

# Progesterone Receptor in Non–Small Cell Lung Cancer—A Potent Prognostic Factor and Possible Target for Endocrine Therapy

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## Abstract

A possible involvement of gender-dependent factors has been postulated in development of human non–small-cell lung cancers (NSCLC), but its details remain unclear. In this study, we examined biological significance of progesterone receptor in NSCLCs. Progesterone receptor immunoreactivity was detected in 106 of 228 NSCLCs (46.5%). Progesterone receptor–positive NSCLC was frequently detected in female and adenocarcinoma, and was inversely associated with tumor-node-metastasis stage and histologic differentiation. Progesterone receptor status was also associated with better clinical outcome of the patients, and a multivariate analysis revealed progesterone receptor status as an independent prognostic factor. Progesterone-synthesizing enzymes were detected in NSCLCs, and tissue concentration of progesterone was higher in these cases ( $n = 42$ ). Immunoblotting analyses showed the presence of progesterone receptor in three NSCLC cell lines (A549, LCSC#2, and 1-87), but not in RERF-LC-OK or PC3. Transcriptional activities of progesterone receptor were increased by progesterone in these three progesterone receptor–positive NSCLC cells by luciferase assays. Cell proliferation was inhibited by progesterone in these progesterone receptor–positive NSCLC cells in a dose-dependent manner, which was inhibited by progesterone receptor blocker. Proliferation of these tumor cells injected into nude mice was also dose-dependently inhibited by progesterone, with a concomitant increase of p21 and p27 and a decrease of cyclin A, cyclin E, and Ki67. Results of our present study suggested that progesterone receptor was a potent prognostic factor in NSCLCs and progesterone inhibited growth of progesterone receptor–positive NSCLC cells. Therefore, progesterone therapy may be clinically effective in suppressing development of progesterone receptor–positive NSCLC patients. (Cancer Res 2005; 65(14): 6450–8)

## Introduction

Lung cancer is one of the leading causes of cancer death throughout the world. Despite recent advances in its treatment, its prognosis remains dismal (1–3). Therefore, it becomes very important to examine the details of biological features of lung carcinoma cell proliferation and to develop the targeted therapies

aimed at the specific proteins involved in these biological behaviors. Lung cancer is histologically classified into small-cell lung carcinoma and non–small-cell lung carcinoma (NSCLC). NSCLC accounts for ~80% of all the lung cancer cases, and represents heterogeneous groups which include the squamous cell carcinoma, adenocarcinoma, and large cell carcinoma. Squamous cell carcinoma is markedly associated with smoking and more frequently detected in males, but adenocarcinoma tends to occur more frequently in female, suggesting a possible involvement of gender-dependent factors in the pathogenesis and/or development of NSCLCs.

It is well known that sex steroids play important roles in various human tissues as gender-dependent factors. Among sex steroids, estrogens greatly contribute to development of hormone-dependent breast and endometrial carcinomas through an initial interaction with estrogen receptor  $\alpha$  and/or  $\beta$  (4), whereas androgens play important roles in the development of prostate cancers. In contrast, progesterone generally promotes differentiation and inhibits cellular proliferation through the progesterone receptor (5). Previous studies showed that progesterone-mediated growth inhibition was mainly preceded by decreased expression of cyclins A, D1, and E and/or induction of cyclin-dependent kinase inhibitors such as p21 and p27 (6–8), and administration of progestins, including medroxyprogesterone acetate, has been currently done as an endocrine therapy in breast and endometrial carcinoma patients (9, 10).

Immunohistochemical examination of sex steroid receptors has been reported in NSCLC tissues by several investigators (11–14). However, the lung tissue is not generally considered a target for sex steroids, and biological significance of sex steroid receptors remains unclear in NSCLCs. In this study, we examined immunolocalization of progesterone receptor and estrogen receptors in 228 cases of NSCLCs, and showed that progesterone receptor was a potent prognostic factor. We also revealed that progesterone inhibited the growth of progesterone receptor–positive NSCLC cell lines, and proposed that progesterone receptor is a possible target for progesterone therapy in NSCLC patients.

## Materials and Methods

**Patients and tissue specimens.** Two-hundred twenty eight specimens of NSCLC were obtained from patients who underwent surgical resection from 1993 to 1995 in the Department of Surgery, Sendai Kousei Hospital, Sendai, Japan. A mean age of the patients was 65.5 years (range 23–82). Patients examined in this study did not receive irradiation or chemotherapy before surgery. The mean follow-up time was 1,600 days (range 17–3,695 days). All specimens were fixed in 10% formalin and embedded in paraffin wax. Frozen tissues were also available in 42 cases. Research protocols for this study were approved by the Ethics Committee at both Tohoku University School of Medicine and Sendai Kousei Hospital, Sendai, Japan.

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**Immunohistochemistry.** The specimens for immunohistochemistry were fixed in 10% formalin and embedded in paraffin wax, and streptavidin-biotin amplification method was employed for immunostaining using a Histofine Kit (Nichirei Co. Ltd., Tokyo, Japan). Monoclonal antibodies for progesterone receptor (MAB429), estrogen receptor  $\alpha$  (6F11), estrogen receptor  $\beta$  (14C8), Ki67 (MIB1), and p21 (15091A) were purchased from Chemicon (Temecula, CA), Novocastra (Newcastle, United Kingdom), GeneTex (San Antonio, TX), DAKO (Carpinteria, CA), and BD Biosciences Pharmingen (San Diego, CA), respectively. p53 (DO-7), p27 (1B4), cyclin A (6E6), cyclin D1 (P2D11F11), and cyclin E (13A3) were purchased from Novocastra. The characteristics of polyclonal antibody for steroidogenic acute regulatory protein (StAR), P450 side chain cleavage (P450scc), and 3 $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ HSD) were described previously (15, 16). The antigen-antibody complex was subsequently visualized with 3,3'-diaminobenzidine solution and counterstained with hematoxylin.

