

Table 1. Representative estrogen deprivation therapies currently used in hormone-receptor positive breast carcinoma

Treatment	Effect	Patients
Ovariectomy	Decrease of estrogen concentration in plasma	Premenopausal women
GnRH agonists* (Goserelin and leuprolide)	Suppression of ovarian function (decrease of estrogen concentration in plasma)	Premenopausal women
Anti-estrogens (Tamoxifen, fulvestrant and toremifene)	Inhibition of estrogenic actions	Premenopausal and postmenopausal women
Aromatase inhibitors (Anastrozole, letrozole and exemestane)	Inhibition of estrogen production	Postmenopausal women

GnRH; gonadotropin releasing hormone

concentrations of bioactive estrogen estradiol were more than ten times higher in breast carcinoma than in plasma, and demonstrated that human breast neoplasms can produce estradiol *in vitro*. Intratumoral estradiol levels were not significantly different between premenopausal and postmenopausal breast carcinoma patients, but the intratumoral estradiol/estrone ratio was significantly higher in postmenopausal than premenopausal breast carcinoma [6]. In addition, the concentration of estradiol was 2.3-times higher in breast carcinoma tissues than in the areas considered as morphologically normal [7]. To date, bioactive estrogens are thought to be locally produced from circulating inactive steroids and to act on breast carcinoma tissue. Since this phenomenon is different from the classical endocrine system, it is called an "intracrine" system (Fig. 1).

In the classical endocrine system, only a small amount of hormone is generally utilized in the target tissues, and thereafter the great majority of it is either metabolized or converted into inactive forms. On the other hand, an intracrine system requires a minimal amount of biologically active hormone to exert its maximum effect. Therefore, the intracrine system is an efficient mode of hormone action and plays important roles especially in the development of hormone dependent neoplasms. A large proportion of estrogens in women (approximately 75% before menopause, and close to 100% after menopause) were synthesized in peripheral hormone-target tissues from abundantly present circulating precursor steroids [8]. Intratumoral production of estrogens plays an important role in the proliferation of breast carcinoma cells, especially in postmenopausal women, and the blockade of this pathway is suggested to lead to the inhibition of growth of breast carcinoma.

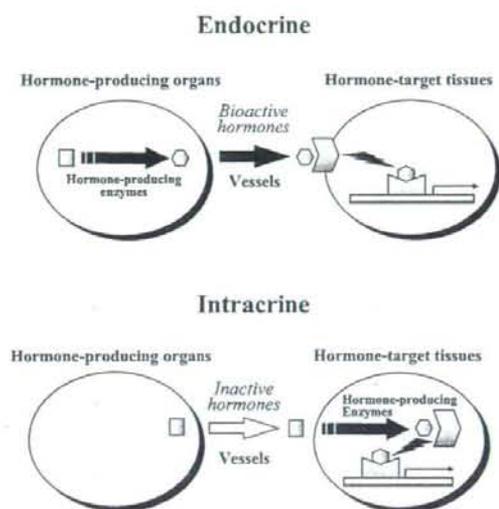


Fig. 1. Summary of endocrine and intracrine actions. In a classical endocrine action (upper panel), biologically active hormones are produced and secreted from endocrine organs, transported through the circulation, and act on their target tissues where their specific receptors are expressed. On the other hand, in an intracrine action (lower panel), bioactive hormones are synthesized in peripheral hormone-target tissues from abundantly present circulating precursor steroids, where hormone-producing enzymes are expressed, and act on the tissue. □; inactive hormone, ○; bioactive hormone, D; receptor, and ▭; promoter region of the target gene.

Aromatase as a potent estrogen-producing enzyme in breast carcinoma

Fig. 2 summarizes the representative pathways of *in situ* production of sex steroids in breast carcinoma tis-

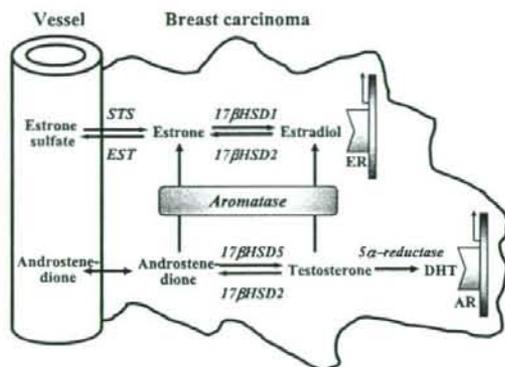


Fig. 2. Scheme representing *in situ* production of sex steroids in human breast carcinoma tissues. High concentrations of circulating inactive steroids, such as estrone sulfate and androstenedione are precursor substrates of local production of estrogens and/or androgens in breast carcinomas. Bioactive sex steroids such as estradiol and 5 α -dihydrotestosterone (DHT), are locally produced, and act on the carcinoma cells through estrogen (ER) and androgen (AR) receptor, respectively. STS; steroid sulfatase, EST; estrogen sulfotransferase, and 17 β HSD; 17 β -hydroxysteroid dehydrogenase.

sues, which are currently postulated. High concentrations of circulating inactive steroids, such as androstenedione and estrone sulfate, are considered major precursor substrates of local estrogen production. Briefly, aromatase catalyzes the conversion of androgens (androstenedione and testosterone) to estrogens (estrone and estradiol, respectively), while steroid sulfatase (STS) hydrolyzes estrone sulfate to estrone. Estrone is subsequently converted to estradiol by 17 β -hydroxysteroid dehydrogenase type 1 (17 β HSD1), and acts locally on breast carcinoma cells through ER. On the other hand, estrogens were metabolized by 17 β HSD2 (oxidation of estradiol to estrone) and estrogen sulfotransferase (EST; designated SULT 1E1) (sulfonation of estrone to estrone sulfate).

