Intratumoral Estrogens and Estrogen Receptors in Human Non–Small Cell Lung Carcinoma

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Abstract

Purpose: The possible involvement of gender-dependent factors has been suggested in human non-small cell lung carcinomas (NSCLC), but their precise roles remain largely unclear. Therefore, we examined intratumoral estradiol concentrations in NSCLC to examine local actions of estrogens in NSCLC.

Experimental Design: Fifty-nine frozen specimens of NSCLC were available for liquid chromatography/electrospray tandem mass spectrometry to study intratumoral estradiol concentrations. In addition, A549 NSCLC cells stably expressing estrogen receptor (ER) α (A549 + ERα) or ERβ (A549 + ERβ) were used *in vitro* studies.

Results: Forty-three (73%) of 59 NSCLC showed higher concentration of estradiol in carcinoma tissues than the corresponding nonneoplastic lung tissues from the same patient, and intratumoral estradiol concentrations were significantly (P = 0.0002 and 2.2-fold) higher than the corresponding nonneoplastic lungs. The intratumoral concentration of estradiol was positively correlated with aromatase expression, tumor size, and Ki-67 status in ERα- or ERβ-positive cases. In *in vitro* studies, estradiol significantly increased cell proliferation of A549 + ERα or A549 + ERβ, which was significantly suppressed by selective ER modulators, tamoxifen or raloxifene. Both A549 + ERα and A549 + ERβ cells expressed aromatase. The cell proliferation level in these cells was significantly increased under treatment with testosterone, and it was inhibited by addition of the aromatase inhibitor letrozole.

Conclusions: These results suggest that estradiol is locally produced in NSCLC mainly by aromatase and plays an important role in the growth of ERα- or ERβ-positive NSCLC. Therefore, use of selective ER modulators and/or aromatase inhibitors may be clinically effective in NSCLC that are positive for both ER and aromatase.

Lung carcinoma is the most leading cause of cancer mortality throughout the world. Despite recent improvements in its treatment, it still remains a highly lethal disease (1–3). Therefore, it is very important to investigate biological features of the lung cancer to develop targeted therapies aimed at specific proteins expressed by the carcinoma cells. Lung carcinoma is histologically classified into small cell carcinoma and non-small cell lung carcinoma (NSCLC). NSCLC accounts for ~80% of the lung carcinomas and is composed of heterogeneous groups such as adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. Squamous cell carcinoma is closely associated with smoking and a higher frequency is detected in men, whereas adenocarcinoma tends to occur more frequently in women, suggesting a possible involvement of gender-dependent factors in the pathogenesis of NSCLC (1, 4, 5).

It is well known that sex steroids play important roles in various human tissues as gender-dependent factors including nonclassic target tissues. Among sex steroids, estrogens are major contributors to cell proliferation of both breast and endometrial carcinomas through an initial interaction with estrogen receptor (ER) α and/or ERβ. Previous studies showed that a great majority of NSCLC expressed ERs (5–8) and estrogen stimulated the growth of NSCLC tumors in nude mouse xenografts. In addition, hormone replacement therapy has been reported to significantly decrease survival in women with lung cancer (9). Therefore, estrogenic actions have been postulated to contribute to the development and/or progression of NSCLC.

The most biologically active estrogen is estradiol; therefore, it is very important to examine the intratumoral concentrations of estradiol in NSCLC to obtain a better understanding of estrogenic actions in NSCLC. However, measurements of intratumoral estrogen concentrations have not been reported

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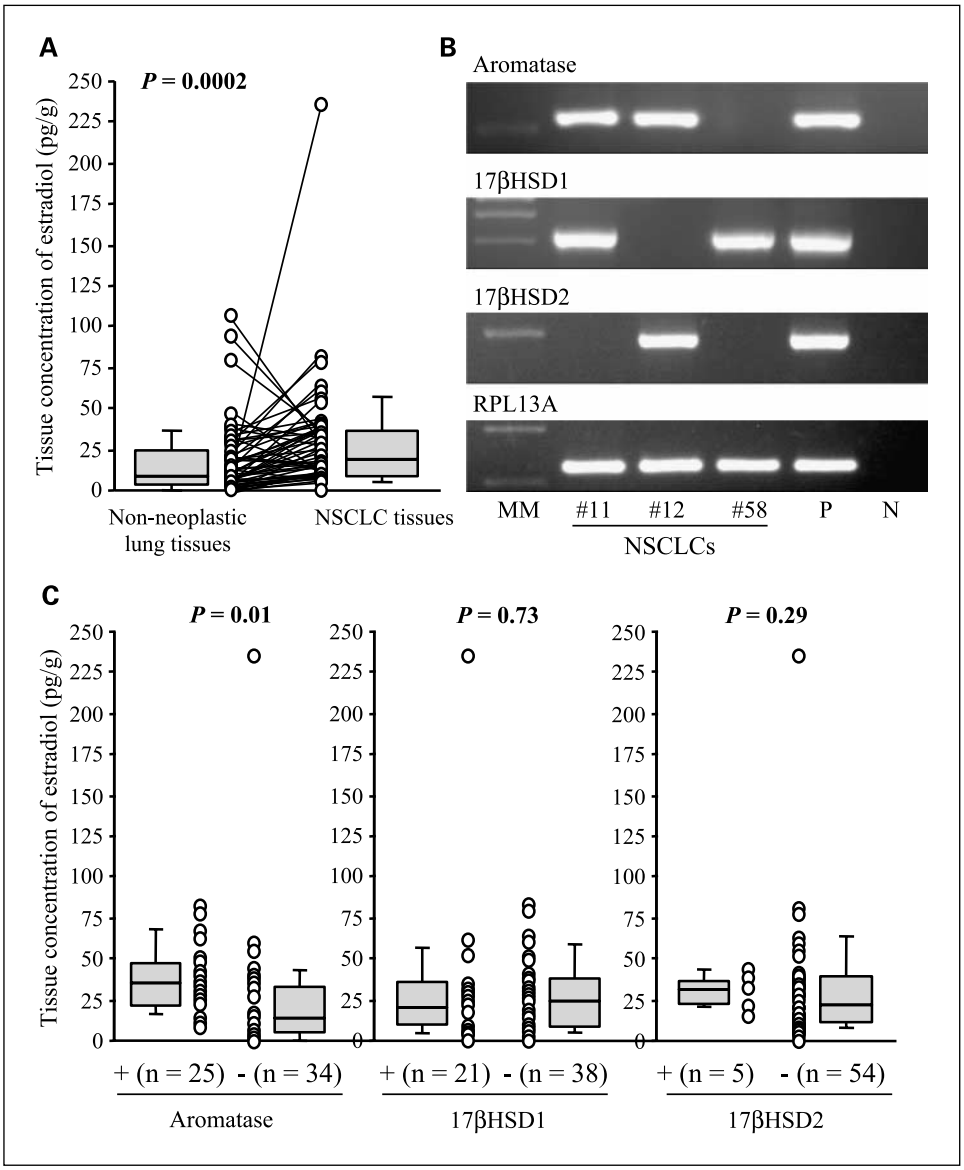

Fig. 1. Intratumoral concentration of estradiol in 59 NSCLC. *A,* tissue concentration of estradiol in NSCLC and corresponding nonneoplastic lung tissues. Each value was shown in an open circle, and the paired values from the same patient were connected in a line. The grouped data are represented as box-and-whisker plots. The median value is shown by a horizontal line in the box plot and the gray box denotes the 75th (upper margin) and 25th percentiles of the values (lower margin). The upper and lower bars indicate the 90th and 10th percentiles, respectively. The statistical analysis was done by a Wilcoxon matched-pair signed-rank test. *B,* RT-PCR for enzymes related to estrogen production in three representative NSCLC tissues. mRNA expression of RPL13A was also evaluated as an internal standard. *MM,* molecular marker; *P,* positive control (placental tissues); *N,* negative control (no cDNA substrate). *C,* association between intratumoral concentration of estradiol and enzymes related to estrogen production in the NSCLC. Values of each case were represented as an open circle and the grouped data were shown as box-and-whisker plots. Expression of aromatase, 17βHSD1, and 17βHSD2 was evaluated by RT-PCR analyses. The statistical analysis was done by a Mann-Whitney's *U* test. *P* values < 0.05 were considered significant and were indicated in boldface.

at all in NSCLC, so the clinical and/or biological significance of the role of estrogens have largely remained unknown in NSCLC. Therefore, in this study, we first measured the tissue concentration of estradiol in 59 cases of NSCLC and correlated these findings with various clinicopathologic factors of the cases. We subsequently characterized the potential biological functions of estrogens in NSCLC cells through the use of cell culture studies.