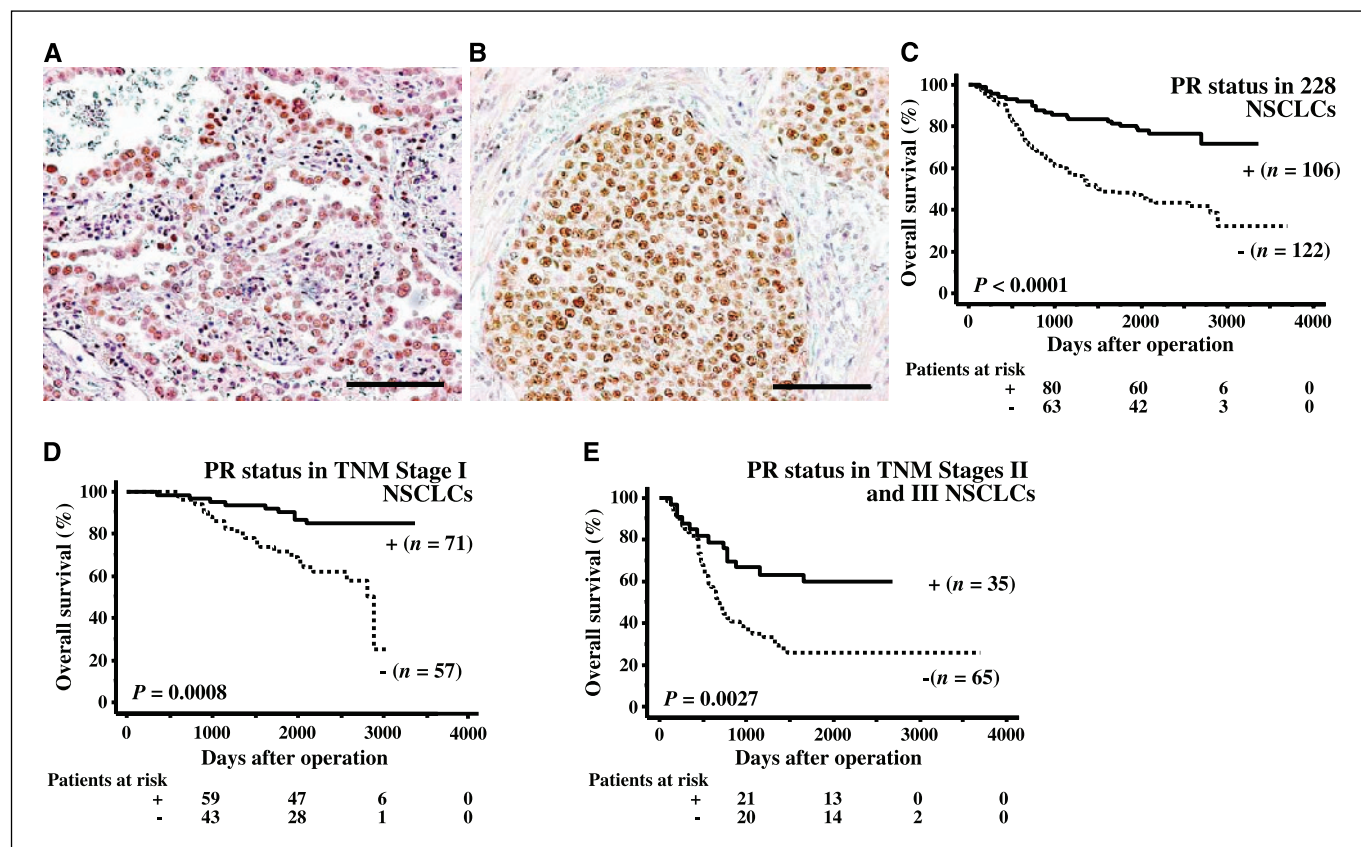
Immunoreactivity for progesterone receptor, Ki67, p21, p27, p53, and cyclins A, D1, and E was scored in more than 1,000 carcinoma cells for each case, and the percentage of immunoreactivity (i.e., labeling index (LI)) was determined. Cases that have progesterone receptor or estrogen receptor LI less than 10% were determined as progesterone receptor or estrogen receptor negative, according to a report by Allred et al. (17). Statistical analyses were done using a one-way ANOVA and Bonferroni test or a cross-table using the  $\chi^2$  test. Overall and disease-free survival curves were generated according to the Kaplan-Meier method. Univariate and multivariate analyses were done by a proportional hazard model (Cox) using PROC PHREG in our SAS software.  $P < 0.05$  was considered significant in this study.

**Tissue concentrations of progesterone.** Tissue concentrations of progesterone were examined in 42 cases of NSCLC in which sufficient amounts of frozen specimens were available for examination. The cytosol

and nuclear fractions were prepared by centrifugation of homogenates (1.0 g specimens in 10 mL cold physiologic saline) at 4°C for 60 minutes at 15,000 rpm in an ultracentrifuge and progesterone concentrations were determined by a Progesterone EIA kit (Cayman Chemical Company, Miami, FL). The minimum concentration of progesterone which can be detected by this kit is 10 pg/mL. The specificity is as follows: progesterone, 100%; pregnenolone, 61%; 5 $\alpha$ -Pregnan-3 $\alpha$ ,20 $\alpha$ -diol, <0.01%; 5 $\beta$ -Pregnan-3 $\alpha$ ,20 $\alpha$ -diol, <0.01%; and 5 $\beta$ -Pregnan-3 $\alpha$ ,20 $\alpha$ -diol glucuronide, <0.01%, according to the information from the manufacturer.

**Cell culture.** Five human NSCLC cell lines [A549, LCSC#2, 1-87, RERF-LC-OK, and PC3, which differs from PC-3 prostate cancer cell line (ATCC CRL-1435); refs. 18, 19] were available in this study. Histologic type of the original tumor was adenocarcinoma in these five cell lines, and patient gender of each cell line was as follows: A549, male; LCSC#2, female; 1-87, male; RERF-LC-OK, female; and PC3, female. These cell lines were cultured in RPMI 1640 (Sigma, St. Louis, MO) with 10% fetal bovine serum (FBS). Immunohistochemistry for progesterone-producing enzymes in NSCLC cells was done in the cell blocks which were fixed in formalin and embedded with paraffin wax.

**Immunoblotting.** The cell protein was extracted in triple detergent lysis buffer (20) at 4°C. Twenty micrograms of the protein (whole cell extracts) were subjected to SDS-PAGE (10% acrylamide gel). Following SDS-PAGE, proteins were transferred onto Hybond P polyvinylidene difluoride membrane (Amersham Biosciences, Piscataway, NJ). The blots were blocked in 5% nonfat dry skim milk for 1 hour at room temperature, and were then incubated with primary antibodies for progesterone receptor, estrogen receptor  $\alpha$ , or estrogen receptor  $\beta$  for 18 hours at 4°C. After incubation with anti-mouse immunoglobulin G horseradish peroxidase (Amersham Biosciences) for 1 hour at room temperature, antibody/protein complexes on



**Figure 1.** Immunohistochemistry of progesterone receptor in NSCLCs. A and B, immunoreactivity for progesterone receptor was detected in the nuclei of carcinoma cells in adenocarcinoma (A) or squamous cell carcinoma (B). Bar, 100  $\mu$ m. C to E, overall survival curves (Kaplan-Meier method) of NSCLC patients showed that progesterone receptor status was significantly associated with a better clinical outcome of the patients ( $P < 0.0001$ ; C), and a similar tendency was also detected in the group of TNM stage I ( $n = 128$ ;  $P = 0.0008$ ; D) or TNM stages II and III ( $n = 100$ ;  $P = 0.0027$ ; E).

**Table 1.** Association between progesterone receptor status and clinicopathologic variables in 228 NSCLCs

	Progesterone receptor status		<i>P</i>
	Positive ( <i>n</i> = 106)	Negative ( <i>n</i> = 122)	
Gender			
Male	67 (29.7%)	98 (42.8%)	0.0045
Female	39 (17.0%)	24 (10.5%)	
Premenopausal	3 (1.3%)	2 (1.6%)	
Postmenopausal	36 (15.8%)	22 (9.6%)	
Age*	65.7 ± 0.9	65.4 ± 0.9	0.7892
TNM stage			
I	71 (31.4%)	57 (25.0%)	0.0085
II	11 (4.8%)	14 (6.1%)	
III	24 (10.5%)	51 (22.3%)	
Histologic type			
Adeno	76 (33.6%)	59 (25.8%)	0.0002
Large	11 (4.8%)	11 (4.8%)	
SCC	19 (8.3%)	52 (22.7%)	
Histologic differentiation†			
Well	31 (15.0%)	21 (10.1%)	0.0355
Moderately	22 (10.6%)	40 (19.3%)	
Poorly	43 (20.8%)	49 (23.8%)	
Estrogen receptor α status			
Positive	47 (20.6%)	40 (17.6%)	0.1780
Negative	59 (25.9%)	82 (36.0%)	
Estrogen receptor β status			
Positive	37 (16.2%)	40 (17.6%)	0.7358
Negative	69 (30.3%)	82 (36.0%)	
Ki67 LI (%)*	18.9 ± 1.7	26.9 ± 1.7	0.0010
p53 status			
Positive	76 (33.3%)	80 (35.1%)	0.3202
Negative	30 (13.2%)	42 (18.4%)	

Abbreviations: Adeno, adenocarcinoma; Large, large cell carcinoma; SCC, squamous cell carcinoma.

