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Original Article

Involvement of 27-hydroxycholesterol on the progression of non-small cell lung cancer via the estrogen receptor

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Abstract: The oxysterol 27-hydroxycholesterol (27HC) promotes the proliferation of breast cancer cells as a selective estrogen receptor modulator (SERM), but it is mostly produced by alveolar macrophages *in vivo.* The present study evaluated hypothesis that 27HC may also promote the proliferation of lung cancer cells. In the tumor and nontumor regions of lung tissue from 23 patients with non-small cell lung cancer (NSCLC) who underwent lung cancer surgery, we compared the 27HC content and its synthetic and catabolic enzyme expressions (*CYP27A1* and *CYP7B1*), the expressions of the estrogen receptor (ER) gene and its target gene *cMYC* by using high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC-ESI-MS/MS), real-time RT-PCR, and immunohistochemical staining. In addition, we evaluated the effects of 27HC and β-estradiol (E2) treatments on the proliferation of a cultured lung cancer cell line (H23 cells) expressing ERβ. In squamous cell carcinoma and in adenocarcinoma, the 27HC content was significantly higher in the tumor region than in the nontumor region, and in cancer grade III than in the other cancer grades. CYP27A1-positive macrophages were histologically detected in the nontumor regions of both cancer types, whereas the gene and protein expressions of ERβ, as well as the *CYP7B1* and *cMYC* genes, were significantly increased in the tumor tissues. In cultured H23 cells, proliferation was significantly increased by 27HC and E2 treatments for 48 h. Similar to breast cancer, the present results supported idea that the 27HC produced from alveolar macrophages promotes the proliferation of lung cancer cells highly expressing ER through the SERM action. Therefore, 27HC should be an important target for cancer therapy of NSCLC.

Keywords: 27HC, alveolar macrophage, ER, NSCLC, SERM

Introduction

Lung cancer remains the most frequent cause of cancer and the leading cause of cancer deaths worldwide [1]. Lung cancer is the second most common cancer in men with an estimated 1,369,000 cases, after prostate cancer, and it is the second most common cancer in women with 725,000 cases, after breast cancer [2]. Lung cancer is histologically classified as small cell lung cancer and non-small cell lung cancer (NSCLC), consisting of adenocarcinoma, squamous cell carcinoma (SCC), and large cell carcinoma, and the latter accounts for >80% of all lung cancers [3, 4].

The incidence of lung cancer has been rising in both sexes. However, women, particularly in the premenopausal period, are more likely to be diagnosed at an advanced stage of lung cancer concomitant with less differentiated tumors, more distant metastases, and a worse prognosis, compared to men and postmenopausal women [5-7]. This difference may be associated with sex hormones that have important roles in the progression of various types of cancer in

the reproductive organs and in nonreproductive organs such as the lung, colon, kidney, and esophagus [8]. In patients with NSCLC, a high level of endogenously circulating estrogen is associated with reduced survival in premenopausal women [9]. In addition, a significantly higher mortality of patients with NSCLC has also been observed in male individuals with a high serum free β-estradiol (E2) level than in male individuals with a low E2 level [10]. These influences of estrogen in the progression of lung cancer should be through the activation of the estrogen receptor (ER), which consist of a and b isoforms.

Umetani *et al.* [11] were the first to identify that an oxysterol, 27-hydroxycholesterol (27HC), is an endogenous selective estrogen receptor modulator (SERM) that acts as an agonist or antagonist of the ER, depending on the tissue and target genes. 27-Hydroxycholesterol has an agonist action and promotes cell proliferation in hepatoma HepG2 cells and colon cancer Caco-2 cells; it also has antagonist action and inhibits nitric oxide production in cardiovascular cells [12]. In breast cancer, 27HC promotes cancer cell growth through ER agonist action [13-15]. Furthermore, a significantly higher 27HC content has been confirmed in the tumor tissue than in the normal breast tissue counterpart of patients with breast cancer and in controls [13]. In a murine study, a high cholesterol diet similarly promoted the growth of melanoma cells transplanted subcutaneously, accompanied by a significant increase in the serum 27HC concentration. The promoted cell growth was alleviated in the gene knockout condition of mitochondrial cholesterol 27-hydroxylase (*Cyp27a1*: EC 1.14.15.15), which catalyzes cholesterol to 27HC [16]. The aforementioned study also demonstrated that the proliferation of melanoma B16-F10 cell lines were promoted by 27HC, and that this effect was canceled by the ERα antagonist tamoxifen. This finding implies that the promotive effect of 27HC on the cell growth through ERα activation [16]. Thus, ERα expression is an essential factor for the promotion of cell growth by 27HC in breast cancer [17] and melanoma [16].

