 1 mmol/L EDTA, 2.5 mmol/L EGTA, 1 mmol/L DTT, 0.5 mmol/L PMSF, 10 μ g/ml aprotinin, 1 mmol/L NaF, and 10 mmol/L β -glycerophosphate. After centrifugation, the supernatants were recovered as protein extracts. Equal volumes of 2X SDS buffer containing 0.05 mol/L Tris (pH 6.8), 2% SDS, 6% β -ME, 5% glycerol, and 0.005% BPB were added, and the mixture was boiled. Fifty micrograms of the extracts were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and then were blotted onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). The membranes were incubated with 1% nonfat milk in 0.01 mol/L Tris-buffered saline containing 0.1% Tween-20 (TBS-T) to block non-immunospecific protein binding, and then with 0.1 μ g/ml primary antibody against K-ras (Transduction Laboratories, Franklin Lakes, NJ), C-met (Fuji Drug, Tokyo, Japan), whole Akt, Ser473-phosphorylated Akt, whole, phosphorylated Erk1/2 (Cell Signaling Technology), C-terminal polyhistidine (Invitrogen), and β -actin (Sigma Japan, Kyoto, Japan). After washing with TBS-T, the membranes were incubated with animal-matched HRP-conjugated secondary antibodies (Amersham). Immunoreactivities were visualized with the enhanced chemiluminescence system (Amersham).

Densitometric Analysis

The expression levels of phosphorylated Akt and Erk1/2 were analyzed using NIH Image computer software. The expression levels were standardized relative to β -actin levels in the same cell lysates.

Human Lung Tumors

One hundred eight cases of lung tumors, which consisted of 5 atypical adenomatous hyperplasia (AAH), 4 bronchioalveolar carcinoma (BAC), 42 well (Well), 34 moderately (Mod), and 23 poorly (Por) differentiated adenocarcinomas were studied. These tumors were surgically resected in the Kanagawa Cardiovascular and Respiratory Disease Center Hospital during the period between 1987-1996. The resected materials were fixed with 10% formalin and embedded in paraffin. The paraffin sections were mounted on MPS-coated glass slides.

Immunohistochemistry

The paraffin sections were deparaffinized and rehydrated, and were then immersed in 3% hydrogen peroxide/methanol to block the endogenous peroxidase activities. After this step, microwave treatment was required to detect Akt, but not phospho Ser473 Akt (p-ser473Akt). Then the sections were briefly washed with TBS-T (described above for Western blotting), and were incubated

Table 1. Scoring of Immunoreactivities for p-ser473Akt

Positive area	Intensity	Score
Negative (0%)		0
Very focal (<5%)		0.5
Small area (<30%)	Modest	1.0
	Strong	1.5
Moderate (<60%)	Modest	2.0
	Strong	2.5
Diffuse (<100%)	Modest	3.0
	Strong	3.5

Positive area, the percentage of positive cells in the observed fields.

with 5% goat serum, followed by endogenous avidin and biotin masking treatment using an Avidin/Biotin Blocking Kit (Vector Laboratories, Burlingame, CA), to block the non-immunospecific protein binding. Rabbit polyclonal antibodies against Akt (Santa Cruz) and p-ser473Akt (Cell Signaling Technology) were incubated with the sections. The immunoreactivity for Akt was visualized by the avidin-biotin complex method using an LSAB 2 Kit (DAKO, Tokyo, Japan). To detect p-ser473Akt, the tyramide-coupled signal amplification procedure using a CSA Kit (DAKO) was used. Immunoreactivity for p-ser473Akt was scored according to the criteria described in Table 1.