Among the pathways of intratumoral production of estrogens, only aromatase route is irreversible, suggesting that aromatase is a key enzyme for the incremental increase of intratumoral estrogen level in breast carcinoma. Inhibition of aromatase is clinically useful for reducing the progression of breast tumors especially in postmenopausal women, and third-generation aromatase inhibitors, such as anastrozole, letrozole and exemestane, are currently available [9] (Table 1). Aro-

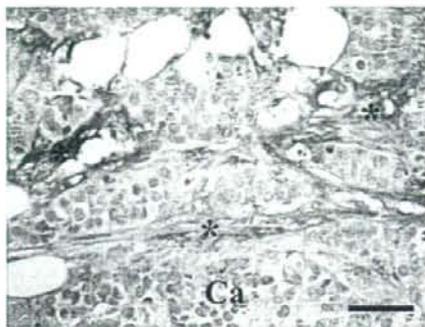
matase inhibitors have been shown to efficiently suppress estrogen levels in plasma [10] and breast carcinoma tissue [11]. Results of large multicenter trials, such as ATAC trial, NCIC MA-17 trial, and Intergroup exemestane study, all demonstrated that aromatase inhibitors are significantly associated with the improved disease-free survival and good tolerability in breast cancer patients [12–16], with anastrozole demonstrating superior efficacy to tamoxifen in the ATAC trial.

Expression and localization of aromatase in breast carcinoma tissue

Previous studies reported an association between aromatase activity in breast carcinoma tissues and the response to treatment with aromatase inhibitors [17, 18]. Therefore, it is very important to obtain a better understanding of aromatase expression in breast carcinoma to improve clinical effects of aromatase inhibitor in the breast carcinoma patients. Approximately 70% of breast carcinoma specimens had aromatase activity comparable with or greater than that found in other tissues [19]. Aromatase mRNA levels were significantly increased in invasive [20] and noninvasive [21] breast carcinoma compared to nonmalignant breast tissue. Therefore, aromatase is abundantly expressed in breast carcinoma tissues.

Immunolocalization of aromatase in breast carcinomas was examined by several groups, but the results reported are inconsistent. Previously, Sasano *et al.* [22] showed aromatase immunoreactivity in stromal cells such as intratumoral fibroblasts and adipocytes in breast carcinoma tissues (Fig. 3A). Santen *et al.* [23] also demonstrated aromatase immunoreactivity predominantly in the stromal cells. On the other hand, Esteban *et al.* [24] and Brodie *et al.* [25] reported aromatase immunoreactivity in breast carcinoma cells. Recently, Sasano *et al.* [26] validated several new aromatase antibodies for immunohistochemistry, and demonstrated that aromatase immunoreactivity was detected in various types of cells such as stromal cells, carcinoma cells and normal duct epithelial cells (Fig. 3B). When we examined localization of aromatase mRNA in breast carcinoma tissues by laser capture microdissection (LCM)/real-time polymerase chain reaction (real-time PCR), aromatase mRNA was detected in both carcinoma and intratumoral stromal

A



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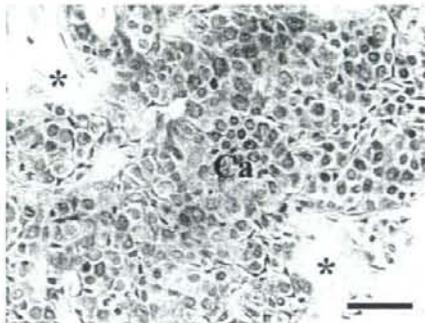


Fig. 3. Immunohistochemistry for aromatase in the breast carcinoma. **A:** Aromatase immunoreactivity was detected in the intratumoral stromal cells (*), but not in the carcinoma cells (Ca), when we used the rabbit polyclonal antibody same as Ref. 22. **B:** On the other hand, aromatase immunoreactivity was detected in the carcinoma cells, when we used the mouse monoclonal antibody same as Ref. 26. Bar = 50 μ m, respectively.

cells [19]. This suggested that aromatase is expressed in various types of cells, such as carcinoma cells, intratumoral stromal cells, and adipose tissues adjacent to the carcinoma.

Discrepant results of aromatase immunolocalization in previous studies may be due to the different nature of the aromatase antibodies employed. Since immunohistochemistry for aromatase is expected to be the most attractive method to evaluate aromatase expression, considering its great success in detecting ER, prostest-

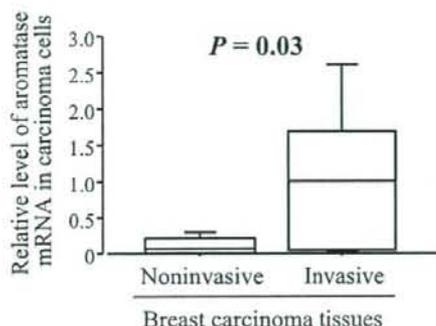
erone receptor (PR) and HER2 in breast carcinoma tissues, further examinations are required to establish a standardized approach, including the determination of aromatase antibody, immunohistochemical procedure and the evaluation system.

Regulation of aromatase expression in breast carcinoma

It remains largely unclear which mechanism increases aromatase expression in various types of cells in breast carcinoma tissues. In a preliminary study we examined expression of aromatase mRNA in breast carcinoma tissues by LCM/real-time PCR analysis, the aromatase mRNA levels both in carcinoma cells and intratumoral stromal cells were significantly higher in invasive breast carcinoma than noninvasive breast carcinoma tissues ($P = 0.03$ and $P = 0.01$, respectively) (Fig. 4). Previous *in vitro* studies demonstrated that breast carcinoma cells secrete various factors that induce aromatase expression in adipose fibroblasts [27], including prostaglandin E2 [28], interleukin (IL)-1, IL-6, IL-11 and tumor necrosis factor [29]. On the other hand, it has also been reported that exogenous growth factors, such as epidermal growth factor [30], transforming growth factor [30], and keratinocyte growth factor [31], stimulated aromatase activity in MCF-7 breast carcinoma cells. Very recently, Miki *et al.* [32] reported that mRNA level and enzymatic activity of aromatase in MCF-7 breast carcinoma cells were significantly increased on coculture with primary stromal cells isolated from breast carcinoma tissue. Therefore, aromatase expression is suggested to be, at least in part, regulated by tumor-stromal interactions in breast carcinoma tissues, which may be promoted by invasion of the carcinoma cells into the stroma.