\*Data were presented as mean ± 95% confidence interval (95% CI), and were statistically evaluated using a one-way ANOVA and Bonferroni test. All other values represented the number of cases and percentage compared with the total number, and were evaluated using a cross-table using the  $\chi^2$ .

†Cases of Large were excluded (*n* = 206, in total). Status of steroid receptors was evaluated by immunohistochemistry.

the blots were detected using ECLplus Western blotting detection reagents (Amersham Biosciences). T47D cells were used as positive controls for progesterone receptor and estrogen receptors (21, 22). Equal loading of protein in each lane was confirmed by probing the membrane with anti-human β-actin monoclonal antibody (Sigma).

**Luciferase assay.** Progesterone-responsive reporter plasmids pHRE (hormone responsive element)-Luc (23) were kindly provided by Dr. Hirotohi Tanaka (Division of Clinical Immunology, Advanced Clinical Research Center, Institute of Medical Science, University of Tokyo, Tokyo, Japan). Estrogen-responsive reporter plasmids pERE (estrogen-responsive element)-Luc (24) were also used in this study. Luciferase assay was done according to a previous report (25) with some modifications. Briefly, 1 μg of pHRE-Luc or pERE-Luc plasmids and 200 ng of pRL-TK control plasmids (Promega Co., Madison, WI) were used to measure the transcriptional activity of endogenous progesterone receptor or estrogen receptor. Transient transfections were carried out using *TransIT-LT* transfection reagents (TaKaRa, Tokyo, Japan), and the luciferase activity of lysates was

measured using a Dual-Luciferase Reporter Assay system (Promega) and Luminescence-PSN (AB-2200; ATTO Co., Tokyo, Japan) after incubation with medium containing 1 μmol/L progesterone or 10 nmol/L estradiol for 24 hours. The cells were also treated with the same volume of ethanol for 24 hours as controls. The transfection efficiency was normalized against Renilla luciferase activity using pRL-TK control plasmids, and the luciferase activity was evaluated as a ratio (%) compared with that of controls. T47D cells were also used as positive controls for the transcriptional activity.

**Analysis for progesterone production.** Progesterone production in the NSCLC cells was examined according to previous reports (26). Briefly, after 2, 4, 8, and 24 hours of treatment with 40 mg cholesterol/dL of low-density lipoprotein, the medium was removed and purified by Oasis HLB Plus cartridges (Waters Corp. Massachusetts), and progesterone concentration was evaluated by a Progesterone EIA kit (Cayman Chemical).

**Cell proliferation assay and cell cycle analysis.** NSCLC cells were cultured in phenol red-free RPMI 1640 without FBS for 24 hours following 48 hours of culture with 10% FBS, and the medium was replaced again with either vehicle (0.1%), an indicated concentration of progesterone (Sigma-Aldrich Co., St. Louis, MO), or progesterone (1,000 nmol/L) with RU 38,486 (1,000 nmol/L). Following 12, 24, 48, and 72 hours of incubation, the cells were counted using a Cell Counting Kit-8 system (Dojindo Technologies, Kumamoto, Japan).

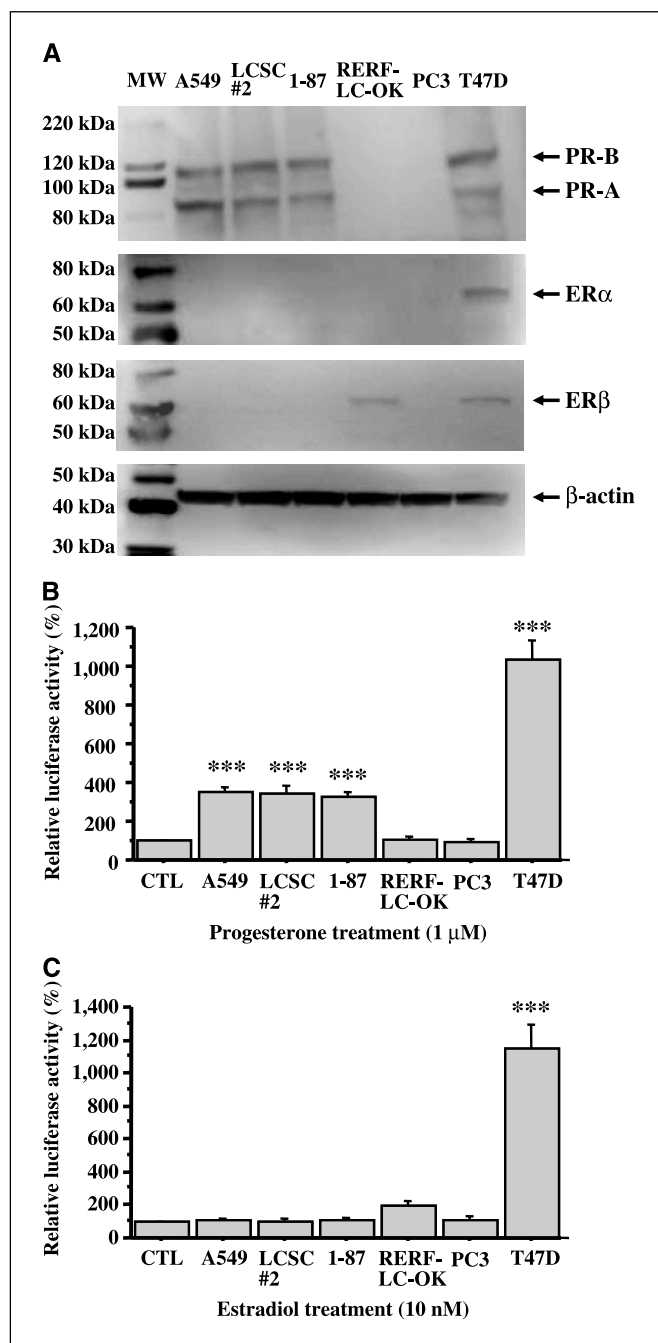
Cell cycle perturbations were determined at 72 hours after treatment with progesterone using FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Data were obtained and processed using the Lysis II software (Becton Dickinson). The percentage of cells in each cell cycle phases was evaluated on a DNA linear plot using the CellFit software (Becton Dickinson).