Determination of the *K-ras* Gene Codon 12 Status

Among the 108 lung tumors described above, 68 tumors were examined for *K-ras* gene status. The mutations were screened by single-strand conformation polymorphism analysis (SSCP), and were confirmed by direct DNA sequencing. The genomic DNA of the lung tumors was extracted from the paraffin sections by the following method. The sections were deparaffinized and stained briefly with toluidine blue. The desired areas were dissected, and the fragments were incubated with DNA lysis buffer containing 10 mmol/L Tris-HCl (pH8.3), 1 mmol/L EDTA, 1% SDS, and 2 μ g/ μ l proteinase K. The sample DNA was purified by phenol/chloroform extraction and ethanol precipitation. One hundred thirty-four-bp DNA fragments surrounding *K-ras* gene codon 12 were amplified by PCR with the primers, 5'-GGCTGCTGAAAAT-GACTGAATATA and 5'-CAAGATTACCTCTATTGTT. Ten μ l of the PCR products were mixed with 5 μ l of denaturing solution containing 95% formamide, 0.05% bromophenol blue, 0.05% xylencyanol, and 20 mmol/L EDTA, and were boiled, then rapidly cooled on ice. The samples were subjected to 6% polyacrylamide gel electrophoresis, and then stained with silver (BioRad, Hercules, CA). For the samples showing abnormal band shifts, the DNA sequence of the PCR products was determined using a DNA sequencing kit (Amersham).

Statistical Analysis

The differences in mean values from the cell migration, cell growth and cell death assays, or of p-ser473Akt

immunoreactive scores were analyzed by the Student's *t*-test. The correlation between the immunoreactive score and lymphatic metastasis (N factor) was analyzed by Spearman's correlation coefficient. To evaluate the relationship between the immunoreactive score and survival distribution, the T1 cases, in which the patients died of causes other than lung adenocarcinoma, were excluded, and the data were examined by Kaplan-Meier's survival analysis with the log rank test. The differences with a *P* value less than 0.05 were considered significant in these analyses.

Results

Mutated *K-ras* Enhances Cell Motility via Akt Activation

The success of *K-ras* or *K-rasV12* gene transfections was confirmed by Western blotting. In the empty vector-transfected cells (HPL-E cell), the amount of endogenous *K-ras* was too small to detect under this experimental condition. Both *K-ras*- and *K-rasV12*-transfected cells (HPL-K and HPL-V12 cells) expressed the corresponding protein products at similar levels (Figure 1a).

Since we used HGF as a chemoattractant for the cell migration assays in the present study, the expression of the HGF receptor, c-met, in these transfectants was examined to exclude the possibility that the *K-ras* gene status could influence its expression. As shown in Figure 1a, the expression of c-met was not modified by *K-ras* or *K-rasV12* transfection.

HGF has been shown to promote the scattering of a wide variety of cells including the airway epithelial cells,²² and to simultaneously activate the ras- and ras-PI3K-Akt pathways.^{23,24} We therefore anticipated that airway cells bearing an activating mutation of the *K-ras* gene would be able to migrate well even in the absence of HGF. As expected, HPL-V12 cells migrated well through the pores of transwell membranes (6.5- and 3.5-fold better than HPL-E and HPL-K cells, respectively) under conditions without HGF stimulation (Figure 1, b and c). This enhanced migration of HPL-V12 cells was similar to that of HPL-K cells stimulated with HGF (Figure 1c). No difference in the cell growth of the transfectants was seen at least during the period of the migration assay (see Figure 2a).

To identify the pathways mediating the cell motility of these transfectants, the phosphorylation status of Akt and Erk1/2 was examined. Irrespective of the presence or absence of HGF, HPL-V12 cells showed a constitutively higher level of Akt phosphorylation compared to either HPL-E or HPL-K cells (Figure 1, d and e). Erk1/2, especially Erk1, phosphorylation was also slightly enhanced by *K-rasV12* transfection (Figure 1, d and e). The HGF treatment increased Akt phosphorylation in HPL-E and HPL-V12 cells. The phosphorylation levels reached the similar level of HPL-V12 cells at 90 minutes after the treatment (Figure 1, d and e), and reduced to the basal level at 6 to 12 hours (data not shown). Erk1/2 phosphorylation was also enhanced by the HGF treatment, but the change was modest in HPL-V12 cells compared to the

Figure 2. A: The effects of inhibitors of ras (manumycin, 50 μ mol/L, Mn), PI3K (LY294002, 10 μ mol/L, LY), and MEK (PD98059, 50 μ mol/L, PD) on the autonomous migration (top), cell growth (middle), and cell death induction (bottom). Three independent assays were performed. The mean values of the migration or growth levels relative to those of HPL-E without treatment (NT), and cell death index (%) were plotted. #1/#2, #1/#5; $P < 0.05$. **Bar**, SD. **B:** Effects of the inhibitors on phosphorylation status of Akt and Erk1/2. The protein extracts from the K-rasV12 cells treated or not with these inhibitors were resolved by SDS-PAGE, and blotted with antibodies against p-ser473Akt, p-Erk1/2, Akt, or Erk1/2.