Materials and Methods

Patients and tissue specimens. Fifty-nine specimens of NSCLC were obtained from patients who underwent surgical resection from 2000 to 2003 in the Department of Surgery at Tohoku University Hospital. Thirty-three patients were men and the mean age was 71 years (range, 45-82 years), whereas 26 cases were from postmenopausal women and the mean age was 71 years (range, 50-81 years). NSCLC tissue from premenopausal women was not available for examination in this study. The patients examined in this study did not receive irradiation or chemotherapy before surgery. Overall survival data were available for all patients examined, with the mean follow-up time of 1,257 days [3.4 years; range, 245-2,414 days (0.7-6.6 years)]. Specimens for estradiol extraction or RNA isolation were snap-frozen and stored at -80°C and those for immunohistochemistry were fixed with 10% formalin and embedded in paraffin wax.

Informed consent was obtained from all the patients before their surgery and examination of the specimens used in this study. Research protocols for this study were approved by the Ethics Committee at Tohoku University School of Medicine.

Liquid chromatography/electrospray tandem mass spectrometry. Concentrations of estradiol were measured by liquid chromatography/electrospray tandem mass spectrometry analysis in Teizo Medical as described previously (10, 11). Briefly, we used a liquid chromatograph (Agilent 1100, Agilent Technologies) coupled with an API 4000 triple-stage quadrupole mass spectrometer (Applied Biosystems) operated with electrospray ionization in the positive-ion mode in this study. The mobile phase consisted of solvents A [0.1% formic acid in water (v/v)] and B (acetonitrile) and delivered at flow rate of 0.4 mL/min. We used mixture of solvents A and B [30:70 (v/v)] as an initial condition. After injection, it was followed by a linear

gradient to 100% solvent B for 4 min, and this condition was maintained for 3 min. The system was returned to the initial proportion within 0.05 min and maintained for the final 2.95 min of each run. For multiple reaction monitoring mode, the instrument monitored the *m/z* 255.3 (I.S. 258.3) from 396.4 (I.S. 399.4) for estradiol derivatives.

In our present study, the lower limit of quantification of estradiol was 0.2 pg. The reproducibility of the experiment was evaluated by intra-assay and interassay (n = 3), and their coefficient variations were 12% and 2%, respectively.

Reverse transcription-PCR. Total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies) and a reverse transcription kit (SuperScript II, Preamplification System, Life Technologies) was used in the synthesis of cDNA. Reverse transcription-PCR (RT-PCR) was done using the Light-Cycler System (Roche Diagnostics) and ribosomal protein L 13a (RPL13A) was also used as an internal standard. The primer sequences used in this study are as follows: aromatase (X13589; forward: cDNA position 691-711 and reverse: cDNA position 766-786; ref. 11), 17β-hydroxysteroid dehydrogenase (17βHSD) type 1 (17βHSD1) (NM_000413; forward: 1,290-1,310 and reverse: 1,604-1,623), 17βHSD type 2 (17βHSD1; NM_002153; forward: 797-816 and reverse: 971-989), ERα (NM_000125; forward: 1,811-1,831 and reverse: 2,080-2,100), ERβ (AB006590; forward: 1,460-1,480 and reverse: 1,608-1,627), and RPL13A (NM_012423; forward: 487-509 and reverse: 588-612; ref. 12). To verify amplification of the correct sequences, the PCR products were purified and subjected to direct sequencing. Negative controls, in which the reaction mixture lacked cDNA template, were included to check for the possibility of exogenous contaminant DNA.

Immunohistochemistry. Monoclonal antibodies for ERα (NCLER-6F11), ERβ (MS-ERβ13-PX1), and Ki-67 (MIB1) were purchased from Novocastra Lab, Gene Tex, and DAKO, respectively. Rabbit polyclonal antibody for 17βHSD1 was kindly provided by Dr. M. Poutanen (University of Oulu; ref. 13). In this study, a Histofine Kit (Nichirei), which employs the streptavidin-biotin amplification method was used for immunohistochemistry. Antigen retrieval was done by heating the slides in an autoclave at 120°C for 5 min in citric acid buffer [2 mmol/L citric acid and 9 mmol/L trisodium citrate dehydrate (pH 6.0)] for ERα, ERβ, and Ki-67 immunostaining. The antigen-antibody complex was visualized with 3,3′-diaminobenzidine solution [1 mmol/L 3,3′-diaminobenzidine, 50 mmol/L Tris-HCl (pH 7.6), and 0.006% H_2O_2] and counterstained with hematoxylin. As a negative control, normal mouse or rabbit IgG was used instead of the primary antibody.

Immunoreactivity for ERα, ERβ, and Ki-67 was detected in nuclei of carcinoma cells and the immunoreactivity was evaluated in >1,000 carcinoma cells for each case. Subsequently, the percentage of immunoreactivity, labeling index (LI), was determined. Cases with ERα or ERβ LI of >10% were considered ERα- or ERβ-positive NSCLC as reported previously (14). Immunoreactivity for 17βHSD1 was detected in the cytoplasm of carcinoma cells, and cases that had >10% of positive carcinoma cells were considered positive (15).

To evaluate relative immunointensity of ERs in NSCLC, immunoreactivity of ERα and ERβ was also evaluated by H scoring system (16). Briefly, ER-positive carcinoma cells were further classified into the strongly or weakly positive cells, and H scores were subsequently generated by adding together 2× % strongly stained cells, 1× % weakly stained cells, and 0× % negative cells, giving a possible range of 0 to 200.

