Amplification and Overexpression of *SKP2* Are Associated with Metastasis of Non-Small-Cell Lung Cancers to Lymph Nodes

Sana Yokoi,* Kohichiroh Yasui,*[†] Miki Mori,*
Toshihiko Iizasa,[‡] Takehiko Fujisawa,[‡] and
Johji Inazawa*[†]

From the Department of Molecular Cytogenetics,* Graduate School of Biomedical Science, Medical Research Institute, Tokyo Medical and Dental University, Tokyo; Core Research for Evolutional Science and Technology,[†] Japan Science and Technology Corporation, Kawaguchi; and the Department of Thoracic Surgery,[‡] Chiba University Graduate School of Medicine, Chiba, Japan

SKP2, an F-box protein constituting the substrate recognition subunit of the SCF^{SKP2} ubiquitin ligase complex, is implicated in ubiquitin-mediated degradation of the cyclin-dependent kinase inhibitor p27^{KIP1}. Our earlier studies revealed *SKP2* as a target gene within the 5p13 amplicon that is often seen in small-cell lung cancers. In the present study we examined amplification status and expression levels of *SKP2* in non-small-cell lung cancer (NSCLC) and investigated its clinicopathological significance in this type of tumor because amplification of DNA at 5p13 is observed frequently in NSCLCs as well as in small-cell lung cancers. *SKP2* exhibited amplification in 5 (20%) of 25 cell lines derived from NSCLC, and the transcript was overexpressed in 11 (44%) of the 25 lines. Moreover, expression of *SKP2* was up-regulated significantly in 60 primary NSCLC tumors as compared to nontumorous lung tissues ($P < 0.0001$). Elevated expression of *SKP2* correlated significantly with positive lymph node metastasis ($P = 0.007$), with stage II or higher of the international TNM classification ($P = 0.014$), with poor or moderate differentiation ($P < 0.001$), and with the presence of squamous cell carcinoma ($P = 0.037$). Reduction of *SKP2* expression by transfection of an anti-sense oligonucleotide inhibited invasion and migration of NSCLC cells in culture. Our results suggest that *SKP2* may be involved in progression of NSCLC, and that targeting this molecule could represent a promising therapeutic option. (Am J Pathol 2004; 165:175–180)

Carcinoma of the lung, one of the most common types of cancer worldwide, consists of two subtypes: small-cell lung cancer (SCLC) and non-small-cell lung cancer

(NSCLC). The latter category, which accounts for 75 to 80% of all lung cancers, includes squamous cell carcinoma, adenocarcinoma, and large-cell carcinoma, and prognosis is generally unfavorable for patients with NSCLC. Identification of specific genetic alterations could suggest optimal molecular targets for clinical management of this heretofore intractable type of cancer.

Amplification of chromosomal DNA is one of the mechanisms capable of activating genes whose overexpression contributes to development and progression of cancer. Recently we identified the gene encoding S-phase kinase-associated protein 2 (*SKP2*) as a target within an amplicon at 5p13 that is often observed in SCLCs;¹ *SKP2* was amplified in 44% and overexpressed in 83% of the primary SCLC tumors we examined. *SKP2*, a member of the F-box family is the substrate recognition subunit of the SCF^{SKP2} ubiquitin ligase complex.² This protein is implicated in ubiquitin-mediated degradation of the cyclin-dependent kinase (CDK) inhibitor p27^{KIP1}, and positively regulates the G₁/S transition.^{3–5} We demonstrated that expression of *SKP2* was inversely correlated with expression of p27^{KIP1} in SCLC cells, and down-regulation of the former by transfection of an anti-sense oligonucleotide inhibited the growth of SCLC cells in culture¹ and induced apoptosis in those cells.⁶ Our comparative genomic hybridization studies⁷ together with others^{8–11} had shown that amplification at 5p13 occurs frequently in NSCLCs as well as in SCLCs. These circumstances prompted us to examine amplification status and expres-

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S.Y. and K.Y. contributed equally to this work.