**Table 2.** Univariate and multivariate analyses for clinical outcome in NSCLC patients

Variable	Overall survival		Relative risk (95% CI)
	Univariate	Multivariate	
	<i>P</i>	<i>P</i>	
TNM stage	<0.0001*	<0.0001*	3.127 (1.959-4.992)
(II and III/I)			
Progesterone receptor status (negative/positive)	<0.0001*	0.0001*	2.717 (1.657-4.459)
Histologic differentiation (poorly/well and moderately)	0.0028*	0.5809	
Estrogen receptor β status (negative/positive)	0.1463		
Ki67 LI (≥10%/<10%)	0.1729		
Gender (male/female)	0.2827		
p53 status (positive/negative)	0.4513		
Histologic type (large/adeno and SCC)	0.6358		
Estrogen receptor α status (positive/negative)	0.7321		

\*Data were considered significant in the univariate analyses, and were examined in the multivariate analyses.





**Figure 2.** Progesterone receptor and estrogen receptor expression in NSCLC cells. **A**, immunoblotting for progesterone receptor, estrogen receptor  $\alpha$ , and estrogen receptor  $\beta$  in A549, LCSC#2, 1-87, RERF-LC-OK, and PC3 cells. Both progesterone receptor-A and progesterone receptor-B immunoreactivities were detected in A549, LCSC#2, and 1-87 cells, whereas estrogen receptor  $\beta$  was present only in RERF-LC-OK cells. Estrogen receptor  $\alpha$  was not detected in any of the NSCLC cell lines examined in this study. T47D breast carcinoma cells were used as positive controls. Immunoblotting for  $\beta$ -actin ( $\sim 42$  kDa) was also done as an internal standard of the experiment. Twenty micrograms of protein were loaded on each lane. **B** and **C**, luciferase analyses for transcriptional activity of progesterone receptor (**B**) or estrogen receptor (**C**) in A549, LCSC#2, 1-87, RERF-LC-OK, and PC3 cells. Cells were transiently transfected with pHRE-Luc plasmids (**B**) or pERE-Luc plasmids (**C**), and treated with 1  $\mu$ M/L progesterone (**B**) or 10 nmol/L estradiol (**C**) for 24 hours. The luciferase activity was evaluated as a ratio (%) compared with that of controls (CTL, treatment without progesterone or estradiol for 24 hours). T47D cells were used as a positive control of this experiment. Columns, mean ( $n = 3$ ); bars, SD. \*\*\*,  $P < 0.001$  versus CTL.

**Athymic mouse model.** A549, LCSC#2, 1-87, or RERF-LC-OK cells were resuspended in phenol red-free Matrigel [Becton Dickinson, Bedford, MA;  $1 \times 10^7$  (0.1 mL)/site] and placed on superior side of ovariectomized BALB/c nu/nu athymic female mice (5 weeks of age; Charles River Laboratories, Tokyo, Japan). A progesterone pellet (5, 50, or 200 mg; Innovative Research of America, Toledo, OH) was implanted when tumors measured 5 mm in diameter. Progesterone pellets of 5, 50, and 200 mg used in this study continuously elevate serum progesterone levels up to  $\sim 40$  nmol/L (equivalent to serum progesterone concentration at the luteal phase of normal cycling premenopausal women), 350 nmol/L, and 1,200 nmol/L, respectively, corresponding to  $\sim 8$  times higher than the third trimester of pregnancy.<sup>5</sup> The tumor volumes were monitored weekly using the formula for a semiellipsoid ( $4/3 \pi r^3/2$ ). Tumor tissues were resected at 5 weeks after treatment, and were subsequently fixed in 10% formalin and embedded in paraffin wax to perform immunohistochemistry.

## Results

**Immunohistochemistry for progesterone receptor and estrogen receptors in non-small-cell lung cancers.** Progesterone receptor immunoreactivity was detected in the nuclei of carcinoma cells (Fig. 1A and B), and progesterone receptor was detected in 106 of 228 (46.5%) NSCLC cases examined in this study. Progesterone receptor immunoreactivity was significantly associated with patient gender ( $P = 0.0045$ ), and was frequently detected in female. Progesterone receptor immunoreactivity was also significantly detected in adenocarcinoma ( $P = 0.0002$ ), and was inversely associated with tumor-node-metastasis (TNM) stage ( $P = 0.0085$ ), histologic differentiation ( $P = 0.0355$ ), and Ki67 LI that indicates activity of carcinoma cell proliferation ( $P = 0.0010$ ; Table 1). Hormone replacement therapy was administered only in three female patients in this study, and two of that three cases were immunohistochemically positive for progesterone receptor. The mean value of progesterone receptor LI in 228 NSCLCs examined was 15.7% (range 0-93%), and similar statistical association was also detected between progesterone receptor LI and clinicopathologic variables examined in this study (data not shown).

Estrogen receptor  $\alpha$  and estrogen receptor  $\beta$  immunoreactivities were detected in the nuclei of carcinoma cells, and the number of estrogen receptor  $\alpha$ -positive and estrogen receptor  $\beta$ -positive NSCLCs was 87 (38.2%) and 77 (33.8%) of 228 cases, respectively. Estrogen receptor  $\beta$  immunoreactivity was significantly associated with female ( $P = 0.0304$ ) or adenocarcinoma ( $P = 0.0133$ ) and was inversely correlated with histologic differentiation of carcinoma ( $P = 0.0484$ ). Estrogen receptor  $\beta$  immunoreactivity tended to be positively associated with estrogen receptor  $\alpha$  immunoreactivity, but this association did not reach a statistical significance ( $P = 0.1139$ ). No significant association was detected between estrogen receptor  $\beta$  immunoreactivity and other clinicopathologic variables examined. Estrogen receptor  $\alpha$  immunoreactivity also tended to be associated with female ( $P = 0.1338$ ), but it was not significantly associated with any clinicopathologic variables of the patients examined in this study.

**Correlation between progesterone receptor and estrogen receptor status and clinical outcome of non-small-cell lung cancer patients.** Progesterone receptor status was significantly associated with better clinical outcome of the 228 NSCLC patients ( $P < 0.0001$ ; Fig. 1C). This association was detected regardless of TNM stage in this study (Fig. 1D and E). Following a univariate analysis, TNM stage ( $P < 0.0001$ ), progesterone receptor status

<sup>5</sup> Unpublished data from Innovative Research of America, Toledo, Ohio.