Cell culture and chemicals. Human NSCLC cell line A549 was purchased from Institute of Development, Aging and Cancer, Tohoku University. The A549 cells were cultured in RPMI 1640 (Sigma-Aldrich)

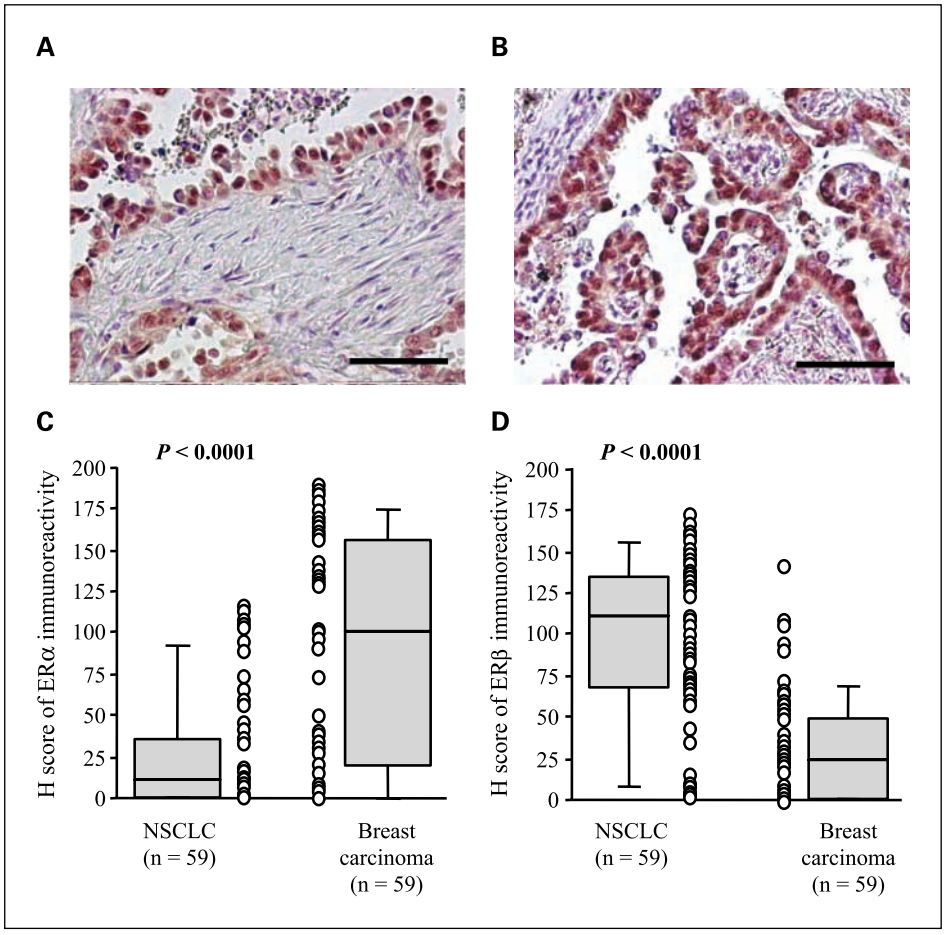

Fig. 2. Immunohistochemistry for ER isoforms in 59 NSCLC. *A* and *B,* immunoreactivity of ERα (*A*) and ERβ (*B*) was detected in the nucleus of adenocarcinoma cells in NSCLC. Bar, 50 μm. *C* and *D,* immunointensity of ERα (*C*) or ERβ (*D*) in NSCLC compared with breast carcinomas. Each value was represented as an open circle and the grouped data were shown as box-and-whisker plots. The statistical analysis was done by a Mann-Whitney's *U* test.

Table 1. Association between intratumoral concentration of estradiol and clinicopathologic variables according to ERα status in 59 NSCLC

Value	Intratumoral concentration of estradiol					
	ERα-positive cases (*n* = 32)			ERα-negative cases (*n* = 27)		
	n	Median (minimum-maximum)	*P*	*n*	Median (minimum-maximum)	*P*
Patient age* [70 (45-82) y]	32	22 (0-234)	0.23	27	18 (0-78)	0.55
Gender						
Men	15	32 (13-234)		18	35 (9-78)	
Postmenopausal women	17	10 (0-50)	**0.0002**	9	6 (0-17)	**0.001**
Stage						
I	19	18 (0-234)		15	15 (0-60)	
II-IV	13	30 (0-65)	0.24	12	33 (4-78)	0.43
Tumor size* [2.8 (1.0-6.5) cm]	32	22 (0-234)	**0.01** (r = 0.45)	27	18 (0-78)	0.70
Histologic type						
Adenocarcinoma	28	20 (0-234)		16	15 (0-78)	
Squamous cell carcinoma	4	31 (6-32)	0.78	11	31 (9-77)	0.20
Lymph node metastasis						
Positive	9	30 (0-65)		6	15 (5-77)	
Negative	23	18 (0-234)	0.41	21	22 (0-78)	0.73
Ki-67 LI* [18% (0-54%)]	32	22 (0-234)	**0.01** (r = 0.47)	27	18 (0-78)	0.19

Data were statistically analyzed using Mann-Whitney's *U* test or the Spearman rank correction. *P* values < 0.05 were considered significant and are indicated in boldface. Indicate which statistical methods were used for which particular data.
*Data were continuous variable and the median with minimum-maximum values was presented.

with 10% fetal bovine serum (JRH Biosciences). In this study, cells were cultured with phenol red–free RPMI 1640 containing 10% dextran-coated charcoal-fetal bovine serum for 3 days before the experiment.

Sex steroids (estradiol and testosterone) and selective ER modulators (SERM) such as tamoxifen and raloxifene were purchased from Sigma-Aldrich. An ERα agonist (propyl-pyrazole-triol; PPT; ref. 17), ERβ agonist (diarylpropionitrile; DPN; ref. 17), and pure ER antagonist (ICI 182,780) were purchased from Tocris. The aromatase inhibitor letrozole was synthesized within laboratories at Novartis Pharma.

Stable transfection. Stable transfection was done according to previous reports with some modifications (5, 18), and ERα or ERβ expression vector for ERα (pRc/CMV-ERα) or ERβ (pRc/CMV-ERβ)

Table 2. Association between intratumoral concentration of estradiol and clinicopathologic variables according to ERβ status in 59 NSCLC

Value	Intratumoral concentration of estradiol					
	ERβ-positive cases (*n* = 53)			ERβ-negative cases (*n* = 6)		
	n	Median (minimum-maximum)	*P*	*n*	Median (minimum-maximum)	*P*
Patient age [70 (45-82) y]	53	20 (0-234)	0.17	6	34 (15-40)	0.80
Gender						
Men	28	34 (9-234)		5	36 (18-40)	
Postmenopausal women	25	9 (0-50)	**<0.0001**	1	15	NA
Stage						
I	30	15 (0-234)		4	37 (15-40)	
II-IV	23	32 (0-78)	0.09	2	24 (18-31)	0.35
Tumor size [2.8 (1.0-6.5) cm]	53	20 (0-234)	**0.04** (r = 0.27)	6	34 (15-40)	0.34
Histologic type						
Adenocarcinoma	42	190 (0-234)		2	26 (15-37)	
Squamous cell carcinoma	11	30 (6-77)	0.51	4	34 (18-40)	0.64
Lymph node metastasis						
Positive	14	30 (0-77)		1	18	
Negative	39	18 (0-234)	0.53	5	36 (15-40)	NA
Ki-67 LI [18% (0-54%)]	53	20 (0-234)	**0.01** (r = 0.37)	6	34 (15-40)	0.78

NOTE: Data were continuous variable, and the median with minimum-maximum values were presented.
NA indicates that the *P* value was not available because of $n = 1$ in one group. Data were statistically analyzed using Mann-Whitney's *U* test or the Spearman rank correction. *P* values < 0.05 were considered significant and are indicated in boldface.

used in this study was described previously (5, 18). Briefly, A549 cells were transfected with ERα or ERβ expression vector with Trans IT LT-1 reagent (Takara), respectively. After 24 h in culture, the cells were grown in fresh RPMI 1640 supplemented with 10% fetal bovine serum containing 1 mg/mL geneticin (G418; Sigma-Aldrich) for 2 weeks. Isolated colonies were trypsinized in metal ring cups and the cells were further cultured in the presence of 200 μg/mL G418. As a negative control, empty vector was also transfected in the A549 cells.