Previous studies also demonstrated the regulation of aromatase expression by various transcriptional factors. Transcription of aromatase is activated by steroidogenic factor 1/adrenal 4 binding protein (SF1; designated NR5A1) in the ovary, which binds to a nuclear receptor half site within their promoter regions to mediate basal transcription and in part cAMP-induced transcription. However, SF1 is not expressed in breast carcinoma tissues [33]. Clyne *et al.* and Zhou *et al.* examined various orphan nuclear receptors known to bind to such a nuclear receptor half site in 3T3-L1 preadipocytes, and reported the induction of aromatase

A



B

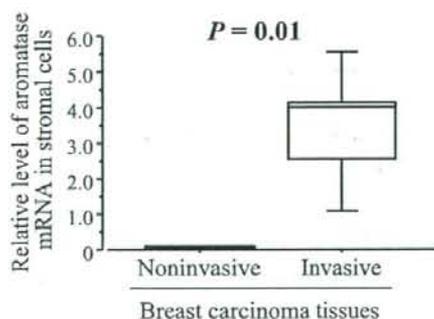


Fig. 4. Expression of aromatase mRNA in carcinoma cells (A) and intratumoral stromal cells (B) in noninvasive ($n = 8$) and invasive ($n = 9$) breast carcinoma tissues. The carcinoma cells and intratumoral stromal cells in breast carcinoma were separately collected by laser capture microdissection (LCM), and subsequently, mRNA level of aromatase was examined by real-time polymerase chain reaction (real-time PCR). Data were summarized as a relative ratio ($\times 10^{-2}\%$) of aromatase mRNA to an internal standard (mRNA of ribosomal protein L 13a). The data were represented as box and whisker plots, and statistical analyses were performed using a Mann-Whitney's U test.

expression by liver receptor homologue-1 (LRH-1; NR5A2) in adipose stromal cells in the breast carcinoma [33, 34]. LRH-1 immunoreactivity was detected in adipocytes adjacent to the carcinoma invasion and car-

cinoma cells, and significant association was detected between LRH-1 and aromatase mRNA levels in the adipose tissues adjacent to the carcinoma [34]. On the other hand, LRH-1 in breast carcinoma cells was not associated with the aromatase expression [35]. Therefore, LRH-1 may mainly regulate aromatase expression in adipose tissue adjacent to the breast carcinoma.

On the other hand, estrogen-related receptor α (ERR α ; NR3B1) has a positive regulatory function of aromatase in SK-BR-3 breast carcinoma cells [32, 36], but not in 3T3-L1 preadipocytes [33]. ERR α was mainly immunolocalized in breast carcinoma cells, but not in the intratumoral stromal cells or adipocytes [37], and expression of ERR α mRNA in carcinoma cells was positively associated with that of aromatase mRNA in breast carcinoma tissues [32]. Therefore, aromatase expression is regulated by various transcriptional factors in breast carcinoma tissues, and the key regulator may be different according to the types of cells expressing aromatase.

Regulation of intratumoral androgen production by aromatase in breast carcinoma tissues

In contrast to estrogens, androgens are considered to exert antiproliferative effects predominantly *via* androgen receptor (AR) in breast carcinoma cells [38, 39], although some divergent findings have been reported. Tissue concentration of androgens was investigated in breast carcinomas by three groups [40–42]. Biologically active and potent androgen, 5 α -dihydrotestosterone (DHT), was significantly higher in breast carcinoma tissues than in plasma [41], and *in situ* production of DHT has been proposed in breast carcinoma tissues (Fig. 2). AR is expressed in a majority of human breast carcinoma tissues [43–45], suggesting important roles of androgens in breast carcinoma as well as estrogenic actions.

The substrates of aromatase, *i.e.* androstenedione and testosterone, are not only precursors of estradiol synthesis but also precursors of DHT production (Fig. 2). DHT itself is nonaromatizable. Intratumoral concentration of DHT was significantly associated with that of testosterone in breast carcinoma tissues [40, 41], suggesting that DHT concentration in breast carcinoma is influenced by the amount of precursor. Spinola *et al.* [46] showed that treatment with an aromatase inhibitor markedly elevated intratumoral test-

osterone concentrations in dimethylbenz(a)anthracene (DMBA)-induced rat mammary tumors, and Sonne-Hansen and Lykkesfeldt [47] reported that aromatase preferred testosterone as a substrate in MCF-7 breast carcinoma cells. In addition, very recently, Suzuki *et al.* [42] demonstrated that aromatase expression was inversely associated with intratumoral DHT concentration in breast carcinoma tissues, and aromatase suppressed DHT synthesis from androstenedione in coculture experiments of MCF-7 cells and primary intratumoral stromal cells isolated from breast carcinoma. Aromatase is thus suggested as a negative regulator for *in situ* production of DHT in breast carcinoma tissues possibly by reducing concentrations or availability of the precursor testosterone.

Administration of androgens combined with antiestrogens has been more effective than that of antiestrogen alone in breast cancer patients, and the additive inhibitory effects were exerted in part by different mechanisms [8]. Results of large multicenter trials demonstrated superior efficacy of aromatase inhibitors compared to antiestrogen tamoxifen [12–16]. Although it might be due to the agonistic effects of tamoxifen in estrogen-deprived environment [14], it is speculated that aromatase inhibitors have additional antiproliferative effects through increasing local DHT concentration with estrogen deprivation. Further examinations are required to clarify the clinical importance of androgenic actions in association with a response to aromatase inhibitors in breast cancer patients.