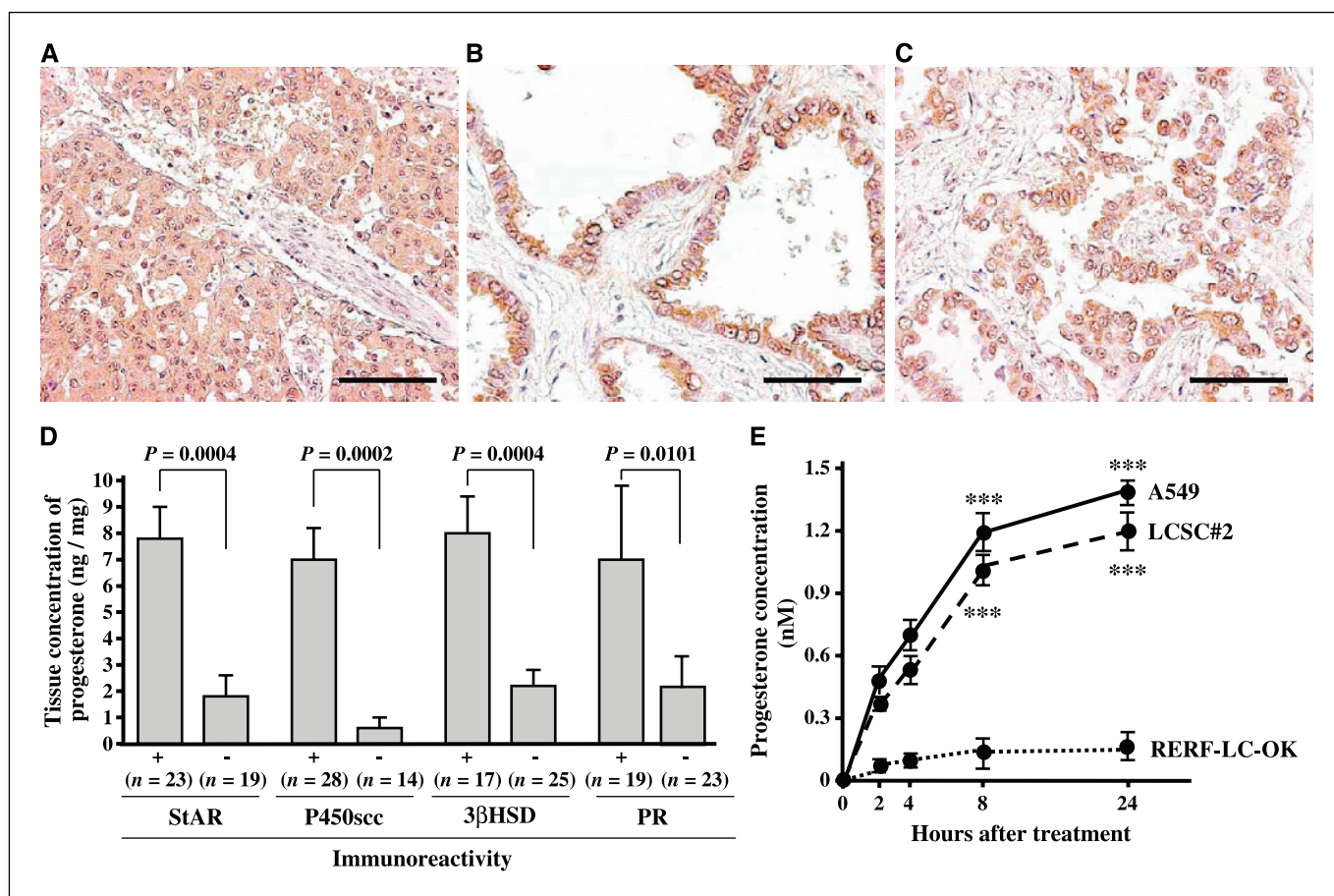
( $P < 0.0001$ ), and histologic differentiation ( $P = 0.0028$ ) were shown as significant prognostic factors for overall survival in this study (Table 2). Estrogen receptor  $\beta$  status tended to be associated with better clinical outcome of the patients ( $P = 0.1463$ ), but this association did not reach a statistical significance. No significant association was detected between estrogen receptor  $\alpha$  status ( $P = 0.7321$ ) or gender ( $P = 0.2827$ ) and clinical outcome of the patients. A multivariate analysis revealed that TNM stage ( $P < 0.0001$ ) and progesterone receptor status ( $P = 0.0001$ ) were independent prognostic factors with relative risks over 1.0.

**Expression of progesterone receptor and estrogen receptors in non-small-cell lung cancer cell lines.** We did immunoblotting analyses in A549, LCSC#2, 1-87, RERF-LC-OK, and PC3 cells to examine the expression of progesterone receptor and estrogen receptors in NSCLC cell lines. Two subtypes of progesterone receptor (progesterone receptor-A and progesterone receptor-B), estrogen receptor  $\alpha$ , and estrogen receptor  $\beta$  were detected as a specific band of approximately 85 kDa (27), 116 kDa (27), 66 kDa (28), and 65 kDa (14), respectively (Fig. 2A). Both progesterone receptor-A and progesterone receptor-B were detected in A549, LCSC#2, and 1-87 cells, but progesterone receptor was not detected in RERF-LC-OK or PC3 cells. Estrogen receptor  $\beta$  was detected only

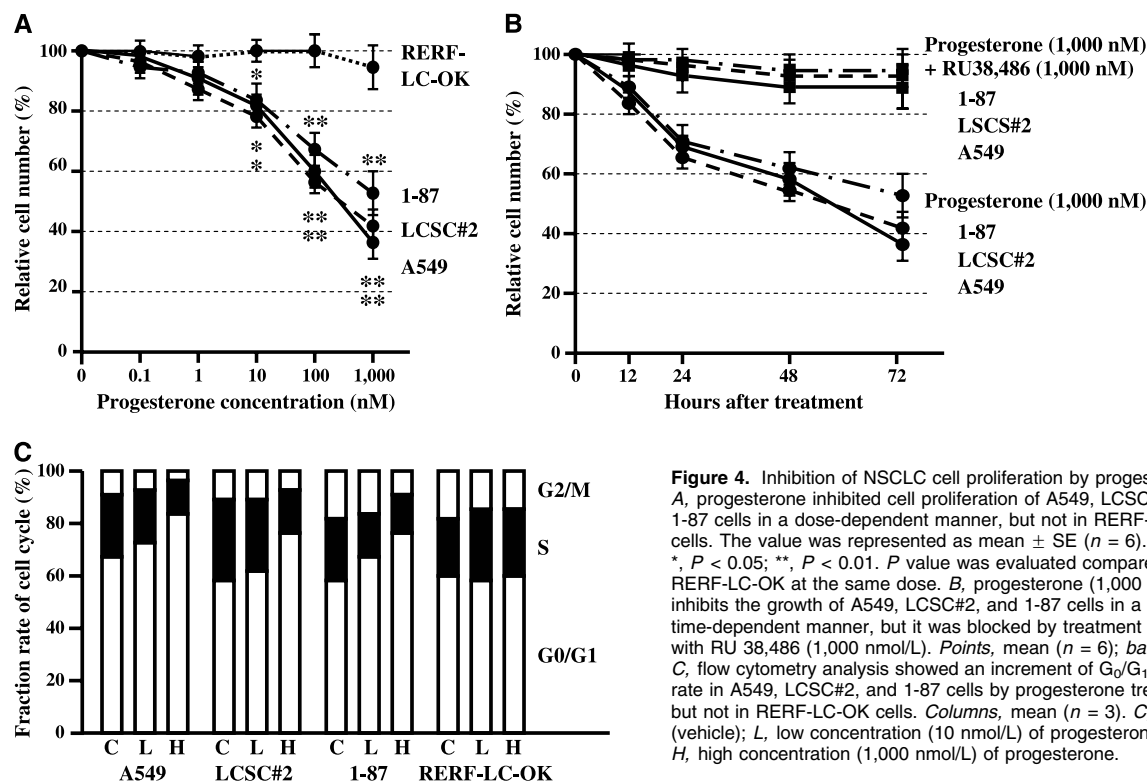
in RERF-LC-OK cells, and estrogen receptor  $\alpha$  was not detected in any of the NSCLC cell lines examined in our study.