Luciferase assay. The luciferase reporter gene assay was done as described previously (19) with some modifications. Briefly, 1 μg ptk-ERE-Luc plasmids and 200 ng pRL-TK control plasmids (Promega) were used to measure the transcriptional activity of ER. Transient transfections were carried out using *Trans*IT-LT Transfection Reagents (TaKaRa) in A549 transformants and the luciferase activity of lysates was measured using a Dual-Luciferase Reporter Assay system (Promega) and Luminescencer-PSN (AB-2200; ATTO). The transfection efficiency was normalized against *Renilla* luciferase activity using pRL-TK control plasmids and the luciferase activity for each sample was evaluated as a ratio (%) compared with that of controls.

Cell proliferation assays. A549 transformants were treated with the indicated compounds for 3 days and the status of cell proliferation was measured by a WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] method using Cell Counting Kit-8 (Dosin Kagaku; ref. 12).

Results

Intratumoral estradiol concentrations in NSCLC. We first examined the tissue concentration of estradiol in NSCLC and corresponding nonneoplastic lung tissues using liquid chromatography/electrospray tandem mass spectrometry. As shown in Fig. 1A, the median with minimum-maximum value of tissue concentration of estradiol was 20 (0-234) pg/g in NSCLC. Forty-three (73%) of 59 NSCLC cases showed higher concentration of estradiol in carcinoma tissues than the corresponding nonneoplastic lung tissues from the same patients, and the intratumoral estradiol concentrations were significantly (P = 0.0002 and 2.2-fold) higher than those found in their corresponding nonneoplastic lung tissues [9 (0-116) pg/g]. This correlation was detected regardless of the gender of the patients

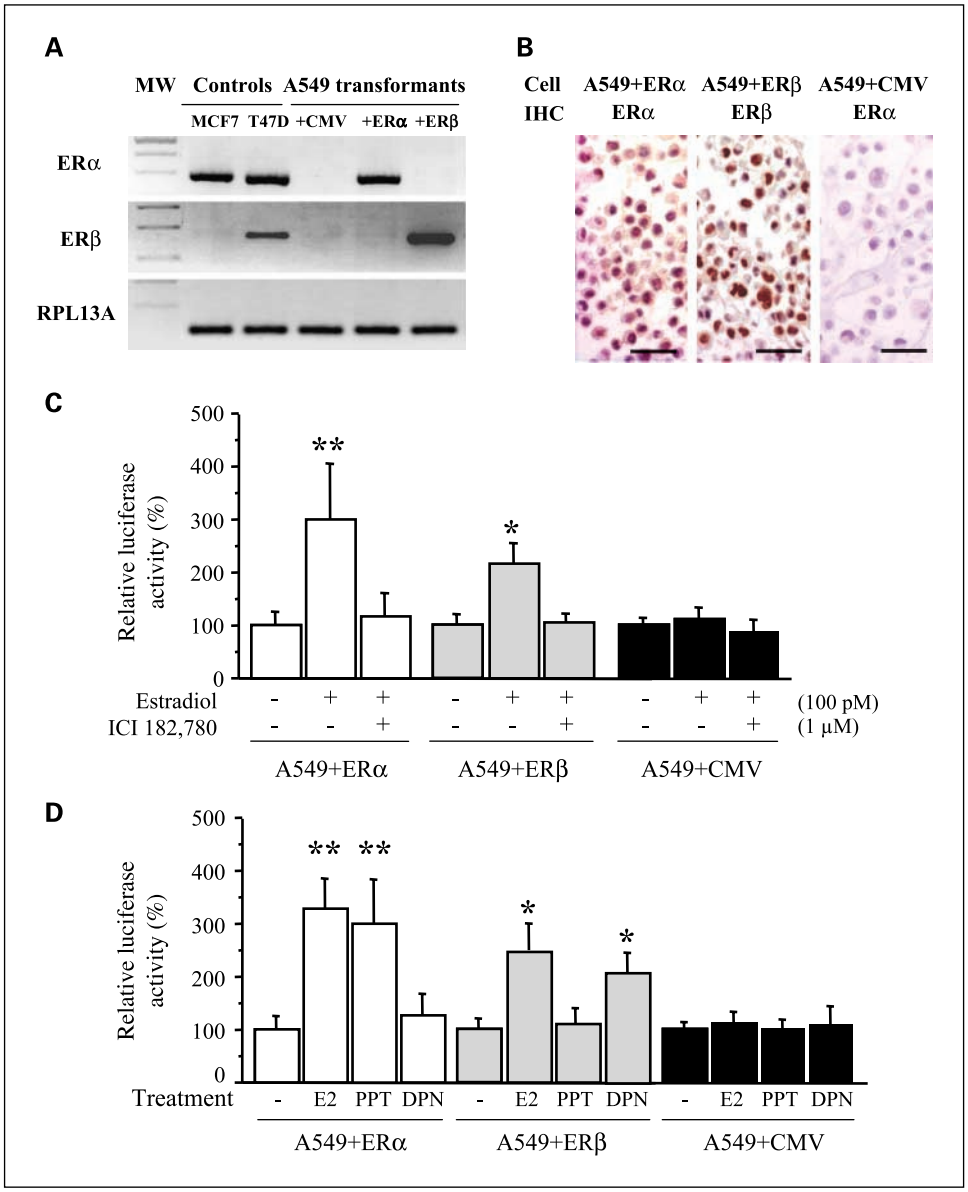

Fig. 3. Expression of ER isoforms in transformant A549 human NSCLC cells. *A,* A549 + ERα cells expressed ERα mRNA, whereas A549 + ERβ cells expressed ERβ mRNA. A549 + CMV cells did not express either ERα or ERβ mRNA. mRNA expression of ER isoforms was examined by RT-PCR analysis. As positive controls, MCF-7 breast carcinoma cells for ERα and T-47D breast carcinomas for ERα and ERβ were used. As an internal standard in each sample, RT-PCR was done for RPL13A. *B,* immunohistochemistry for ER isoforms in the A549 transformants. Immunoreactivity of ERα was detected in the nucleus of A549 + ERα cells (*left*), whereas that of ERβ was positive in the nucleus of A549 + ERβ cells (*middle*). No significant immunoreactivity for ER isoforms was detected in A549 + CMV cells (*right*). Immunoreactivity was done using cell blocks from formalin-fixed and paraffin-embedded specimens. Bar, 25 μm. *C* and *D,* estrogen-responsive element – dependent transactivation by estradiol in A549 cells stably expressing ER isoforms. A549 transformants were transiently transfected with ptk-ERE-Luc plasmids and treated with estradiol (100 pmol/L) and/or an ER antagonist ICI 182,780 (1 μmol/L; *C*), or estradiol (100 pmol/L), ERα agonist PPT (100 pmol/L), or ERβ agonist DPN (100 pmol/L; *D*) for 24 h. Subsequently, the luciferase assay was done. The luciferase activity was evaluated as a ratio (%) compared with that of controls (no treatment with either estradiol or ICI 182,780 for 24 h). Mean ± SD (n = 3). *, $P < 0.05$; **, $P < 0.01$ versus controls, respectively (one-way ANOVA and Fisher's protected least significant difference test).