Estrogen deprivation therapies in breast carcinoma patients

Aromatase is a key enzyme of intratumoral production of estrogen in breast carcinomas. However, it is

also true that other enzymes such as STS and 17 β HSD1 are involved in the intratumoral production of estrogen in breast carcinoma (Fig. 2). STS immunoreactivity was detected in carcinoma cells in 60% to 90% of breast carcinoma tissues [48, 49]. STS immunoreactivity was correlated with the tumor size, and it was significantly associated with an increased risk of recurrence in breast carcinoma patients [49]. On the other hand, 17 β HSD1 immunoreactivity was detected in carcinoma cells in approximately 60% of the cases [50, 51], and 17 β HSD1 mRNA levels and intratumoral estradiol/estrone ratios were significantly higher in postmenopausal than premenopausal breast carcinoma [6]. Therefore, other estrogen-producing enzymes with the exception of aromatase, including STS and 17 β HSD1, may also have important therapeutic potential as an endocrine therapy for total blockade of local estrogen in breast cancer tissues.

Reed *et al.* [52] proposed that the sulfatase pathway might be more important than the aromatase route for intratumoral estrogen synthesis in breast carcinomas, because aromatase mRNA expression was reported to have no significant prognostic value. STS inhibitors are currently being developed by several groups, and the results of a phase I study suggested that STS inhibitor may be effective in hormone-dependent breast carcinoma including those that progressed on aromatase inhibitors [53]. The design of 17 β HSD1 inhibitors has also been attempted [54–56]. In addition, Pasqualini [57] reported that progestin medrogestone stimulates EST in breast carcinoma cells through decreasing the estrogen-dependent cell proliferation. Therefore, induction of estrogen-metabolizing enzymes is also considered to result in a decrement of estrogenic actions in breast carcinoma tissues, which may eventually contribute to an improved prognosis in breast cancer patients.

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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 + ER α) or ER β (A549 + ER β) 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 ER α - or ER β -positive cases. In *in vitro* studies, estradiol significantly increased cell proliferation of A549 + ER α or A549 + ER β , which was significantly suppressed by selective ER modulators, tamoxifen or raloxifene. Both A549 + ER α and A549 + ER β 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 ER α - or ER β -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 ER β . 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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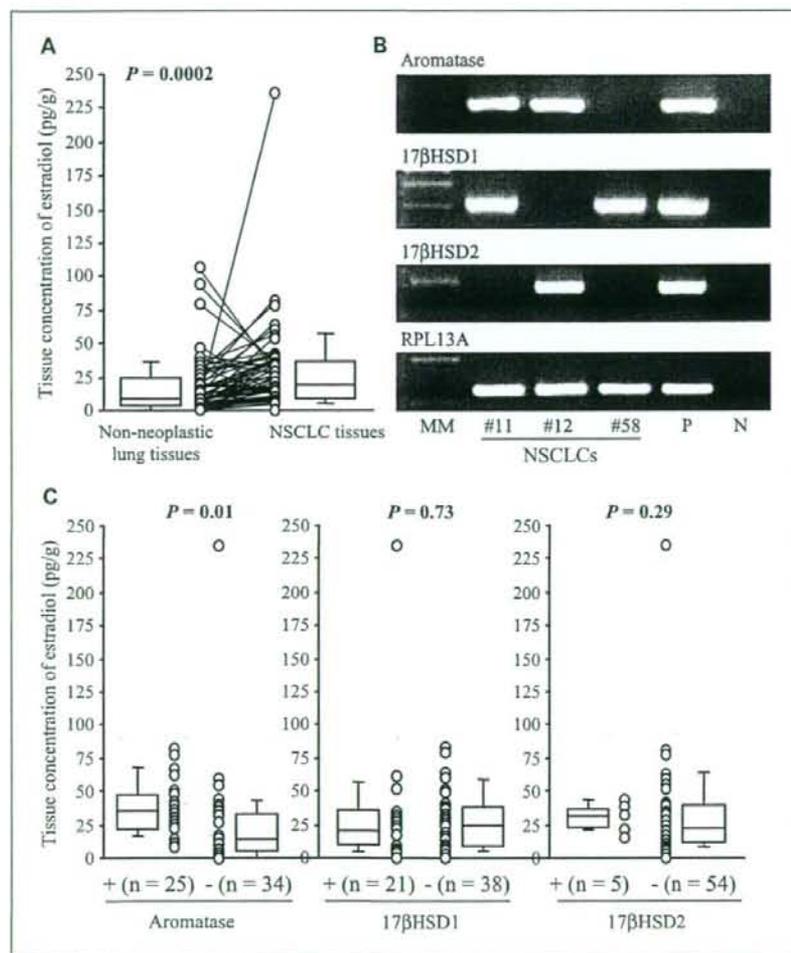


Fig. 1. Intratumoral concentration of estradiol in 59 NSCLC. **A**, tissue concentration of estradiol in NSCLC and corresponding nonneoplastic lung tissues. Each value was shown in an open circle, and the paired values from the same patient were connected in a line. The grouped data are represented as 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, Pre-amplification 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), 17 β -hydroxysteroid dehydrogenase (17 β HSD) type 1 (17 β HSD1) (NM_000413; forward: 1,290-1,310 and reverse: 1,604-1,623), 17 β HSD type 2 (17 β HSD2; NM_002153; forward: 797-816 and reverse: 971-989), ER α (NM_000125; forward: 1,811-1,831 and reverse: 2,080-2,100), ER β (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 ER α (NCLER-F11), ER β (MS-ER β 13-PX1), and Ki-67 (MIB1) were purchased from

Novocastra Lab, Gene Tex, and DAKO, respectively. Rabbit polyclonal antibody for 17 β HSD1 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 ER α , ER β , 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% H₂O₂] and counterstained with hematoxylin. As a negative control, normal mouse or rabbit IgG was used instead of the primary antibody.