To further study transcriptional activities of progesterone receptor or estrogen receptor in NSCLC cells, we did luciferase analyses in five NSCLC cell lines. When NSCLC cells were transiently transfected with pHRE-Luc plasmids and were treated with 1  $\mu$ mol/L progesterone for 24 hours, relative luciferase activity was significantly increased in A549, LCSC#2, and 1-87 cells, compared with the basal level (3.5-fold in A549, 3.4-fold in LCSC#2, and 3.3-fold in 1-87,  $P < 0.001$ , respectively), but not in RERF-LC-OK or PC3 cells (Fig. 2B). A 1.5-fold increase of relative luciferase activity of estrogen-responsive element was detected in RERF-LC-OK cells, when cells were treated with 10 nmol/L estradiol for 24 hours, but this increment did not reach a statistical significance ( $P = 0.1128$ ; Fig. 2C).

**In situ production of progesterone in non-small-cell lung cancers.** Progesterone is synthesized from cholesterol through cascades of several steroidogenic enzymes, including StAR, P450scc, and  $3\beta$ HSD. To examine possible *in situ* production of progesterone in NSCLCs, we examined tissue concentration of progesterone and immunolocalization of these enzymes in 42 cases of NSCLC (Fig. 3). Immunoreactivity for StAR, P450scc, and



**Figure 3.** *In situ* production of progesterone in NSCLCs. A to C, immunoreactivity for StAR (A), P450scc (B), and  $3\beta$ HSD (C) was detected in the cytoplasm of carcinoma cells in the NSCLC. Bar, 100  $\mu$ m. The specimens used for immunohistochemistry were fixed in 10% formalin and embedded in paraffin wax. D, tissue concentration of progesterone in the NSCLC was significantly associated with immunoreactivity for StAR ( $P = 0.0004$ ), P450scc ( $P = 0.0002$ ),  $3\beta$ HSD ( $P = 0.0004$ ), and progesterone receptor ( $P = 0.0101$ ;  $n = 42$ ). Columns, mean of tissue concentrations of progesterone in the group of cases positive (+) or negative (-) for indicated immunoreactivity; bars, 95% CI. E, A549, LCSC#2, and RERF-LC-OK cells were treated with 40 mg cholesterol/dL of low-density lipoprotein, and subsequently, progesterone concentrations in the medium were evaluated. Points, mean ( $n = 6$ ); bars, SE. \*\*\*,  $P < 0.001$ , compared with that of RERF-LC-OK at the same treatment period.



**Figure 4.** Inhibition of NSCLC cell proliferation by progesterone. **A**, progesterone inhibited cell proliferation of A549, LCSC#2, and 1-87 cells in a dose-dependent manner, but not in RERF-LC-OK cells. The value was represented as mean  $\pm$  SE ( $n = 6$ ). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .  $P$  value was evaluated compared with RERF-LC-OK at the same dose. **B**, progesterone (1,000 nmol/L) inhibits the growth of A549, LCSC#2, and 1-87 cells in a time-dependent manner, but it was blocked by treatment with RU 38,486 (1,000 nmol/L). Points, mean ( $n = 6$ ); bars, SE. **C**, flow cytometry analysis showed an increment of G<sub>0</sub>/G<sub>1</sub> fraction rate in A549, LCSC#2, and 1-87 cells by progesterone treatment, but not in RERF-LC-OK cells. Columns, mean ( $n = 3$ ). C, control (vehicle); L, low concentration (10 nmol/L) of progesterone; H, high concentration (1,000 nmol/L) of progesterone.

3 $\beta$ HSD was detected in the cytoplasm of carcinoma cells in 23 (54.8%), 28 (66.7%), and 17 (40.5%) of 42 cases, respectively (Fig. 3A-C). Tissue concentration of progesterone was positively correlated with immunoreactivity of these enzymes ( $P = 0.0004$  for StAR,  $P = 0.0002$  for P450scc, and  $P = 0.0004$  for 3 $\beta$ HSD) and progesterone receptor status ( $P = 0.0101$ ; Fig. 3D). Immunoreactivity of these enzymes was also significantly associated with progesterone receptor status ( $P = 0.0035$  for StAR,  $P = 0.0005$  for P450scc, and  $P = 0.0104$  for 3 $\beta$ HSD). We then evaluated progesterone synthesis using three NSCLC cell lines. Progesterone was synthesized from cholesterol contained in low-density lipoprotein in A549 and LCSC#2 cells, which were immunohistochemically positive for StAR, P450scc, and 3 $\beta$ HSD, in contrast to RERF-LC-OK cells, which were immunohistochemically negative for these enzymes, after 8 and 24 hours of treatment (Fig. 3E;  $P < 0.001$ , respectively).

**Inhibition of non-small-cell lung cancer cell proliferation by progesterone through progesterone receptor.** We then examined the effects of progesterone on NSCLC cell proliferation. Because results of immunoblotting and luciferase assays showed that PC3 cells did not respond to sex steroids in this study, we used three progesterone receptor-positive NSCLC cell lines (A549, LCSC#2, and 1-87) as well as progesterone receptor-negative RERF-LC-OK cells in the experiments. Progesterone significantly suppressed cell proliferation in progesterone receptor-positive A549, LCSC#2, and 1-87 NSCLC cells at 10 nmol/L ( $P < 0.05$  versus RERF-LC-OK, respectively) in a dose-dependent manner (Fig. 4A). However, no such effects were detected in the growth of progesterone receptor-negative RERF-LC-OK cells. Progesterone did not inhibit the cell proliferation of A549, LCSC#2, and 1-87 cells

when these cells were treated with progesterone together with a potent progesterone receptor blocker, RU 38,486 (ref. 29; Fig. 4B). Flow cytometry analysis showed that G<sub>0</sub>/G<sub>1</sub> fraction was increased in A549, LCSC#2, and 1-87 cells according to the concentration of progesterone, but not in RERF-LC-OK cells (Fig. 4C).

**Inhibition of non-small-cell lung cancer cell proliferation by progesterone in athymic mice.** To further characterize inhibitory effects of progesterone in NSCLC cell proliferation, female nude mice were injected with NSCLC cells, ovariectomized, and treated with a progesterone pellet. Development of progesterone receptor-positive A549 cells was marked (Fig. 5A) and the tumor volume reached  $\sim 10$ -fold of the original volume in 5 weeks after treatment (Fig. 5B). A progesterone pellet, however, significantly reduced the tumor volume of A549 in a dose-dependent manner (Fig. 5A and B;  $P < 0.001$ , control versus 200 mg progesterone pellet, and  $P < 0.05$ , control versus 50 mg progesterone pellet). Similar results were also obtained in other progesterone receptor-positive LCSC#2 (Fig. 5C) and 1-87 (Fig. 5D) cells, but progesterone pellets did not exert any significant effects on the cell proliferation of progesterone receptor-negative RERF-LC-OK cells (data not shown).

We also did immunohistochemistry for p21, p27, cyclin A, cyclin D1, and cyclin E, as well as Ki67, in NSCLC cells in tumor tissues obtained from nude mice at 5 weeks after treatment with a progesterone pellet (Fig. 6). In A549, 1-87, and LCSC2 cells, LI was dose-dependently increased in p21 (Fig. 6A) and p27 (Fig. 6B), and was decreased in cyclin A (Fig. 6C), cyclin E (Fig. 6E), and Ki67 (Fig. 6F), with no significant change in cyclin D1 LI (Fig. 6D). These changes were not detected in progesterone receptor-negative RERF-LC-OK cells.