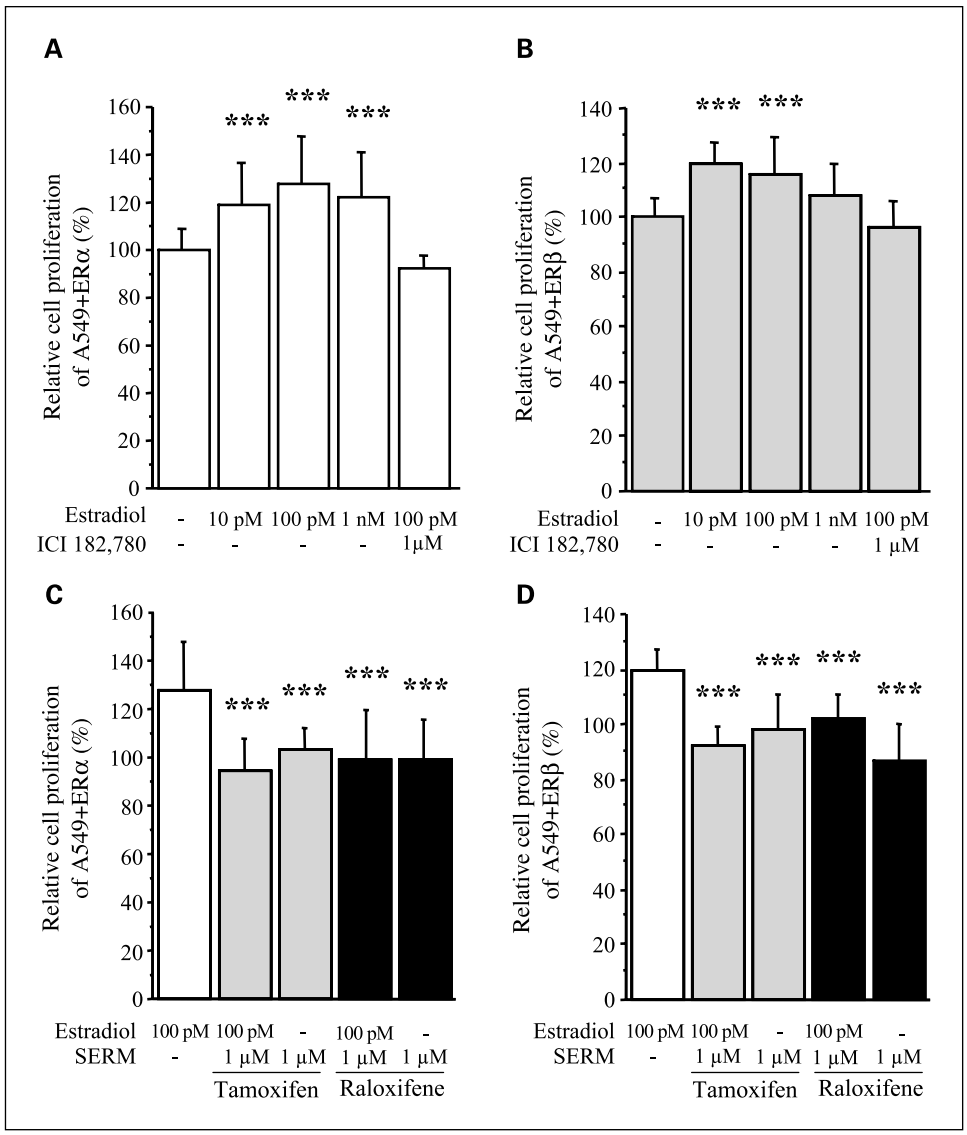

Fig. 4. Effects of ER isoform expression on the estrogen-mediated proliferation of A549 transformants. A549 + ERα (*A*) and A549 + ERβ (*B*) cells were treated with the indicated concentrations of estradiol and/or ICI 182,780 (1 μmol/L) in phenol red – free RPMI 1640 containing 10% dextran-coated charcoal-fetal bovine serum for 3 d. The status of cell proliferation was measured using a WST-8 method. The cell number was evaluated as a ratio (%) compared with controls (no treatment with either estradiol or ICI 182,780). Mean ± SD (n = 6). ***, $P < 0.001$ versus controls (one-way ANOVA and Fisher's protected least significant difference test). To examine the effects of SERMs on the estradiol-mediated proliferation of A549 cells expressing ER isoforms, A549 + ERα (*C*) and A549 + ERβ (*D*) cells were treated with estradiol (100 pmol/L) and/or SERM [tamoxifen (1 μmol/L) or raloxifene (1 μmol/L)] for 3 d. The cell number was evaluated as a ratio (%) compared with controls (no treatment with either estradiol or SERM). Mean ± SD (n = 6). ***, $P <$ 0.001 versus estradiol (100 pmol/L) alone (one-way ANOVA and Fisher's protected least significant difference test).

(P = 0.004 and 1.7-fold in men and P = 0.01 and 2.3-fold in postmenopausal women). Tissue concentrations of estradiol in men were significantly higher than that found in postmenopausal women both in NSCLC ($P < 0.0001$ and 3.9-fold [35 (9-234) pg/g in men and 9 (0-50) pg/g in postmenopausal women]) and nonneoplastic lung tissues ($P < 0.0001$ and 5.3-fold [21 (0-116) pg/g in men and 4 (0-32) pg/g in postmenopausal women]).

We then examined the association between the intratumoral estradiol concentration and expression of enzymes related to estrogen production in NSCLC tissues. mRNA expression for aromatase, 17βHSD1, 17βHSD2, and RPL13A was detected as a specific single band (115, 326, 192, and 125 bp, respectively) by RT-PCR analyses (Fig. 1B), and the results for 17βHSD1 were confirmed by immunohistochemistry (data not shown). As shown in Fig. 1C, the intratumoral estradiol concentration was significantly associated with aromatase (P = 0.01) but not with 17βHSD1 (P = 0.73) or 17βHSD2 (P = 0.29).

Association between intratumoral concentration of estradiol and clinicopathologic variables according to ER status in NSCLC. We subsequently examined an association between intratumoral estradiol concentration and clinicopathologic factors according to ER status in NSCLC, because estrogenic actions are mediated through an interaction with estradiol and ER isoforms. Immunoreactivity for both ERα (Fig. 2A) and ERβ (Fig. 2B) was detected in 32 (54%) and 53 (90%) of the 59 NSCLC cases, respectively, and 54 (92%) of 59 cases were ER (ERα or ERβ) positive. The intratumoral concentration of estradiol was not significantly associated with the ERα (P = 0.96) or ERβ status (P = 0.28).

When we evaluated relative immunointensity of ERs by H score in NSCLC and correlated with that in the same number of breast carcinomas, the relative immunointensity of ERα was significantly ($P < 0.0001$ and 9.3-fold) lower in NSCLC than the breast carcinomas (Fig. 2C), whereas ERβ immunointensity in NSCLC was significantly ($P < 0.0001$ and 4.7-fold) higher (Fig. 2D).

As shown in Table 1, the intratumoral estradiol concentration was positively associated with tumor size (P = 0.01) and Ki-67 LI (P = 0.01) in ERα-positive NSCLC but not in

ERα-negative cases (P = 0.70 in tumor size and P = 0.19 in Ki-67 LI). The intratumoral concentration of estradiol was significantly higher in men than postmenopausal women regardless of ERα status (P = 0.0002 in ERα-positive group and P = 0.001 in ERα-negative group). No significant association was detected between intratumoral estradiol concentration and other clinicopathologic factors examined regardless of the ERα status of the carcinoma cells. Estradiol concentration in NSCLC was also positively associated with tumor size (P = 0.04) and Ki-67 LI (P = 0.01) in ERβ-positive NSCLC and was significantly higher in male patients than postmenopausal women (P < 0.0001; Table 2).