other two cells (Figure 1, d and e). Our preliminary study showed that the elevation of Erk1/2 phosphorylation in HPL-V12 cells was very transient and the phosphorylation levels was reduced to almost the basal level by 30 minutes after the treatment (data not shown), while in contrast, it continued for 120 minutes in HPL-E and HPL-K cells (data not shown). The expression levels of whole Akt

Figure 1. A: Expression of K-ras and c-met in mock (HPL-E), K-ras (HPL-K) and K-rasV12 (HPL-V12) transfectants. **B:** Autonomous migrations of the transfectants. The cells that migrated through the pores of the transwell membranes were stained with hematoxylin; magnification, $\times 100$. **C:** The results of assays for autonomous and HGF-induced migration. Three independent assays were performed. The mean values of the ratio of stimulated to the autonomous migration of HPL-E cells (HGF (-)) are plotted. #/*; $P < 0.05$. **D:** Phosphorylation status of Akt and Erk1/2. The transfectants were treated with (HGF) or without (Ctl) 20 η g/ml HGF for 90 minutes. The protein extracts were resolved by SDS-PAGE, and blotted with antibodies against p-ser473Akt, p-Erk1/2, Akt, Erk1/2, or β -actin. **E:** Relative expression levels of p-ser473Akt (top) and p-Erk (Erk1, middle; Erk2, bottom). Three independent assays were performed. The expression levels were analyzed as described in Materials and Methods. The mean values of the expression level relative to that of HPL-E cells (HGF (-)) were plotted. #/*; $P < 0.05$. **Bar**, SD.

and Erk1/2 were not changed by K-ras or K-rasV12 gene transfection, or by HGF treatment (Figure 1d, the results of densitometric analysis are not shown).

To confirm the participation of the K-ras-PI3K and -MEK pathways in the cell migrations, the potential effects of inhibitors of ras (manumycin), PI3K (LY294002), and MEK (PD98059) were examined. Manumycin treatment dramatically reduced the autonomous cell migration in all of the transfectants (Figure 2a, top). Treatment with either LY29004 or PD98059 alone also reduced the cell migration, but had modest effects (Figure 2a, top). The combination of LY294002 and PD98059 showed results comparable to those with the manumycin treatment (Figure 2a, top). To exclude the possibility that their effects on cell growth and cell death influenced the results of the migration assays, we examined these cellular events under the same conditions. The treatments with the inhibitors also reduced and induced cell growth and cell death respectively, but the effects were mild and not significant compared to those on cell migration (Figure 2a). The suitability of the doses of inhibitors used was confirmed by Western blotting for p-ser473Akt and p-Erk1/2 (Figure 2b) in HPL-V12 cells.

We next examined the participation of Akt, rather than Erk1/2, to elucidate further the downstream mechanism mediating the enhancement of autonomous motility induced by the mutated K-ras, because Akt was constitutively and highly phosphorylated in HPL-V12 cells, as described above (Figure 1d). Polyhistidine-tagged Akt or dominant-negative mutant Akt (dnAkt)-transfected clones were generated from HPL-V12 cells. The success of the gene transfections was confirmed by Western blotting for Akt and the polyhistidine tag (His) (Figure 3a). The dnAkt-transfected HPL-V12 cells (V12/dnAkt) showed significant reductions of the autonomous migration by about 50% compared to the mock-transfected HPL-V12 cells (V12/Emp), while Akt-transfected HPL-V12 cells (V12/Akt) showed increased levels of migration compared to V12/Emp cells (Figure 3, b and c, top). No marked difference in cell growth or cell death was observed (Figure 3c, middle and bottom).

We further examined the potential roles of the K-ras-Akt pathway in the autonomous motility of lung adenocarcinoma cells (A549, H820, TKB6, and TKB14). A549 and H820 cells had *K-ras* gene mutations, but the others did not. Consistently, A549 and H820 cells migrated well compared to TKB6 and TKB14 cells (Figure 4a, top and b). Phosphorylated Akt was detected in A549 and H820 cells, but not in TKB6 or TKB14 cells (Figure 4c). No correlation between the cell growth and the cell migration was found among these cell lines (Figure 4b, bottom).