Immunoreactivity for ER α , ER β , 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 ER α or ER β LI of >10% were considered ER α - or ER β -positive NSCLC as reported previously (14). Immunoreactivity for 17 β HSD1 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 ER α and ER β 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 ER α (**A**) and ER β (**B**) was detected in the nucleus of adenocarcinoma cells in NSCLC. Bar, 50 μ m. **C** and **D**, immunointensity of ER α (**C**) or ER β (**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.

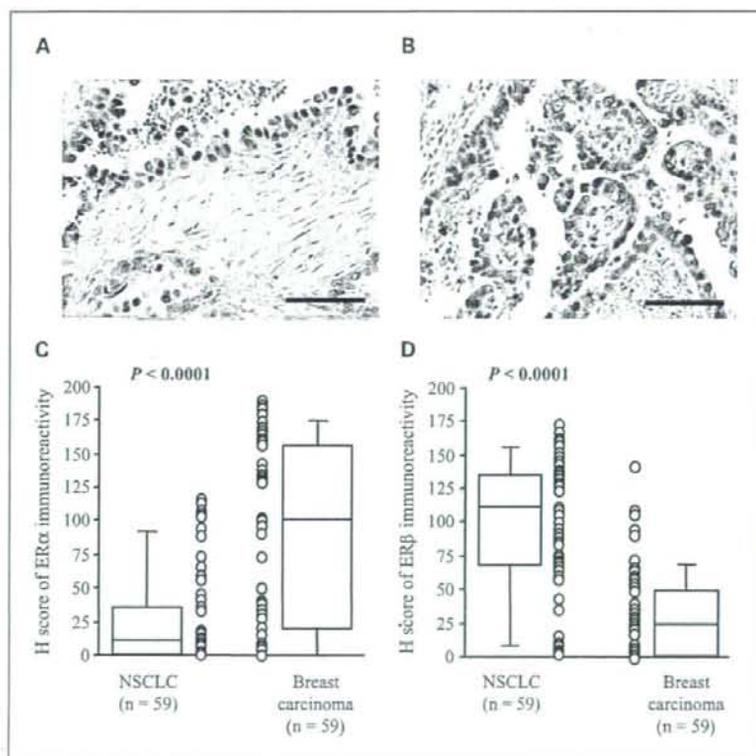


Table 1. Association between intratumoral concentration of estradiol and clinicopathologic variables according to ER α status in 59 NSCLC

Value	Intratumoral concentration of estradiol					
	ER α -positive cases (n = 32)			ER α -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 ER α agonist (propyl-pyrazole-triol; PPT; ref. 17), ER β

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 ER α or ER β expression vector for ER α (pRc/CMV-ER α) or ER β (pRc/CMV-ER β)

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

Value	Intratumoral concentration of estradiol					
	ER β -positive cases (n = 53)			ER β -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 ER α or ER β expression vector with TransIT-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 μ g/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 μ g 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 TransIT-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.

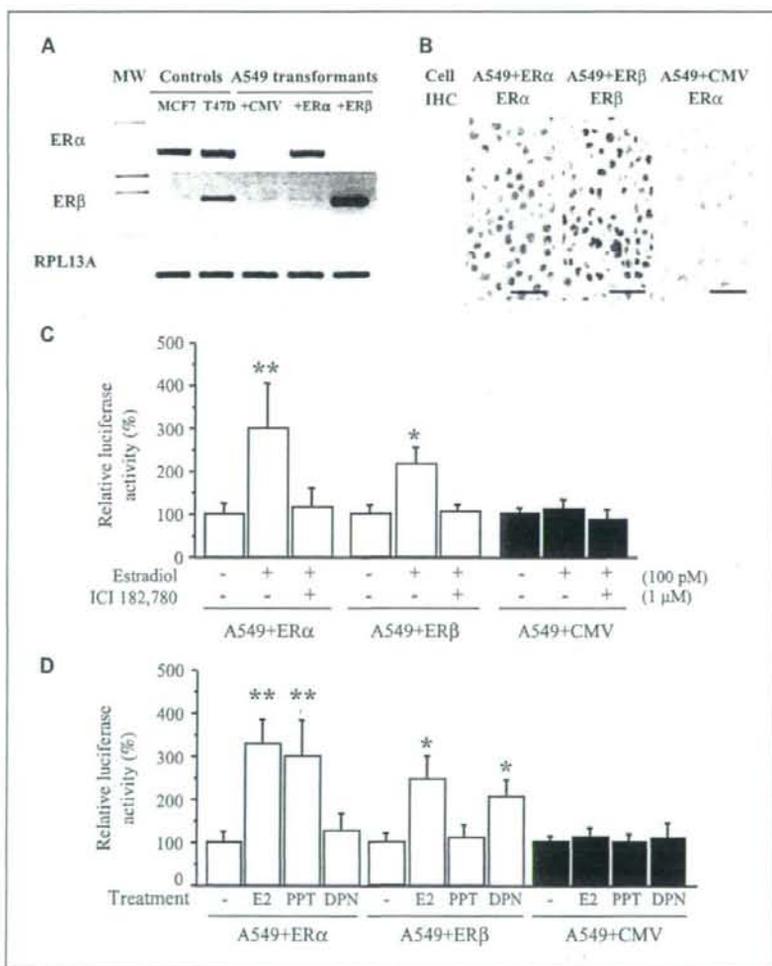
Cell proliferation assays. A549 transformants were treated with the indicated compounds for 3 days and the status of cell proliferation was

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 + ER α cells expressed ER α mRNA, whereas A549 + ER β cells expressed ER β mRNA. A549 + CMV cells did not express either ER α or ER β mRNA. mRNA expression of ER isoforms was examined by RT-PCR analysis. As positive controls, MCF-7 breast carcinoma cells for ER α and T-47D breast carcinomas for ER α and ER β 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 ER α was detected in the nucleus of A549 + ER α cells (left), whereas that of ER β was positive in the nucleus of A549 + ER β 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 μ m. **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 μ M/L; C), or estradiol (100 pmol/L), ER α agonist PPT (100 pmol/L), or ER β 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 \pm SD ($n = 3$). * $P < 0.05$, ** $P < 0.01$ versus controls, respectively (one-way ANOVA and Fisher's protected least significant difference test).