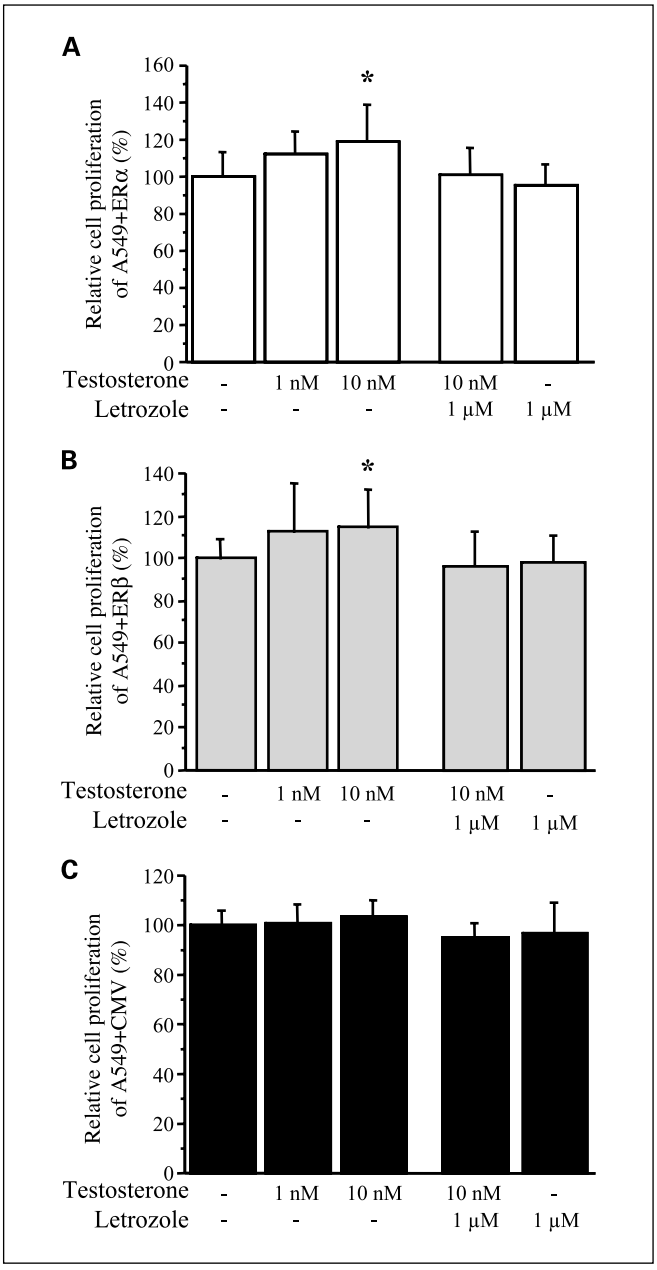

Fig. 5. Effects of the aromatase inhibitor letrozole on the cell proliferation of A549 cells expressing ER isoforms. A549 + ERα (*A*), A549 + ERβ (*B*), and A549 + CMV (*C*) cells were treated with indicated concentration of testosterone and/or letrozole for 3 d. The status of cell proliferation was measured using a WST-8 method. The cell number was evaluated as a ratio (%) compared with controls (no treatment with either testosterone or letrozole). Mean ± SD (n = 4). *, P < 0.001 versus controls (one-way ANOVA and Fisher's protected least significant difference test).

When the intratumoral concentration of estradiol was categorized into two groups according to their median values, the higher concentration group tended to be associated with worse prognosis in ER-positive NSCLC cases, although the association did not reach significant level (P = 0.12, log-rank test) but not in ER-negative patients (P = 0.59) in this study.

Establishment of A549 NSCLC cells expressing ERα or ERβ. To further characterize the biological functions of ER isoforms in NSCLC, transformed A549 NSCLC cells expressing ERα (A549 + ERα) or ERβ (A549 + ERβ) were established (Fig. 3A), because the parental A549 cells examined in this study did not express ERα or ERβ at both mRNA and protein levels in our study (data not shown). As a control, we also isolated a clone named A549 + CMV, which was stably transfected with empty vector in the A549 cells. mRNA expression of ER isoforms was not detected in the A549 + CMV cells (Fig. 3A). The patterns of protein expression of ER isoforms in these cells were confirmed by immunohistochemistry (Fig. 3B).

We subsequently examined the effects of ERα or ERβ expression in these cells on the transcriptional activity mediated through estrogen-responsive element using luciferase reporter gene assays. When the A549 transformants were transiently transfected with ptk-ERE-Luc plasmids and treated with estradiol (100 pmol/L), the luciferase activity was significantly increased in A549 + ERα (P < 0.01 and 3.2-fold) and A549 + ERβ (P < 0.05 and 2.1-fold) cells but not in A549 + CMV cells (1.1-fold) when compared with their basal levels (no estradiol treatment; Fig. 3C). The estrogen-responsive element–dependent transactivation by estradiol in A549 + ERα or A549 + ERβ cells was significantly inhibited (P < 0.01 and P < 0.05, respectively) by addition of the ER antagonist ICI 182,780. The luciferase activity was significantly (P < 0.01 and 3.0-fold) increased by the treatment with ERα agonist PPT (100 pmol/L), but not ERβ agonist DPN (100 pmol/L), in A549 + ERα cells, whereas the activity was significantly (P < 0.05 and 2.0-fold) induced by DPN, but not PPT, in A549 + ERβ cells (Fig. 3D).

When parental A549 cells were treated with estradiol, PPT, or DPN (100 pmol/L, respectively), the estrogen-responsive element–dependent transactivation was not significantly increased (1.1-, 1.0-, or 1.1-fold) compared with the basal level in our present study.

Effects of ERα or ERβ expression on estrogen-mediated proliferation in A549 cells. The number of A549 + ERα cells was significantly increased following the treatment with estradiol over the concentration range of 10 pmol/L to 1 nmol/L for 3 days (Fig. 4A). The cell proliferation of A549 + ERα cells treated with 100 pmol/L estradiol was 1.3-fold higher than the basal proliferative level measured in the absence of estradiol (P < 0.001). Estradiol-induced cell proliferation was significantly inhibited (P < 0.001) by addition of ICI 182,780 (1 μmol/L), with proliferation comparable with the basal levels being observed.

Estradiol-mediated cell proliferation was also detected in A549 + ERβ cells and was significantly induced following treatment with 10 and 100 pmol/L estradiol (P < 0.001; Fig. 4B). The estradiol-mediated cell proliferation of A549 + ERβ cells was significantly inhibited (P < 0.001) by the addition of ICI 182,780 (1 μmol/L) with proliferation comparable with

the basal levels being observed. In the A549 + CMV and parental A549 cells, estradiol, PPT, or DPN (100 pmol/L, respectively) did not significantly change the basal cell proliferation (data not shown).

Effects of SERMs on estrogen-mediated proliferation in A549 cells expressing ER isoform. As shown in Fig. 4C, the estradiol-mediated proliferation of A549 + ERα cells was significantly suppressed by addition of SERMs such as tamoxifen (1 μmol/L) or raloxifene (1 μmol/L; $P < 0.001$). Tamoxifen or raloxifene alone did not significantly change the status of cell proliferation of estradiol-untreated A549 + ERα cells.

Similarly, the estradiol-mediated proliferation of A549 + ERβ cells was also significantly inhibited by tamoxifen ($P < 0.001$) or raloxifene ($P < 0.001$; Fig. 4B). Tamoxifen or raloxifene did not significantly alter the cell proliferation level of estradiol-untreated A549 + ERβ cells. The cell proliferation of A549 + CMV or parental A549 cells was not significantly influenced following treatment with estradiol (100 pmol/L) and/or SERM (1 μmol/L; data not shown).

Aromatase in A549 cells expressing ER isoform and its correlation with cell proliferation. Using liquid chromatography/electrospray tandem mass spectrometry analysis of NSCLC, the intratumoral estradiol concentration was positively associated with the status of aromatase expression (Fig. 1C). Therefore, intratumoral aromatase may play an important role in contributing to the endogenous estrogen-mediated cell proliferation in NSCLC. To further validate this hypothesis, we used the A549 transformants, because these cells all expressed aromatase mRNA (data not shown) as reported previously (20).