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Address reprint requests to Dr. Johji Inazawa, Department of Molecular Cytogenetics, Medical Research Institute, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan. E-mail: johinaz.cgen@mri.tmd.ac.jp.

sion levels of *SKP2* in NSCLC, and to investigate its clinicopathological significance in this type of tumor.

Materials and Methods

Cell Lines and Primary Samples

The 25 NSCLC cell lines chosen for this study included 10 derived from squamous-cell carcinomas (EBC1, HS-24, LK-2, PC10, HUT15, VMRC-LCP, LC-1 sq, ACC-LC-73, SK-MES-1, and Sq-1); nine from adenocarcinomas (11-18, A549, ABC-1, RERF-LC-MS, RERF-LC-OK, VMRC-LCD, PC14, HUT29, and SK-LC-3); and six from large-cell carcinomas (86-2, LU65, PC13, ACC-LC-33, NCI-H460, and LU99A). We obtained primary samples from 60 patients with NSCLC at the Chiba University Hospital, Chiba, Japan. Adjacent nontumorous lung tissues from 20 of those patients were used as controls. Before initiation of the present study, informed consent was obtained in the formal style approved by ethics committees.

Fluorescence in Situ Hybridization (FISH)

We performed FISH experiments for *SKP2*, using as a probe a bacterial artificial chromosome (BAC; RP11-36A10), as described previously.¹ Briefly, the probe was labeled by nick translation with biotin-16-dUTP (Roche Diagnostics, Tokyo, Japan) and hybridized to metaphase chromosomes. Hybridization signals for biotin-labeled probes were detected with fluorescein isothiocyanate-avidin (Boehringer Mannheim, Tokyo, Japan).

Southern and Northern Blot Analyses

Southern and Northern hybridizations of *SKP2* were performed as described previously.¹

Real-Time Quantitative Polymerase Chain Reaction (PCR)

We quantified genomic DNA and mRNA of *SKP2* using a real-time fluorescence detection method described previously.¹² Briefly, genomic DNA was isolated using the Puregene DNA isolation kit (Gentra, Minneapolis, MN). Total RNA was obtained using Trizol (Invitrogen, Carlsbad, CA). Residual genomic DNA was removed by incubating the RNA samples with RNase-free DNase I (Takara, Tokyo, Japan) before reverse transcription (RT)-PCR. Single-stranded complementary DNA (cDNA) was generated using Superscript II reverse transcriptase (Invitrogen) following the manufacturer's directions. Real-time quantitative PCR experiments were performed with an ABI Prism 7900 sequence detection system (Applied Biosystems, Foster City, CA), using SYBR Green PCR Master Mix according to the manufacturer's protocol. The primers were as follows: *SKP2* DNA (forward, 5'-ACTCTGCCTGCCACTCACTT-3' and reverse, 5'-CTCCACGGC-ATACTGTCTCA-3'); *SKP2* mRNA (forward, 5'-CGCTGC-

CCACGATCATTTAT-3' and reverse, 5'-TGCAACTTGGAACTAGAGACA-3'). *RNaseP* (Applied Biosystems) and *GAPDH* (Applied Biosystems) were used as the endogenous controls for genomic DNA and mRNA levels, respectively. Duplicate PCR amplifications were performed for each sample.

Anti-Sense Experiments

Anti-sense experiments targeting *SKP2* were performed as described previously.¹ Briefly, two oligonucleotides containing phosphorothioate backbones were synthesized (Espec Oligo Service Corp., Tsukuba, Japan): AS, 5'-CCTGGGGGATGTTCTCA-3' (the anti-sense direction of human *SKP2* cDNA nucleotides 180 to 196) and SC, 5'-GGCTTCCGGGCATTAG-3' (a scrambled control for AS).^{4,13} The oligonucleotides (AS or SC), 200 nmol/L each, were delivered into PC10 cells using oligofectamine reagent (Invitrogen) according to the manufacturer's instructions. Cells were harvested by trypsinization 48 hours after transfection to serve for invasion, migration, and viability assays. Western blotting of *SKP2* protein was performed as described previously.¹ Blots were reprobed with monoclonal antibody against β -actin (Sigma-Aldrich, St. Louis, MO) for internal control.