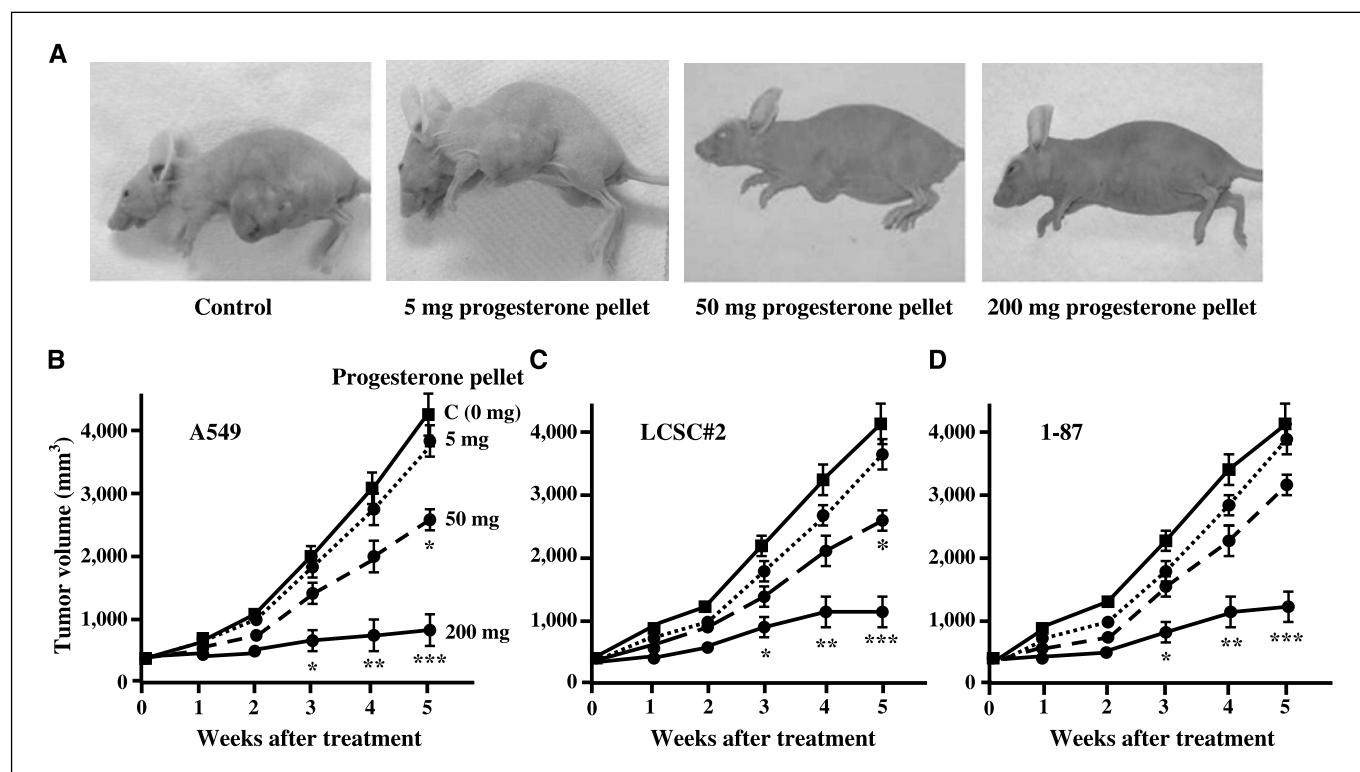
## Discussion

Several groups of investigators previously reported immunolocalization of estrogen receptors and progesterone receptor in NSCLCs (11, 12, 14) but their biological and/or clinical significance still remains unclear. In our present study, estrogen receptor  $\alpha$ , estrogen receptor  $\beta$ , and progesterone receptor immunoreactivities were all positive in 38.2%, 33.8%, and 46.5% of the NSCLCs, respectively, but no significant association was detected among these sex steroid receptors. Transcription of progesterone receptor gene is well known to be regulated by estrogenic actions through estrogen receptor in human breast cancers. Positive progesterone receptor status is generally regarded as one of the markers of functional estrogenic pathways in breast cancers (30), although it does not reflect the patient's circulating progesterone levels (31). However, Su et al. (11) reported that a progesterone receptor (+) estrogen receptor (−) status was detected in 16 of 49 (33%) NSCLC tissues, whereas only one case (2%) was double positive for estrogen receptor and progesterone receptor. Results of our present study are consistent with the study of Su et al. (11), which suggests non-estrogen-induced progesterone receptor expression in NSCLCs. However, it is also true that previous immunohistochemical studies for progesterone receptor have shown great variability of results in NSCLC tissues, and Di Nunno et al. (13) found no immunoreactivity of progesterone receptor in 248 NSCLC tissues. These discrepancies may be partly due to a difference in primary antibody for progesterone receptor used (13).

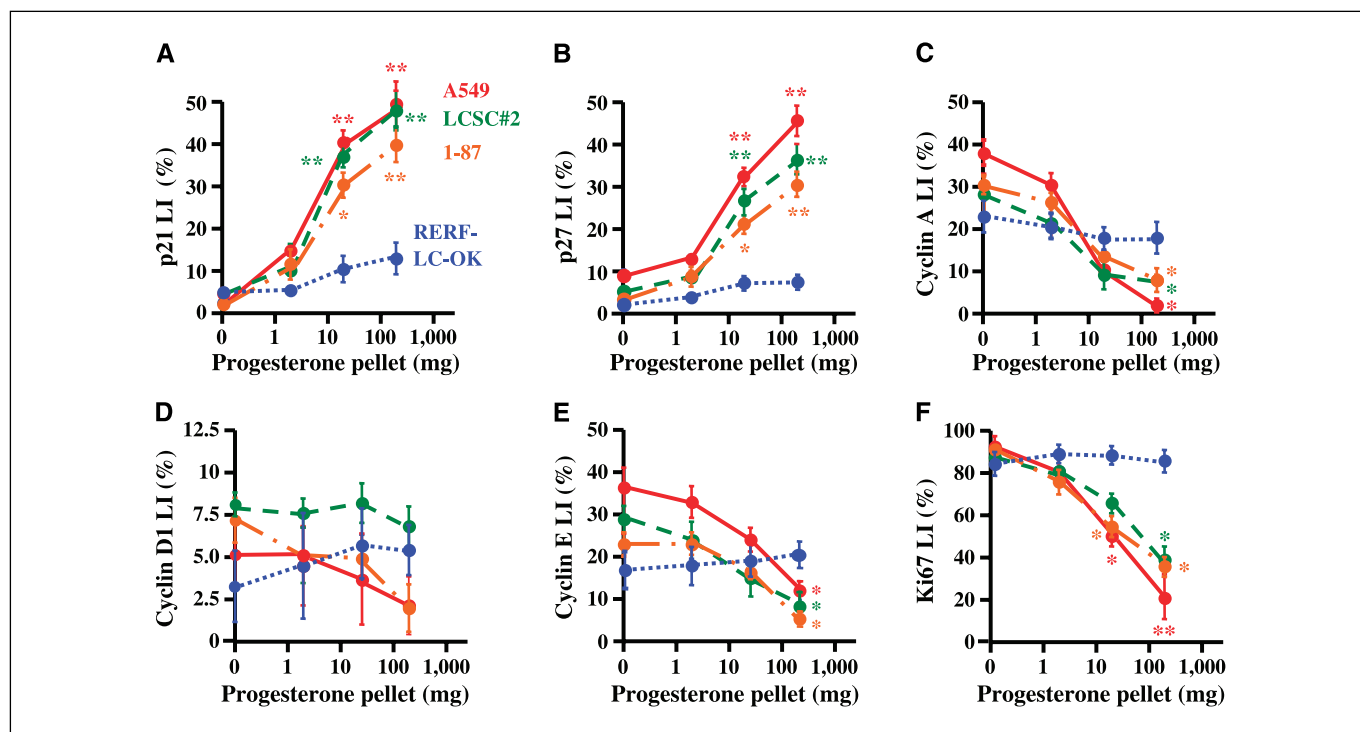
Progesterone is mainly secreted into circulation from the ovary or placenta in premenopausal women. However, a great majority of NSCLC patients (97.8% in this study) occurs postmenopausal women or men, and the serum progesterone level is in general