To confirm that Akt activity could facilitate the autonomous migration of A549 and H820 cells, Akt or dnAkt were transiently transfected using the pIRES-EGFP vector. In this experimental system, enhanced green fluorescence protein (EGFP) and the desired genes, Akt or dnAkt, could be independently expressed at once. The cells expressing EGFP were counted under fluorescence microscopy, and the numbers of cells successfully transfected were equalized before the migration assays. As shown in Figure 5, a significant reduction in the auto-

Figure 3. A: The protein extracts were resolved by SDS-PAGE, and blotted with antibodies against polyhistidine (His) or Akt. The faster migrating bands (#) are endogenous Akt, and the slower migrating bands (*) are exogenously expressed His-tagged Akt. V12/Emp, HPL-V12 transfected with the empty vector; V12/dnAkt, HPL-V12 with dominant-negative Akt; V12/Akt, HPL-V12 with wild-type Akt. **B:** Autonomous migration of V12/Emp, V12/dnAkt, and V12/Akt. The cells that migrated through the pores of the membranes were stained with hematoxylin; magnification $\times 100$. **C:** The cell migration (top), cell growth (middle), and cell death (bottom) of the transfectants. Three independent experiments were performed. The mean values of the ratio against the autonomous migration or growth value relative to those of HPL-E cells (described in Figure 1), and cell death index (%) are plotted. Bar, SD. #1/#2, #2/#3; $P < 0.05$.

nomous migration was observed in dnAkt-transfected A549 cells (Figure 5, a and b, top). Conversely, Akt transfection increased the levels of migration (Figure 5, a and b, top). The cell growth and (apoptotic) cell death were also modified by these gene transfections, but the changes were slight and not significant (Figure 5b, middle and bottom). Similar results were observed in an experiment using H820 cells (not shown).

Figure 4. **A:** Autonomously migrating lung adenocarcinoma cells with the K-ras gene mutation (A549 and H820) and those without the mutation (TKB6 and TKB14). The cells that migrated through the membranes were stained with hematoxylin; magnification $\times 100$. **B:** The autonomous migration (**top**) and cell growth (**bottom**) of the cell lines. Three independent experiments were performed. The mean values of the autonomous migration or growth value relative to those of A549 cells are plotted. **Bar**, SD. #1/#3, #1/#4, #2/#3, #2/#4; $P < 0.05$. **C:** Phosphorylation status of Akt in the cell lines. The protein extracts were immunoblotted with antibodies against p-ser473Akt, Akt, or β -actin.

Akt Activation Is an Early Event during Carcinogenesis

The expression of Akt was immunohistochemically detected in all of the 108 lung tumors examined (Figure 6a),

Figure 5. Effects of Akt/dnAkt on autonomous migration, growth, and apoptosis in A549 cells. The desired genes inserted into pIRES-EGFP vector ($10 \mu\text{g}$) were transfected into A549 cells by using lipofectamine plus reagent. After 18 hours, the cells were trypsinized, and 5×10^5 cells were subjected to the migration assay. The same numbers of cells were seeded onto 6-well plates to examine the influence of Akt and dnAkt on cell growth. A part of the cell suspension was centrifuged using cytopsin (Sakura, Tokyo, Japan) to mount cells on glass slides, and the cells were stained with 4,6-diamino-2-phenylindole (DAPI). Under fluorescent microscopic observation, cells expressing EGFP were counted. Transfection efficiencies were about 5 to 10%, and were not different among the mock, Akt, and dnAkt groups (data not shown). The cells that migrated through the pores of the membranes were observed by fluorescence microscopy, after 18 hours of incubation (**A**). The number of cells that expressed EGFP and passed through the membrane pores were counted and standardized by the transfection efficiency, and taken as migration values. Migration ratio (/mock cells) is plotted (**B, top**). The number of cells expressing EGFP in the 6-well plate after 18 hours of incubation were counted and standardized by the transfection efficiency, and were taken as growth values. Growth ratio (/mock cells) is plotted (**B, middle**). The percentage of cells showing the characteristics of apoptosis (nuclear condensation or fragmentation) among EGFP-expressing cells was determined as an apoptotic index (**B, bottom**). **Bar**, SD. #1/#2; $P < 0.05$.