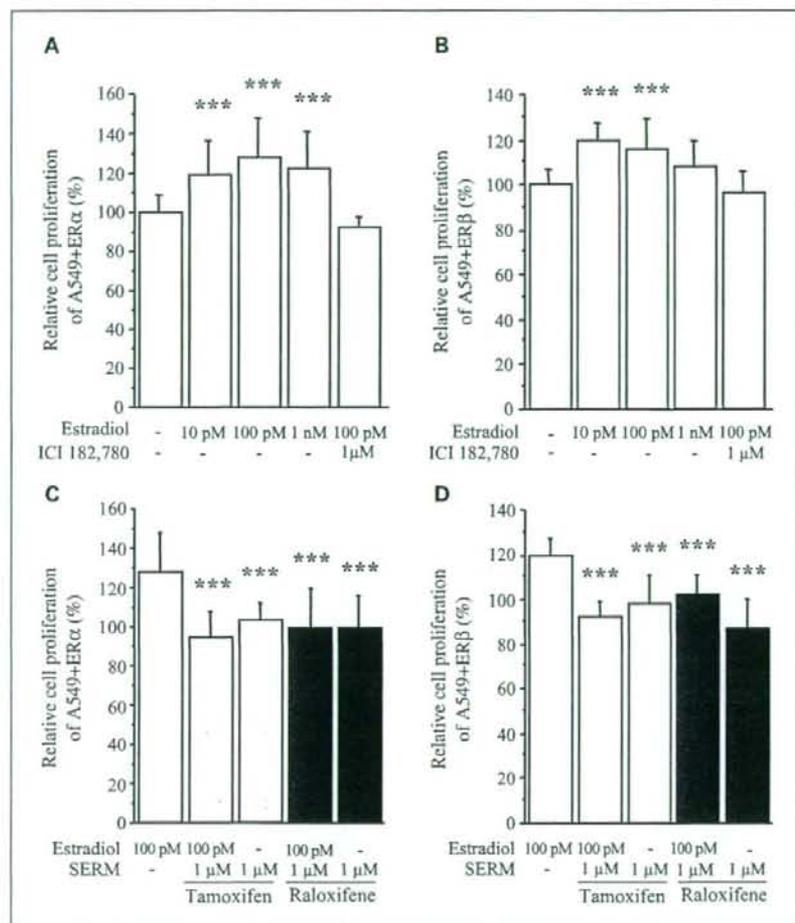


Fig. 4. Effects of ER isoform expression on the estrogen-mediated proliferation of A549 transformants. A549 + ER α (A) and A549 + ER β (B) cells were treated with the indicated concentrations of estradiol and/or ICI 182,780 (1 μ M/L) in phenol red-free RPMI 1640 containing 10% dextran-coated charcoal-fetal 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 \pm 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 + ER α (C) and A549 + ER β (D) cells were treated with estradiol (100 pmol/L) and/or SERM [tamoxifen (1 μ M/L) or raloxifene (1 μ M/L)] for 3 d. The cell number was evaluated as a ratio (%) compared with controls (no treatment with either estradiol or SERM). Mean \pm 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, 17 β HSD1, 17 β HSD2, 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 17 β HSD1 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 17 β HSD1 ($P = 0.73$) or 17 β HSD2 ($P = 0.29$).

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 ER α (Fig. 2A) and ER β (Fig. 2B) was detected in 32 (54%) and 53 (90%) of the 59 NSCLC cases, respectively, and 54 (92%) of 59 cases were ER (ER α or ER β) positive. The intratumoral concentration of estradiol was not significantly associated with the ER α ($P = 0.96$) or ER β 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 ER α was significantly ($P < 0.0001$ and 9.3-fold) lower in NSCLC than the breast carcinomas (Fig. 2C), whereas ER β 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 ER α -positive NSCLC but not in

ER α -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 ER α status ($P = 0.0002$ in ER α -positive group and $P = 0.001$ in ER α -negative group). No significant association was detected between intratumoral estradiol concentration and other clinicopathologic factors examined regardless of the ER α 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 ER β -positive

NSCLC and was significantly higher in male patients than postmenopausal women ($P < 0.0001$; Table 2).

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 ER α or ER β . To further characterize the biological functions of ER isoforms in NSCLC, transformed A549 NSCLC cells expressing ER α (A549 + ER α) or ER β (A549 + ER β) were established (Fig. 3A), because the parental A549 cells examined in this study did not express ER α or ER β 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 ER α or ER β 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 + ER α ($P < 0.01$ and 3.2-fold) and A549 + ER β ($P < 0.05$ 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 + ER α or A549 + ER β 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 ER α agonist PPT (100 pmol/L), but not ER β agonist DPN (100 pmol/L), in A549 + ER α cells, whereas the activity was significantly ($P < 0.05$ and 2.0-fold) induced by DPN, but not PPT, in A549 + ER β 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 ER α or ER β expression on estrogen-mediated proliferation in A549 cells. The number of A549 + ER α 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 + ER α 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 μ mol/L), with proliferation comparable with the basal levels being observed.