27-Hydroxycholesterol is a cholesterol intermediate in the hepatic bile acid biosynthetic pathway [18], but it is also in extrahepatic tissues and cells such as the vascular endothelium and macrophages [19, 20]. In particular, the fact that 27HC is mostly produced by alveolar macrophages, which abundantly express CYP27A1, has been ascertained [19, 20]. Therefore, lung tissue contains a high 27HC level, and most 27HC in blood circulation is derived from alveolar macrophages [19]. In patients with NSCLC, ERβ but not ERα had a significantly higher expression in the nucleus of adenocarcinoma cells, whereas ERα expression was significantly dominant in breast carcinoma [21]. Hiramitsu *et al.* [22] reported in their *in vitro* experiment that 27HC treatment promoted the proliferation of ERβ-positive lung cancer cell lines but not ERα-positive or ER-negative cell lines.

Based on the evidence that 27HC produced mostly in alveolar macrophages increased the proliferation of lung cancer cell lines expressing ERβ through a SERM action, we hypothesized that 27HC is abundantly contained in tumor region of lung tissue and would be associated with the exacerbation of lung cancer. However, 27HC content in lung cancer tissue as well as the difference between tumor and nontumor regions have never evaluated. The purpose of the present study was to evaluate the relationship between the 27HC content and the pathology in lung cancer tissue by comparing the 27HC content in the tumor and the paired nontumor regions resected from lung tissue in patients with NSCLC in parallel with an *in vitro* experiment using an ERβ-positive lung cancer cell line. The findings of this study on the role of 27HC as a SERM promoting lung cancer cell proliferation, obtained using the specimens collected from the patients with lung cancer and using the cultured cells, are expected to clarify the relationship between lung cancer disease progression and ER activation of cancer cells and contribute to the strategies of lung cancer treatment.

Methods

Patients and sample collection

Twenty-three patients with NSCLC diagnosed as having adenocarcinoma or SCC who underwent surgical lung resection from January 2018 to February 2022 in Tokyo Medical University Ibaraki Medical Center (Ibaraki, Japan), were enrolled for the present study (**Table 1**). Cancer stage and cancer grade were histologically determined by pathologists (**Table 1**). For each grade, the number of patients was as follows:

Table 1. Information of the patients with NSCLC enrolled in the present study

	ADC	SCC
Number of patients [male/female; number]	15 (6/9)	8 (7/1)
Age [mean ± SE; years]	71.6 ± 2.8	74.6 ± 1.8
BMI	21.7 ± 0.6	21.1 ± 1.7
Smoking history [yes/no/(unknown)]	9/3/(4)	4/0/(3)
Cancer Stage [0/IA1/IA2/IA3/IB/IIA/IIIA; number]	1/4/2/2/2 1/3	0/1/0/1/3/2/1
Cancer Grade [I/II/III/(undecidable); number]	5/8/1/(1)	2/3/3/(0)

ADC, adenocarcinoma; SCC, squamous cell carcinoma.

the Grade I (i.e., well-differentiated) group had 5 patients with adenocarcinoma and 2 patients with SCC; the Grade II (i.e., moderately differentiated) group had 8 patients and 3 patients, respectively; and the Grade III (i.e., poorly differentiated) group had 1 patient and 3 patients, respectively. No patient had Grade IV (i.e., undifferentiated) in both cancer types. However, the grade of one patient with adenocarcinoma was undetermined (**Table 1**). Body mass index (BMI) was 21.5 ± 0.6 (mean ± the standard error; range 16.0-27.4), 2 patients each were in the range of underweight and pre-obesity classes, and the rest were in normal weight class according to WHO criteria, and no patients in obesity classes. There was no difference in BMI between the cancer types (**Table 1**). Thirteen patients (9 and 4, adenocarcinoma and SCC, respectively) had smoking history and 3 patients with adenocarcinoma had no smoking history, but the history could not be confirmed in 7 patients (**Table 1**).