and was also seen in non-neoplastic airway cells (data not shown). p-ser437Akt was expressed in 74 of the 108 lung tumors (68.5%, Figure 6a). The immunoreactive score for p-ser473Akt was considerably higher in the lung tumors compared to the non-neoplastic airway cells (Table 2). The pre-malignant and early lesions (AAH/BAC) also highly expressed p-ser473Akt with similar frequency and reactive score to those of advanced carcinomas (Table 2). No positive correlation between the p-ser473Akt expression and the lymphatic/organ metastasis (Table 2) or the 5-year survival was observed (Figure 6b).

Figure 6. A: Immunohistochemistry for p-ser473Akt and Akt. A positive signal for Akt was observed in the cytoplasm of both cases with (case 55) and without (case 39) K-ras gene mutations. Diffuse strong immunoreactivity for p-ser473Akt could be seen in case 55, but not in case 39. **B:** P-ser473Akt expression and prognosis. Seventy-two cases of T1 (TNM classification) were selected and divided into two groups; cases with low (score <2.0, **dashed line**, 49 cases) and cases with high (score $\geq$ 2.0, **solid line**, 23 cases) immunoreactivity. Kaplan-Meier survival analysis was performed ($P = 0.4307$).

K-ras gene mutations were seen in 7 of the 68 lung adenocarcinomas examined (10.3%). p-ser473Akt was expressed in 6 of 7 cases with the K-ras gene mutations (85.7%) and in 41 of 61 cases without such mutations (67.2%). The reactive score tended to be higher in adenocarcinomas with K-ras gene mutations compared to those without such mutations although the difference was not significant (Table 3).

Table 3. K-ras Gene Status and p-Akt Expression

Wild (61)	1.12 ± 1.13 (69%)*
Mutant (7)	1.91 ± 0.86 (87%)*
ND (40)	1.04 ± 1.08 (65%)

p-Akt score, mean value $\pm$ standard deviation; (), number of cases; (%), frequency of p-Akt positive cases; ND, not determined. *; $P = 0.137$.

Discussion

K-rasV12-transfected HPL1D cells (HPL-V12) showed significant increases in autonomous cell migration compared to either the mock- or wild-type K-ras-transfected cells (HPL-E or HPL-K), suggesting that the activating mutation of the K-ras gene could act as substitute for growth factor stimulation (Figure 1). Consistently, a downstream factor of K-ras, Akt, was constitutively and highly phosphorylated in the HPL-V12 cells even under conditions without HGF stimulation. On the other hand, the effect of K-rasV12 on Erk1/2 phosphorylation was modest (Figure 1). A previous study showed that the two pathways interact with each other, and that the Akt could negatively regulate the MEK-Erk1/2 pathway,²⁵ supporting our observation that the Akt pathway was predominantly activated in the HPL-V12 cells. We therefore concluded that the enhancement of autonomous migration induced by the mutated K-ras was more likely mediated through the PI3K-Akt pathway than MEK-Erk1/2 pathway. The results of the experiments using a PI3K inhibitor and/or Akt/dnAkt transfection confirmed the notion that participation of the PI3K-Akt in the enhancement of autonomous migration (Figure 3), and also supported this pathway functions in the lung adenocarcinoma cells bearing K-ras gene mutations (Figures 4 and 5). However, among these results, two discrepancies were noticed. First, K-rasV12 increased cell motility almost sevenfold, but the enhancement of Akt phosphorylation level was rather small (about 1.5-fold, Figure 1). It has been reported that Akt can interact with or activate several factors mediating cell motility such as Rac/cdc42, EDG-1, and so on.^{26,27} Thus, Akt may amplify the motility signal input from the K-ras-PI3K pathway by activating such different factors. The second discrepancy is that the dnAkt transfection more markedly reduced the cell migration than the treatment with PI3K inhibitor in the HPL-V12 cells (Figures 2 and 3). Although it could not be ruled out that difference in experimental conditions between