Estradiol-mediated cell proliferation was also detected in A549 + ER β 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 + ER β cells was significantly inhibited ($P < 0.001$) by the addition of ICI 182,780 (1 μ mol/L) with proliferation comparable with

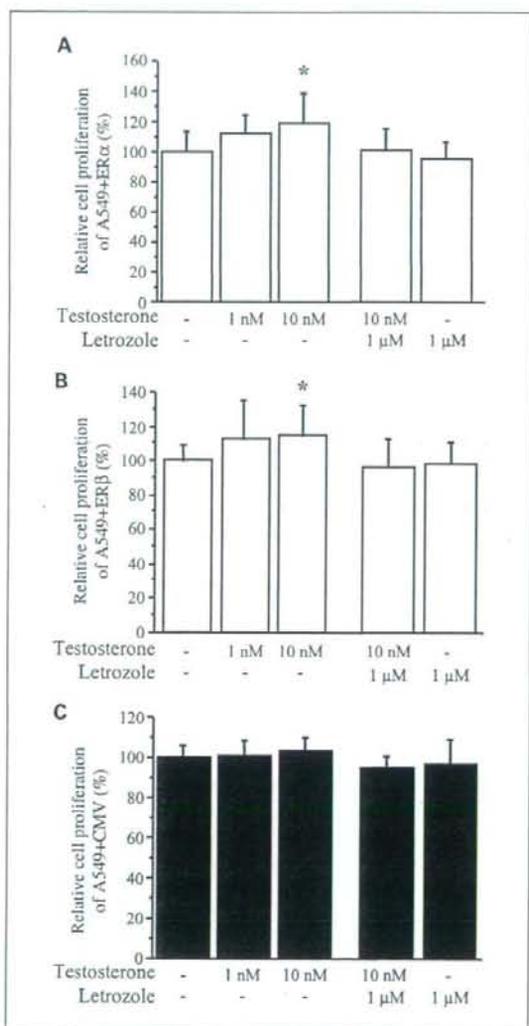


Fig. 5. Effects of the aromatase inhibitor letrozole on the cell proliferation of A549 cells expressing ER isoforms. A549 + ER α (A), A549 + ER β (B), and A549 + CMV (C) cells were treated with indicated concentration of testosterone and/or letrozole for 3 d. The status of cell proliferation was measured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

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 + ER α cells was significantly suppressed by addition of SERMs such as tamoxifen (1 μ mol/L) or raloxifene (1 μ mol/L; $P < 0.001$). Tamoxifen or raloxifene alone did not significantly change the status of cell proliferation of estradiol-untreated A549 + ER α cells.

Similarly, the estradiol-mediated proliferation of A549 + ER β 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 + ER β 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 μ mol/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 + ER α or A549 + ER β 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 + ER α ($P < 0.05$) and A549 + ER β ($P < 0.01$) cells (these significance levels are not indicated in the figure). Letrozole alone did not significantly change the status of the cell proliferation of A549 + ER α or A549 + ER β 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 \sim 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 androstenedione are higher (\sim 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 17 β HSD activity in A549 cells and reported that 17 β HSD5 was the predominant enzyme of the measured 17 β HSD 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 ER α and ER β in humans (3, 24, 25) and ER α is considered to mainly mediate estrogenic actions (26). A great majority of breast carcinomas are positive for ER α and SERMs such as tamoxifen or aromatase inhibitors such as letrozole are being used clinically as antiendocrine therapies for ER α -positive breast carcinoma patients. In NSCLC, ER β immunoreactivity has been found to be frequently positive (6, 14, 27, 28), whereas the status of ER α 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 ER α antibody employed in our present study (clone 6F11) was used in three groups, in which ER α positivity was 0% (30), 38% (14), and 66% (29). In addition, Dabbs et al. (29) reported that nuclear ER α immunoreactivity was detected with the 6F11 clone but not with the 1D5 clone, and these authors suggested that variability in ER α immunoreactivity might be due to different epitope recognized by the antibodies used in the study. In our study, ER α immunoreactivity was detected in 54% of NSCLC, but the relative immunointensity of ER α 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 ER α 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 ER α and ER β , 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 ER α or ER β . In addition, the intratumoral concentration of estradiol was significantly associated with tumor size and Ki-67 LI in both ER α - and ER β -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 G₀ (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 ER α or ER β , 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 μ mol/L SERMs such as tamoxifen and raloxifene significantly suppressed the estradiol-mediated cell proliferation in both A549 + ER α or A549 + ER β cells back to basal levels. Optimal concentrations of tamoxifen were generally considered to be 10 nmol/L to 10 μ mol/L for *in vitro* studies (34) and serum concentrations of tamoxifen were reported to be 1.8 μ mol/L in patients who received high-dose tamoxifen therapy (320 mg), although 20 mg tamoxifen is usually administered 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 + ER α and A549 + ER β 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 ER α - or ER β -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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Intratumoral estrogen production in breast carcinoma: significance of aromatase

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Abstract It is well known that estrogens are closely involved in the growth of human breast carcinoma, and that the great majority of breast carcinomas express estrogen receptor. Recent studies have demonstrated that estrogens are locally produced in breast carcinoma by several enzymes. Among these, aromatase is generally considered the most important enzyme, and aromatase inhibitors are currently used in the treatment of breast carcinoma in postmenopausal women as an estrogen deprivation therapy. Therefore, in this review, we summarize the results of recent studies on aromatase in breast carcinoma, and we discuss its biological and/or clinical significance. Aromatase was expressed in various cell types in breast carcinoma, such as carcinoma cells, intratumoral stromal cells and adipocytes adjacent to the carcinoma, and the aromatase expression was regulated by various factors, including carcinoma cell–stromal cell interactions, cytokines and nuclear receptors, depending on the cell types. Aromatase was involved not only in local estrogen production but also the inhibition of intratumoral androgen synthesis in breast carcinoma. Finally, tissue concentrations of sex steroids were significantly higher in noninvasive breast carcinoma, regarded as a precursor lesion to invasive carcinoma, than in non-neoplastic breast tissue, and various sex steroid-producing enzymes (including aromatase) were abundantly expressed in noninvasive breast carcinoma tissue.

Therefore, sex steroids are locally produced in noninvasive breast carcinoma as well as invasive carcinoma, and endocrine therapies may be clinically effective in a select group of noninvasive breast carcinoma patients.