Invasion and Migration Assays

Membrane invasion assays were performed in Matrigel-coated chambers containing 8- μ m pores (BD Biosciences, Bedford, MA), according to the manufacturer's protocol. Forty-eight hours after treatment with *SKP2* AS or SC, PC-10 cells were harvested, counted, and resuspended in serum-free RPMI 1640 at the same concentration for each condition. RPMI 1640 supplemented with 10% fetal calf serum was added in the lower chamber of a 24-well culture plate as a chemoattractant, and the resuspended cells (4×10^4) were plated in the upper chamber. After a 22-hour incubation, noninvading cells were mechanically removed from the upper surface of the membrane with a cotton swab; cells attached to the lower surface of the membrane (invading cells) were fixed with 70% ethanol and stained with Giemsa. The invading cells were examined by light microscopy at $\times 200$ magnification, and 10 fields were counted per membrane. Each assay was performed in triplicate and the experiment was repeated at least three separate times. Cell migration was assayed by the same procedure, except that cells were seeded on 8- μ m-pore membranes that were not coated with Matrigel. Cells that were viable after the 22-hour incubation procedure were counted by the MTT assay (cell-counting kit-8; Dojindo Laboratories, Kumamoto, Japan) in 96-well plates, as previously described.¹

Statistical Analysis

All statistical analyses were performed using Stat-View 4.5 software (SAS Institute, Cary, NC). The relationship between the relative copy number and the relative expression level

Figure 1. Representative images of FISH for *SKP2* on metaphase chromosomes from two NSCLC cell lines, PC-10 (A) and HS-24 (B). Twin-spot FISH signals on sister chromatids numbered eight in PC-10 and five in HS-24.

was calculated using Spearman's test, with determination of correlation coefficients (r) and associated probability (P). Wilcoxon signed-rank tests or Mann-Whitney tests were used to compare mRNA levels of *SKP2* between tumorous and nontumorous tissues. Chi-square tests (and Fisher's exact probability tests when necessary) were used to evaluate associations between clinicopathological parameters and the expression level of *SKP2*. Ordinary one-way analysis of variance was used to determine significance within data groups in cell invasion, migration, and viability assays. P values of <0.05 were considered significant.

Results

Amplification and Overexpression of *SKP2* in NSCLC Cell Lines

Our earlier comparative genomic hybridization studies had shown that four NSCLC cell lines (ABC1, RERF-LC-

OK, HS-24, and LK-2) exhibited high-level gains, indicative of gene amplification, at 5p13 where *SKP2* is located.⁷ Therefore we determined copy numbers of *SKP2* by FISH in a larger panel of 25 NSCLC cell lines. In five lines including the above four and PC-10, the numbers of FISH signals ranged from five to seven, as representatively shown in Figure 1. The other 20 cell lines showed four or fewer FISH signals.

We next examined amplification of *SKP2* in all 25 NSCLC cell lines by Southern blotting (Figure 2A) and real-time quantitative PCR (Figure 2B). The gene exhibited amplification (ie, more than a twofold increase) in 5 (RERF-LC-MS, RERF-LC-OK, HS-24, LK-2, and PC-10) (20%) of the 25 lines compared with normal genomic DNA (Figure 2B). Expression levels of *SKP2* were determined by Northern blotting (Figure 2C) and real-time quantitative RT-PCR (Figure 2D). The transcript showed overexpression (ie, more than a fourfold increase) in 11 (44%) of the 25 lines compared with normal lung tissues (Figure 2D). The expression pattern of *SKP2* accorded with its amplification pattern (Figure 2; A to D). Significant correlation between amplification and expression was observed (Spearman's rank correlation test, $r = 0.544$; $P = 0.0078$; Figure 2E).