negligible (less than 6 nmol/L) in these patients. On the other hand, *in situ* biosynthesis of progesterone has been reported in the central nervous system (32). In our present study, we indicated immunolocalization of progesterone-producing enzymes, such as StAR, P450scc, and 3 $\beta$ HSD, in NSCLCs, which were correlated with the tissue concentration of progesterone or progesterone receptor status. Therefore, positive progesterone receptor status may be partly associated with *in situ* production and actions of progesterone, rather than estrogenic actions, in NSCLCs, in contrast to estrogen-dependent breast cancers. Both univariate and multivariate analyses showed that progesterone receptor status is a potent prognostic predictor in NSCLC patients in our study (Table 2). In addition, its effect was similar to that of TNM stage, a well-established diagnostic modality in NSCLCs (33). If progesterone is mainly involved in the growth inhibition through progesterone receptor, residual cancer cells following surgical treatment in progesterone receptor–positive NSCLC possibly grow slowly in the presence of locally produced progesterone, which subsequently results in a better clinical outcome of these patients.

In this study, progesterone significantly induced protein levels of p21 and p27, and decreased those of cyclin A, cyclin E, and Ki67 in progesterone receptor–positive NSCLC cells injected into nude mice (Fig. 6). Previous studies showed that progesterone-mediated growth inhibition was mainly preceded by decreased expression of cyclins A and E and/or induction of p21 and p27 (6–8). Reduction of cyclin D1 protein level by progesterone has been also reported in breast cancer cells (8) but not in smooth muscle cells (34). Results of our study were generally consistent with those of previous reports mentioned earlier, and molecular mechanism of progesterone-induced antiproliferation in NSCLCs



**Figure 5.** Reduction of NSCLC growth by progesterone in nude mice. **A**, tumor size of A549 in nude mice 5 weeks after treatment with a progesterone pellet. **B** to **D**, tumor volumes of A549 (**B**), LCSC#2 (**C**), and 1-87 (**D**) were dose-dependently reduced by the progesterone pellet. Points, mean obtained from six mice ( $n = 6$ ); bars, SE. C, control. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .  $P$  value was evaluated compared with control at the same time.



**Figure 6.** Immunohistochemistry of cell cycle regulators in NSCLC in nude mice. A, p21; B, p27; C, cyclin A; D, cyclin D1; E, cyclin E; F, Ki67. The immunoreactivity was evaluated as LI (%) in NSCLCs in nude mice 5 weeks after treatment with a progesterone pellet. Points, mean; bars, SE ( $n = 6$ ). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .  $P$  value was evaluated compared with control.

is considered to be generally similar to that previously reported in other progesterone receptor-positive cells. Favorable prognostic values of p21 (35) and p27 (36) or unfavorable values of cyclin A (37) and cyclin E (37) have been reported in NSCLCs. Therefore, the expression of these cell cycle regulators may partly be modulated by *in situ* progesterone actions in NSCLCs.

Inhibition of progesterone receptor-positive NSCLC cell proliferation by progesterone was significantly detected at 10 nmol/L in our *in vitro* study (Fig. 4A), similar to that in T47D breast cancer cells (38) or endometrial adenocarcinoma cells (39). Results from our *in vivo* study using nude mice (Fig. 5A-C) also showed that progesterone significantly reduced the growth of progesterone receptor-positive NSCLC cells starting from 50 mg of progesterone pellet (~350 nmol/L serum progesterone concentration). Progesterone treatment is an established endocrine therapy in human hormone-dependent breast (9) and endometrial cancers (40), and at present, oral progestins such as medroxyprogesterone acetate are widely employed. Thigpen et al. (10) reported that serum level of medroxyprogesterone acetate became 600 and 1,900 nmol/L in endometrial cancer patients who have received oral medroxyprogesterone acetate in a dose of 200 mg/d (low dose;  $n = 145$ ) and 1,000 mg/d (high dose;  $n = 154$ ), respectively, and the overall clinical response rate was not significantly different in these groups (25% in the low-dose group and 15% in the high-dose group). Effects of medroxyprogesterone acetate were also reported in breast cancer patients when medroxyprogesterone acetate plasma levels were higher than ~500 nmol/L (41), and the overall clinical response rate of patients who received high-dose medroxyprogesterone acetate was 54% (42). In addition, Nishimura et al. (43) reported that the clinical response rate for oral medroxyprogesterone acetate was not significantly different between 400 mg/d (40%) and 800 mg/d (58%) in breast cancer patients treated with

medroxyprogesterone acetate, but four of the six patients who did not respond to the 600 or 800 mg/d dose achieved a clinical response when given 1,200 mg/d of medroxyprogesterone acetate. Therefore, results from our present study as well as from previous reports mentioned above suggest that progestin administration may be effective in suppressing development of the progesterone receptor-positive NSCLC in patients in a therapeutic dose similar to that used in the endocrine treatment of breast and endometrial cancer patients.

Estrogen plays an essential and/or pivotal role in the progression of hormone-dependent breast carcinomas and, therefore, antiestrogens, which block estrogen receptor, have been used in endocrine therapy of patients with breast carcinomas. Tamoxifen is administered to patients with breast cancer, which generally resulted in a 30% to 35% reduction in clinical symptoms, and a 20% to 25% reduction in mortality (44). In NSCLCs, estrogen has been reported to induce estrogen-responsive element activity of RERF-LC-OK cells (14), and to increase cell proliferation of BEAS-2B and DB354 cells (45). In addition, antiestrogen, ICI182,780 or tamoxifen, significantly reduced the colony formation (14) or cell proliferation (45) of these cell lines above. These *in vitro* data suggest a biological function for estrogen in some NSCLC cells, and that estrogen receptors may also have therapeutic potential as an endocrine therapy in NSCLC patients. It awaits further examinations for clarification.

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## Progesterone Receptor in Non–Small Cell Lung Cancer—A Potent Prognostic Factor and Possible Target for Endocrine Therapy

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