Keywords Androgen · Aromatase · Breast carcinoma · Estrogen

Introduction: aromatase as a potent intratumoral estrogen-producing enzyme in breast carcinoma

Estrogens contribute immensely to the development of hormone-dependent human breast carcinoma by binding with the estrogen receptor (ER). Circulating estrogens are mainly secreted from the ovaries in premenopausal women, but the majority of breast carcinomas arise after menopause, when the ovaries have ceased to be functional. Previous investigations have demonstrated that tissue concentrations of bioactive estrogen, estradiol, are more than ten times higher in breast carcinoma tissue than in plasma, and human breast neoplasms can produce estradiol *in vitro* [1]. Tissue concentrations of estradiol are approximately two times higher in breast carcinoma tissue than in areas considered morphologically normal [2]. Intratumoral concentrations of estradiol are not significantly different for premenopausal and postmenopausal breast carcinomas, but the estradiol/estrone ratio was significantly higher in postmenopausal breast carcinoma [3]. Therefore, to date, a large proportion (approximately 75% before menopause and close to 100% after menopause) of the biologically active estrogen is considered to be produced locally in the breast carcinoma [4]. Considering that breast carcinoma occurring after menopause frequently expresses ER, intratumoral production of estrogens plays an

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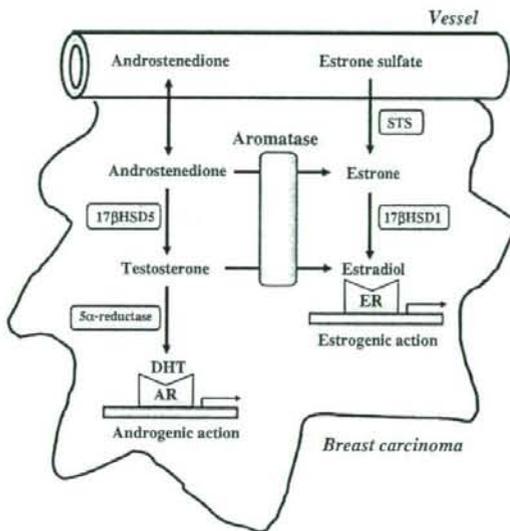


Fig. 1 Scheme representing the intratumoral production of sex steroids in breast carcinoma. High concentrations of circulating inactive steroids, such as androstenedione and estrone sulfate, are precursor substrates to the intratumoral production of estrogens and/or androgens in breast carcinoma. Biologically active sex steroids, such as estradiol and 5 α -dihydrotestosterone (DHT), are produced and act on the breast carcinoma cells through estrogen (ER) and androgen (AR) receptors, respectively. STS, steroid sulfatase; 17 β HSD, 17 β -hydroxysteroid dehydrogenase

important role in the proliferation of breast carcinoma cells, especially in postmenopausal women.

Figure 1 summarizes representative pathways of the intratumoral production of sex steroids in breast carcinoma. High concentrations of circulating inactive steroids, such as androstenedione and estrone sulfate, are major precursor substrates to intratumoral estrogen production. Aromatase catalyzes the conversion of androgens (androstenedione and testosterone) to estrogens (estrone and estradiol, respectively), while steroid sulfatase (STS) hydrolyzes estrone sulfate to estrone. Estrone is subsequently converted to estradiol by 17 β -hydroxysteroid dehydrogenase type 1 (17 β HSD1), and locally acts on the breast carcinoma cells through ER.

Among these pathways, aromatase is considered the most important enzyme in estrogen biosynthesis, and the inhibition of aromatase is clinically useful for reducing the progression of breast carcinomas in postmenopausal women. Two types of aromatase inhibitors have been developed, and these have different mechanisms of action (Table 1). Agents that interfere with the substrate-binding sites of aromatase are androgen analogs known as *steroidal* (or "type 1") aromatase inhibitors, while agents that block the electron transfer chain via the cytochrome P450

Table 1 Characteristics of representative aromatase inhibitors

Generation	Type 1 (steroidal inhibitor)	Type 2 (nonsteroidal inhibitor)
First	Testolactone	Aminoglutethimide
Second	Formestane	Fadrozole
Third	Exemestane Atamestane	Anastrozole Letrozole

prosthetic group of aromatase are termed *nonsteroidal* (or "type 2") inhibitors [5]. Third-generation aromatase inhibitors, such as anastrozole, letrozole and exemestane, are currently available, and these have been shown to efficiently suppress estrogen levels in plasma [6] and breast carcinoma tissue [7]. Results from large multicenter trials, such as the ATAC trial for anastrozole, the NCIC MA-17 trial for letrozole and the Intergroup exemestane study, have all demonstrated that aromatase inhibitors are significantly associated with improved disease-free survival and good tolerability in breast carcinoma patients [8–11].

Expression and localization of aromatase in breast carcinoma

It is very important to obtain a better understanding of aromatase expression in breast carcinoma in order to improve the clinical effects of aromatase inhibitors in breast carcinoma patients, because previous studies have demonstrated an association between aromatase activity in breast carcinoma and response to treatment with aromatase inhibitors [12].

Approximately 70% of breast carcinoma specimens have aromatase activities that are comparable with or that are greater than those found in other tissues [12], and the aromatase mRNA level was significantly increased in breast carcinoma compared to that in nonmalignant tissue [13]. In our study, the expression level of aromatase mRNA was significantly higher in breast carcinoma and adipose tissue adjacent to the carcinoma than in non-neoplastic breast tissue ($P < 0.05$, respectively) (Fig. 2a). When we further examined the localization of aromatase mRNA in breast carcinoma by laser capture microdissection (LCM)/real-time polymerase chain reaction (real-time PCR), aromatase mRNA was detected in both carcinoma and intratumoral stromal cells in breast carcinoma tissues (Fig. 2b), and the aromatase mRNA level was significantly ($P < 0.01$) higher in intratumoral stromal cells than in carcinoma cells (Fig. 2c). Therefore, aromatase is expressed in various types of cells in breast carcinoma, such as carcinoma cells, intratumoral stromal cells, and adipose tissues adjacent to the carcinoma.