Expression of *SKP2* in Primary NSCLC Tumors

We determined expression levels of *SKP2* in paired tumor and nontumor tissues from 20 patients with NSCLC, using real-time quantitative RT-PCR. *SKP2* was significantly overexpressed in 19 (95%) of the tumors compared with their nontumorous counterparts (Wilcoxon signed-rank test, $P < 0.0001$) (Figure 3A). We quantified its transcript

Figure 2. Amplification and overexpression of *SKP2* in NSCLC cell lines, indicated by asterisks. **A:** Southern blots containing genomic DNA derived from 18 of the 25 cell lines examined and normal peripheral lymphocyte (N1). *GAPDH* was used as a control probe to eliminate loading differences on the blots. **B:** Relative copy number of *SKP2* determined by real-time PCR in the same cell lines shown in A and normal peripheral lymphocytes (N1 to N5). Results are presented as the ratio between *SKP2* and a reference gene (*RNase P*), and normalized in such a manner that the average ratio in five normal DNAs (N1 to N5) equals a value of 2. Two copies are indicated by a dotted line. More than four copies are defined as amplification. **C:** Northern blot analyses using total RNA from the same NSCLC cell lines; *GAPDH* again served as a quantity control probe. **D:** Relative expression levels of *SKP2* mRNA determined by real-time RT-PCR in the same cell lines and normal lung tissues (NL1, NL2). Results are presented as the ratio between *SKP2* and a reference gene (*GAPDH*), and normalized in such a manner that the average ratio in two normal lung tissues (NL1, NL2) equals a value of 1. A value of 4 was used to determine the cutoff for overexpression, shown as a dotted line. **E:** Significant correlation ($r = 0.54$, $P = 0.0078$) between copy numbers and relative expression levels of *SKP2* was observed in 25 NSCLC cell lines.

Figure 3. Overexpression of *SKP2* in primary NSCLC tumors. Levels of *SKP2* mRNA were evaluated by real-time quantitative RT-PCR and normalized according to levels of *GAPDH*. **A:** Relative expression of *SKP2* in paired tumor and nontumor tissues from 20 patients with NSCLC. **B:** Expression of *SKP2* in 60 primary NSCLC tumors relative to expression in 20 nontumorous lung tissues. **Solid horizontal lines** indicate the means of expression levels. The mean + 2 × SD of nontumorous tissues was used to determine the cutoff value for overexpression, shown as a **dotted line**.

in 40 additional tumors as well; *SKP2* was significantly up-regulated among the total of 60 tumors as compared to 20 nontumorous lung tissues (Mann-Whitney test, $P < 0.0001$) (Figure 3B).

Relationship between Expression Levels of *SKP2* and Clinicopathological Parameters

To clarify any potential relationship between elevated expression of *SKP2* and various clinicopathological parameters, we examined available data from 54 NSCLC patients, whose tumors were divided into high- and low-expression groups at the mean plus twice SD of levels of *SKP2* mRNA in 20 nontumorous controls (Figure 3B). Elevated expression of *SKP2* was significantly associated with positive lymph node metastasis ($P = 0.007$), stage II or higher in the international TNM classification ($P = 0.014$), poor or moderate differentiation ($P < 0.001$), and the presence of squamous cell carcinoma ($P = 0.037$) (Table 1). We observed no significant link with any other parameter such as patient's age and gender, smoking, or tumor status (international TNM classification).