When A549 + ERα or A549 + ERβ cells were treated with 10 nmol/L testosterone as a substrate for estrogen production by aromatase for 3 days, the number of the cells was significantly increased compared with the basal level (no treatment with testosterone; $P < 0.05$; Fig. 5A and B). This increased cell proliferation was significantly inhibited by the addition of the aromatase inhibitor letrozole in both A549 + ERα ($P < 0.05$) and A549 + ERβ ($P < 0.01$) cells (these significance levels are not indicated in the figure). Letrozole alone did not significantly change the status of the cell proliferation of A549 + ERα or A549 + ERβ cells. The cell proliferation of the A549 + CMV (Fig. 5C) or parental A549 (data not shown) cells was not significantly influenced by addition of testosterone and/or letrozole.

Discussion

To the best of our knowledge, this is the first report to have shown intratumoral concentrations of estradiol in NSCLC. In our present study, the median value of estradiol concentrations in NSCLC was 20 pg/g and was significantly higher (2.2-fold) than that found in the nonneoplastic lung tissues. Previously reported studies showed that estradiol is significantly (2.3-fold) higher in breast carcinomas in postmenopausal women (388 pg/g) than in the areas considered as morphologically normal (172 pg/g) in the same patients (21). Estradiol is considered to be locally produced from circulating inactive steroids found within the breast carcinoma tissues (22). The intratumoral concentration of estradiol in NSCLC was ~20 times lower than that detected in the breast carcinomas of postmenopausal women (21). However, the relative ratio of the intratumoral estradiol concentration to the corresponding nonneoplastic tissue of the same patients was similar between these two carcinomas (2.2 in NSCLC and 2.3 in breast carcinoma). Therefore, it is suggested that estradiol is also locally synthesized in NSCLC as in the great majority of breast carcinomas.

Results of our present study also showed that estradiol concentration in NSCLC tissues was significantly higher (3.7-fold) in men than postmenopausal women. Plasma concentrations of testosterone and androstendione are higher (~10 and 3 times, respectively) in men than postmenopausal women, whereas the plasma concentration of estrogens are similar (Endocrinology Databases; http://www.il-st-acad-sci.org/data2.html). Therefore, circulating androgens are considered to be the major precursor substrates of local estradiol production in NSCLC. In breast carcinoma tissues, estradiol is known to be locally produced from circulating inactive steroids by several sex steroid-producing enzymes including aromatase (conversion from circulating androstenedione to estrone or testosterone to estradiol; ref. 22). In our present study, the intratumoral estradiol concentration in NSCLC was positively associated with aromatase expression but not with the other examined enzymes that can potentially contribute to the production of intratumoral estrogens. Weinberg et al. (20) recently reported that aromatase was expressed in NSCLC cells at both mRNA and protein levels. Provost et al. (23) detected high level of 17βHSD activity in A549 cells and reported that 17βHSD5 was the predominant enzyme of the measured 17βHSD activity that is mainly involved in the regulation of intracellular androgen levels. Results of these studies as well as those in our present study indicate that the great majority of intratumoral estradiol is locally produced from circulating androgens by aromatase in NSCLC.

ER consists of ERα and ERβ in humans (3, 24, 25) and ERα is considered to mainly mediate estrogenic actions (26). A great majority of breast carcinomas are positive for ERα and SERMs such as tamoxifen or aromatase inhibitors such as letrozole are being used clinically as antiendocrine therapies for ERα-positive breast carcinoma patients. In NSCLC, ERβ immunoreactivity has been found to be frequently positive (6, 14, 27, 28), whereas the status of ERα immunoreactivity showed marked variability in its frequency of immunopositivity (0-73%) among the different studies reported (5, 14, 27, 29–31). In these previously reported investigations, the same ERα antibody employed in our present study (clone 6F11) was used in three groups, in which ERα positivity was 0% (30), 38% (14), and 66% (29). In addition, Dabbs et al. (29) reported that nuclear ERα immunoreactivity was detected with the 6F11 clone but not with the 1D5 clone, and these authors suggested that variability in ERα immunoreactivity might be due to different epitope recognized by the antibodies used in the study. In our study, ERα immunoreactivity was detected in 54% of NSCLC, but the relative immunointensity of ERα was much (9.3-fold and $P < 0.0001$) lower than that in the breast carcinoma examined. Thus, these results suggest that immunointensity of nuclear ERα is weak in NSCLC, which may result in variable interpretation of nuclear positivity and subsequently in inconsistent findings among the reports.

In previous *in vitro* studies, estrogens induced the proliferation of ER-expressing NSCLC cells, such as DB354, H23, and 201T cells (5, 6, 32). Stabile et al. (6) reported that estrogens stimulated tumor growth of H23 cells when injected into severe

combined immunodeficient mice. However, these cells all expressed both ERα and ERβ, so the biological significance of the different ER isoforms has still remained unclear in patients with NSCLC. In our present study, estradiol significantly increased the cell proliferation of A549 cells in the presence of ERα or ERβ. In addition, the intratumoral concentration of estradiol was significantly associated with tumor size and Ki-67 LI in both ERα- and ERβ-positive NSCLC but not in ER-negative cases. The MIB1 antibody for Ki-67 recognizes cells in all phases of the cell cycle except the G_0 (resting) phase (33), and Ki-67 LI is known to reflect the proliferative activity. Therefore, estrogens are reasonably postulated to contribute to the cell proliferation or other estrogen-dependent biological processes of NSCLC being mediated through both ERα or ERβ, which primarily occur in NSCLC tissues that are positive for both aromatase and ER.

If intratumoral estrogens promote the growth of NSCLC, antiestrogenic therapies would be considered to be effective in a selective group of NSCLC patients as in the breast carcinoma patients. In our present study, 1 μmol/L SERMs such as tamoxifen and raloxifene significantly suppressed the estradiol-mediated cell proliferation in both A549 + ERα or A549 + ERβ cells back to basal levels. Optimal concentrations of tamoxifen were generally considered to be 10 nmol/L to 10 μmol/L for *in vitro* studies (34) and serum concentrations of tamoxifen were reported to be 1.8 μmol/L in patients who received high-dose tamoxifen therapy (320 mg), although 20 mg tamoxifen is usually administrated in the great majority of breast carcinoma patients. In addition, the aromatase inhibitor letrozole also decreased the cell proliferation back to basal level in both A549 + ERα and A549 + ERβ cells treated with testosterone (Fig. 5). Weinberg et al. (20) showed that administration of the aromatase inhibitor anastrozole significantly reduced tumor growth of A549 cells in ovariectomized nude mouse xenografts, and very recently, Mah et al. (35) reported an association between aromatase expression and worse prognosis in women with early-stage NSCLC. Results of our present study using letrozole were in good agreements with those of the studies above. Therefore, tamoxifen and/or aromatase inhibitors would be considered to be clinically effective in ER-positive and aromatase-expressing NSCLC. The value of using antiendocrine therapies in NSCLC patients requires further examination.

In summary, the intratumoral estradiol concentration was significantly higher in NSCLC than nonneoplastic lung tissues of 59 examined cases. The estradiol concentration in NSCLC was associated with intratumoral aromatase and was correlated with both tumor size and Ki-67 in ERα- or ERβ-positive cases. In *in vitro* cell studies, estradiol significantly increased the cell proliferation of A549 cells stably expressing ER isoforms and this could be suppressed by addition of SERMs. The proliferation of these cells was also increased in the presence of testosterone and this was inhibited by the aromatase inhibitor, letrozole. Results from our present study suggest that estradiol is locally produced in NSCLC mainly through intratumoral aromatase and plays an important role in the growth of ER-positive NSCLC. Therefore, SERMs and/or aromatase inhibitors may be clinically effective in NSCLC patients who are positive for both ER and aromatase.