Table 2. Relationship between p-Akt Reactive Score and Clinicopathologic Data

Histological grade		Clinical grade (TNM classification)					
		T factor		N factor		M factor	
Nor (10)	$0.06 \pm 0.18^*$ (10%)	1 (43)	0.83 ± 0.87 (72%)	0 (56)	1.21 ± 1.09 (80%)	0 (95)	1.20 ± 1.11 (69%)
A/B (9)	$1.11 \pm 1.45^*$ (60%)	2 (36)	1.65 ± 1.29 (69%)	1 (15)	1.46 ± 1.15 (80%)	1 (2)	0.25 ± 0.35 (100%)
Wel (42)	$1.39 \pm 1.15^*$ (25%)	3 (12)	1.42 ± 1.43 (75%)	2 (23)	0.91 ± 1.03 (43%)	2 (0)	—
Mod (34)	$0.94 \pm 1.04^*$ (67%)	4 (6)	0.42 ± 0.49 (50%)	3 (4)	0.94 ± 1.04 (50%)	3 (1)	1.00 ± 0.00 (100%)
Por (23)	$1.15 \pm 1.13^*$ (59%)	ND (2)	—	ND (1)	—	ND (1)	—

p-Akt score, mean value $\pm$ standard deviation; (), number of cases; (%), frequency of positive cases; Nor, no-tumor lung epithelia; A/B, AAH + BAC; Wel, well; Mod, moderately; ND, not determined; Por, poorly differentiated carcinoma; *: $P < 0.05$ (Student's t test).

the inhibitor treatment and the gene transfection influenced the results, the downstream pathway of PI3K likely consists of different factors either facilitating or inhibiting cell motility, among which Akt is one of the major facilitating factors. Several recent studies have suggested that Akt could facilitate filamentous actin organization^{26–28} or could transactivate expression of the extracellular matrix and activate matrix metalloproteinases.¹⁸ We are interested in examining the participation of these events in the vigorous autonomous migration seen in HPL-V12 and adenocarcinoma cells bearing K-ras gene mutations. Aside from the PI3K-Akt pathway, the MEK pathway may be also involved in the mechanisms mediating cell motility, as the treatment with MEK inhibitors also reduced the migration of the immortalized airway cells (Figure 2). We thus are very interested in elucidating downstream factors of the MEK pathway involved in the cell motility in the airway cells, and are now examining possible contributions of Erk1, Erk2, Elk1, etc.

The present study further investigated the K-ras-Akt pathway *in vivo*. The results supported our *in vitro* observation that the mutated K-ras could constitutively activate Akt, as most of the adenocarcinomas with K-ras gene mutations showed p-ser473Akt expression (Table 3). However, this expression was also observed in many of those without the mutation (Table 2). This result suggests that there may be different factors other than the K-ras gene mutation activating Akt. The overexpression of c-erbB2,²⁹ and inactivating mutations or promoter methylation of phosphatase and tensin homologue deleted on chromosome 10 (PTEN).^{30–32} are thought to be involved in such K-ras gene mutation-independent Akt activation. Aside from the mechanism mediating its activation, the activated Akt was expected to contribute to pathological events such as expansion, invasion, or metastasis, since it can facilitate the motility of neoplastic cells. However, no correlation was found between the p-ser473Akt expression and the lymphatic/organ metastasis or the prognosis (Figure 6b). The activation of Akt should be an early event during the carcinogenic process, as p-ser473Akt was also expressed in pre-malignant or early lesions, and not only in advanced lung adenocarcinomas (Table 2). We have therefore concluded that the activation of Akt likely contributes to non-invasive expansion of the neoplastic cells along the alveolar septa, as is observed in the AAH and BAC. Additional genetic abnormalities are likely needed to acquire invasive or metastatic properties during the progression process.

In summary, the present study has provided two interesting findings. The first is that the K-ras-Akt pathway can also transmit signals mediating the cell motility, and not only cell survival or proliferation as is generally known.^{13,33,34} The second is that the Akt activation is one of the early events during the development of lung adenocarcinoma, and might support the non-invasive expansion of the neoplastic cells. There have only been a few studies examining the role of the K-ras-Akt pathway in the carcinogenic process of human malignancy.^{35–38} We believe that the present study is the first report describing the role of K-ras-PI3K-Akt pathway in the carcinogenesis of lung adenocarcinoma.

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