Inhibition of Invasiveness and Migration of NSCLC Cells by Reduction of *SKP2* Expression with Anti-Sense Oligonucleotide

Because we had correlated elevated expression of *SKP2* with lymph node metastasis, we examined whether excess *SKP2* is involved in invasiveness. An anti-sense oligonucleotide (AS), but not a control oligonucleotide with scrambled sequence (SC), induced a decrease in *SKP2* protein in PC-10, a cell line that had shown amplification and consequent overexpression of the *SKP2* gene, 48 to 72 hours after transfection (Figure 4A). Invasion assays were performed during 22 hours (48 to 70 hours after transfection) as described in Materials and Methods. Because *SKP2* anti-sense treatment inhibited the growth of PC-10 cells,⁶ PC-10 cells treated with AS or SC were resuspended at the same concentration on the start in the invasion assay (48 hours after transfection). Reduction of *SKP2* protein inhibited the ability of cells to

Table 1. Relationship between Clinicopathological Features and Levels of Expression of *SKP2* in 54 NSCLCs

	<i>SKP2</i> mRNA		P^*
	Low (n = 15)	High (n = 39)	
Mean age ± SD	63 ± 12	65 ± 9	0.42
Sex			
Male	11	31	0.72
Female	4	8	
Smoking			
Smoker	9	33	0.72
Nonsmoker	6	6	
Tumor status			
T1	6	16	0.95
T2–4	9	23	
Lymph node status			
Negative	13	18	0.007
Positive	2	21	
Stage			
I	11	14	0.014
II–IIIb	4	25	
Grade			
Poorly and moderately differentiated	10	39	<0.001
Well differentiated	5	0	
Histology			
Adenocarcinoma	12	19	0.037
Squamous cell carcinoma	3	20	

* Chi-square test.

invade surrounding tissues, as determined by Matrigel assay (Figure 4, B and C). We examined cell migration as well, because it is an important component of the invasive process. PC-10 cells treated with AS demonstrated decreased ability to migrate through membranes of 8-μm-pore size that were not coated with Matrigel (Figure 4, B and D). AS and SC treatment had no significant effect on cell viability during the 22 hours of invasion and migration assays (Figure 4E), indicating that the anti-invasive and anti-migratory effects of AS were not because of suppression of cell growth.

Discussion

In the work reported here we showed that *SKP2* was amplified and consequently overexpressed in multiple NSCLC cell lines (Figures 1 and 2). The gene was always overexpressed in the cell lines in which it was amplified, as well as in some lines without amplification. Moreover, our results clearly showed that expression of *SKP2* was significantly up-regulated in primary NSCLC tumors compared with nontumorous lung tissues (Figure 3). These findings suggest that DNA amplification is one of the mechanisms capable of activating *SKP2* and that this gene is a probable target of the amplification event at 5p13 not only in SCLCs¹ but also in NSCLCs.

Several lines of evidence have suggested an oncogenic potential for *SKP2*.^{3, 14–16} Overexpression of *SKP2* has been observed in various types of human tumors, including colorectal carcinoma,¹⁷ oral squamous cell carcinoma,^{18,19} laryngeal squamous cell carcinoma,²⁰ lymphoma,^{15,21} gastric cancer,²² various soft-tissue sar-

Figure 4. Inhibition of invasion and migration of PC-10 cells by anti-sense oligonucleotide mediates reduction of SKP2 expression. **A:** Western blot analysis of SKP2 and β -actin as an internal control. PC-10 cells were treated with an anti-sense oligonucleotide (AS) targeting *SKP2*, a control oligonucleotide with scrambled sequence (SC), or oligofectamine alone (oligofectamine). Cells were harvested 48 hours, 60 hours, and 72 hours after transfection. Ten- μ g aliquots of each whole cell lysate were separated by 12.5% dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by immunoblotting. **B:** Invasive and migratory ability of PC-10 cells treated with AS, SC, or oligofectamine alone (control). The cells were placed in Matrigel-coated (for invasion assay; **top**) or uncoated (for migration assay; **bottom**) chambers containing 8- μ m pores. Invaded or migrated cells were stained with Giemsa and counted. **C, D, E:** Effect of AS or SC on the invasion (**C**), migration (**D**), and viability (**E**) of PC-10 cells. Results are expressed as percentages of oligofectamine alone (control), and values are represented as the mean $\pm$ SD of at least three independent experiments.