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References

1. Sekine I, Nishiwaki Y, Yokose T, Nagai K, Suzuki K, Kodama T. Young lung cancer patients in Japan: different characteristics between the sexes. Ann Thorac Surg 1999;67:1451–5.
2. Greenlee RT, Murray T, Bolden S, Wingo PA. Cancer statistics, 2000. CA Cancer J Clin 2000;50:7–33.
3. Jemal A, Chu KC, Tarone RE. Recent trends in lung cancer mortality in the United States. J Natl Cancer Inst 2001;93:277–83.
4. Rom WN, Hay JG, Lee TC, Jiang Y, Tchou-Wong KM. Molecular and genetic aspects of lung cancer. Am J Respir Crit Care Med 2000;161:1355–67.
5. Omoto Y, Kobayashi Y, Nishida K, et al. Expression, function, and clinical implications of the estrogen receptor β in human lung cancers. Biochem Biophys Res Commun 2001;285:340–7.
6. Stabile LP, Davis AL, Gubish CT, et al. Human non-small cell lung tumors and cells derived from normal lung express both estrogen receptor α and β and show biological responses to estrogen. Cancer Res 2002;62:2141–50.
7. Mollerup S, Jorgensen K, Berge G, Haugen A. Expression of estrogen receptors α and β in human lung tissue and cell lines. Lung Cancer 2002;37:153–9.
8. Pietras RJ, Márquez DC, Chen HW, Tsai E, Weinberg O, Fishbein M. Estrogen and growth factor receptor interactions in human breast and non-small cell lung cancer cells. Steroids 2005;70:372–81. Epub 2005 Mar 25.
9. Ganti AK, Sahmoun AE, Panwalkar AW, Tendulkar KK, Potti A. Hormone replacement therapy is associated with decreased survival in women with lung cancer. J Clin Oncol 2006;24:59–63. Epub 2005 Nov 28.
10. Suzuki T, Miki Y, Moriya T, et al. 5α-Reductase type 1 and aromatase in breast carcinoma as regulators of *in situ* androgen production. Int J Cancer 2007;120:285–91.
11. Miki Y, Suzuki T, Tazawa C, et al. Aromatase localization in human breast cancer tissues: possible interactions between intratumoral stromal and parenchymal cells. Cancer Res 2007;67:3945–54.
12. Suzuki T, Inoue A, Miki Y, et al. Early growth responsive gene 3 (EGR3) in human breast carcinoma: a regulator of estrogen-meditated invasion and a potent prognostic factor. Endocr Relat Cancer 2007;14:279–92.
13. Suzuki T, Moriya T, Ariga N, Kaneko C, Kanazawa M, Sasano H. 17β-Hydroxysteroid dehydrogenase type 1 and type 2 in human breast carcinoma: a correlation to clinicopathological parameters. Br J Cancer 2000;82:518–23.
14. Ishibashi H, Suzuki T, Suzuki S, et al. Progesterone receptor in non-small cell lung cancer—a potent prognostic factor and possible target for endocrine therapy. Cancer Res 2005;65:6450–8.
15. Suzuki T, Miki Y, Moriya T, et al. 5α-Reductase type1 and aromatase in breast carcinoma as regulators of *in situ* androgen production. Int J Cancer 2007;120:285–91.
16. Suzuki T, Takahashi K, Darnel AD, et al. COUP-TFII in the human adrenal cortex and its disorders. J Clin Endocrinol Metab 2000;85:2752–7.
17. Gottfried-Blackmore A, Croft G, McEwen BS, Bulloch K. Transcriptional activity of estrogen receptors ERα and ERβ in the E(t)C.1 cerebellar granule cell line. Brain Res 2007;1186:41–7. Epub 2007 Oct 23.
18. Omoto Y, Eguchi H, Yamamoto-Yamaguchi Y, Hayashi S. Estrogen receptor β1 and ERβcx/β2 inhibit ERα function differently in breast cancer cell line MCF7. Oncogene 2003;22:5011–20.
19. Sakamoto T, Eguchi H, Omoto Y, Ayabe T, Mori H, Hayashi S. Estrogen receptor-mediated effects of tamoxifen on human endometrial cancer cells. Mol Cell Endocrinol 2002;192:93–104.
20. Weinberg OK, Marquez-Garban DC, Fishbein MC, et al. Aromatase inhibitors in human lung cancer therapy. Cancer Res 2005;65:11287–91.
21. Chetrite GS, Cortes-Prieto J, Philippe JC, Wright F, Pasqualini JR. Comparison of estrogen concentrations, estrone sulfatase and aromatase activities in normal, and in cancerous, human breast tissues. J Steroid Biochem Mol Biol 2000;72:23–7.
22. Suzuki T, Miki Y, Nakamura Y, et al. Sex steroid-producing enzymes in human breast cancer. Endocr Relat Cancer 2005;12:701–20.
23. Provost PR, Blomquist CH, Godin C, et al. Androgen formation and metabolism in the pulmonary epithelial cell line A549: expression of 17β-hydroxysteroid

dehydrogenase type 5 and 3α-hydroxysteroid dehydrogenase type 3. Endocrinology 2000;141:2786–94.
24. Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA. Cloning of a novel receptor expressed in rat prostate and ovary. Proc Natl Acad Sci U S A 1996;93:5925–30.
25. Enmark E, Pelto-Huikko M, Grandien K, et al. Human estrogen receptor β-gene structure, chromosomal localization, and expression pattern. J Clin Endocrinol Metab 1997;82:4258–65.
26. Korach KS. Insights from the study of animals lacking functional estrogen receptor. Science 1994;266:1524–7.
27. Kawai H, Ishii A, Washiya K, et al. Estrogen receptor α and β are prognostic factors in non-small cell lung cancer. Clin Cancer Res 2005;11:5084–9.
28. Wu CT, Chang YL, Shih JY, Lee YC. The significance of estrogen receptor β in 301 surgically treated non-small cell lung cancers. J Thorac Cardiovasc Surg 2005;130:979–86.
29. Dabbs DJ, Landreneau RJ, Liu Y, et al. Detection of estrogen receptor by immunohistochemistry in pulmonary adenocarcinoma. Ann Thorac Surg 2002;73:403–5; discussion 406.
30. Schwartz AG, Prysak GM, Murphy V, et al. Nuclear estrogen receptor β in lung cancer: expression and survival differences by sex. Clin Cancer Res 2005;11:7280–7.
31. Lau SK, Chu PG, Weiss LM. Immunohistochemical expression of estrogen receptor in pulmonary adenocarcinoma. Appl Immunohistochem Mol Morphol 2006;14:83–7.
32. Hershberger PA, Vasquez AC, Kanterewicz B, Land S, Siegfried JM, Nichols M. Regulation of endogenous gene expression in human non-small cell lung cancer cells by estrogen receptor ligands. Cancer Res 2005;65:1598–605.
33. Gerdes J, Schwab U, Lemke H, Stein H. Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. Int J Cancer 1983;31:13–20.
34. Vendrell JA, Bieche I, Desmetz C, et al. Molecular changes associated with the agonist activity of hydroxy-tamoxifen and the hyper-response to estradiol in hydroxy-tamoxifen-resistant breast cancer cell lines. Endocr Relat Cancer 2005;12:75–92.
35. Mah V, Seligson DB, Li A, et al. Aromatase expression predicts survival in women with early-stage non small cell lung cancer. Cancer Res 2007;67:10484–90.