comas,²³ breast cancer,²⁴ and prostate cancer.²⁵ Expression of *SKP2* correlates with the grade of malignancy of lymphomas¹⁵ and oral squamous cell carcinomas.¹⁴ Elevated expression of *SKP2* indicates poor prognoses for patients with oral squamous cell carcinoma,^{18,19} laryngeal squamous cell carcinoma,²⁰ gastric cancer,²² soft-tissue sarcoma,²³ or prostate cancer.²⁵ Immunohistochemical analyses by other investigators have revealed that overexpression of *SKP2* was associated with lymph node metastasis in oral squamous cell carcinoma¹⁹ and laryngeal squamous cell carcinoma.²⁰

The present study is the first to show that elevated expression of *SKP2* is associated with indicators of malignancy in NSCLCs such as positive lymph node metastasis, advanced stage, and poor or moderate differentiation (Table 1). Metastasis to lymph nodes is a major obstacle for successful treatment of NSCLC and our results suggest that *SKP2* may be a useful diagnostic biomarker for metastatic potential.

We invoked an anti-sense strategy to investigate the effect of *SKP2* on the invasive ability of NSCLC cells that were overexpressing the gene, because invasion is an essential part of the metastatic process. Transfection of an anti-sense oligonucleotide (AS) into cultured PC-10 cells induced a decrease in SKP2 proteins and consequent inhibition of invasion and migration (Figure 4). Because *SKP2* anti-sense treatment inhibited the growth of PC-10 cells,⁶ we examined whether the anti-invasive and anti-migratory effects of AS were because of suppression of cell growth. Although viability of PC-10 cells treated with AS was reduced 96 hours after transfection,⁶ AS treatment had no significant effect on cell viability during a relatively short time (22 hours; 48 to 70 hours after transfection) of invasion and migration assays (Figure 4E). Other investigators have shown that cell lines derived from gastric carcinomas also gain a high potential for invasiveness and motility when transfected with *SKP2*.²² These findings strongly suggest that agents designed to inhibit SKP2 may represent a valid therapeutic option for patients with NSCLC.

The PTEN tumor suppressor negatively controls the phosphoinositide 3-kinase signaling pathway by dephosphorylating the 3 position of phosphoinositides.^{26,27} Interestingly, PTEN deficiency in mouse embryonic stem cells causes a decrease of p27 levels with concomitant increase of *SKP2*.²⁸ It has been shown that PTEN had an effect on the cytoskeleton and had a role in controlling cell migration. Introducing PTEN into PTEN^{-/-} human tumor cells alters actin fibers and inhibits cell migration.²⁹ Migration is increased in mouse embryo fibroblasts that lack PTEN. In these cells, elevated PtdIns(3,4,5)P₃ leads to activation of small GTPase mediators of cellular migration.³⁰ *SKP2* may function as a critical component in the PTEN/PI 3-kinase pathway for invasion and migration through an unknown mechanism.

Among the primary NSCLC tumors we examined, elevated expression of *SKP2* was more frequent in squamous cell carcinomas than in adenocarcinomas (Table 1). However previous comparative genomic hybridization studies of NSCLCs had shown that DNA copy number gains at 5p13 were equally frequent in adenocarcino-

mas.¹¹ A larger study will be necessary for assessing differences in *SKP2* expression between these two types of lung cancer.

The quantitative real-time RT-PCR method is accurate and sensitive, with a wide dynamic range, and it enables us to evaluate levels of *SKP2* mRNA using even a small number of samples from primary NSCLCs. On the other hand, immunohistochemistry usually results in semiquantitative data only, and its reliability depends on the antibody and detection system used. However, as immunohistochemical analysis does reveal protein stability, intratissue distribution, or subcellular localization, we will use this method for investigating expression of *SKP2* protein in primary NSCLCs. Our results suggest that overexpression of *SKP2* plays an important role in progression of NSCLC and that its product could represent an optimal target for development of novel therapies for this widespread type of cancer.

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