Nontumor and tumor regions were obtained from the remaining lung tissue after collection for histological diagnosis under the judgment of the pathologists. The nontumor region was collected from the margin region far from the tumor tissue by avoiding around the tumor tissue. Both regions were immediately frozen and stored at -80°C until the analyses.

For the following analysis of the tumor region, normal tissue that was attached to the tumor tissue was carefully removed, and the center part of the tumor tissue was used. The preserved nontumor tissue was directly used as the nontumor region. The methods and purpose of the study were explained to the patients, and their informed consent was obtained before enrollment. The study protocol was approved by the Ethics Committee of Tokyo Medical University (Tokyo, Japan; IRB approval no., IB1721) and was conducted in accordance with the 1964 Helsinki Declaration.

27HC analysis

The 27HC level in the nontumor and tumor regions was measured using a high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC-ESI-MS/MS) system, as described in our previous reports [23-25]. Fifty-milligram wet weight of both regions was washed several times with ice-cold phosphate-buffered saline (PBS) until the adhered blood was completely removed. The tissue was sonicated with 10-times volume (w/v) of PBS and centrifuged at 3500×*g* for 10 min at 4°C. Ten microliters of the supernatant were added to 27HC-d6 (Avanti Polar Lipids, Birmingham, AL, USA) as the internal standard. Alkaline hydrolysis was achieved in 1N ethanolic potassium hydroxide with butylated hydroxytoluene for 1 hour at 37°C. Sterols were extracted with *n*-hexene and derivatized to picolinyl esters. The sample was injected into the HPLC-ESI-MS/MS system. For purification, all samples were maintained in nitrogen to avoid autoxidation during the assays.

Total RNA extraction and RT-PCR analysis

Fifty-milligram wet weight of the nontumor and tumor tissues was homogenized with a 10-times volume of lysis buffer, and H23 cells per well were lysed with 600 μL of the buffer. Total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen K.K., Tokyo, Japan). cDNA was obtained from 500 ng of total RNA by reverse transcription using the PrimeScript RT reagent kit (Takara Bio, Inc., Shiga, Japan). The mRNA expressions of *ERα, ERβ, CYP27A1*, cytochrome P450 7B1 (*CYP7B1:* EC 1.14.14.29), MYC proto-oncogene (bHLH transcription factor [*cMYC*]), hypoxia-inducible factor-1α (*HIF1α*),

Table 2. Gene sequences of the PCR primers

Gene	Accession number		Sequence (5'-3')	Product Size (*bp*)
18SrRNA	X03205	F	GTAACCCGTTGAACCCCATT	151
		R	CCATCCAATCGGTAGTAGCG	
cMYC	NM_002467	F	CCTGGTGCTCCATGAGGAGAC	128
		R	CAGACTCTGACCTTTTGCCAGG	
CYP27A1	BC051851	F	CACAAACTCCCGGATCAT	121
		R	AGGCTCAGAGAAGGCAGT	
CYP7B1	AF127090	F	CATCTTTCCTCCAGTCCTAC	141
		R	GGCATTAGGTAACACTTCAG	
ERα	NM_000125	F	GAAGCTACTGTTTGCTCCTAACT	122
		R	GCAGATTCATCATGCGGAAC	
ERβ	NM_001437	F	AAGATCGCTAGAACACACCTTAC	71
		R	CGCAACGGTTCCCACTAA	
HIF1α	NM_001243084	F	TATGAGCCAGAAGAACTTTTAGGC	145
		R	CACCTCTTTTGGCAAGCATCCTG	
LXR	NM_005693	F	GACCGACTGATGTTCCCACG	187
		R	CCATCCGGCCAAGAAAACAG	
SREBP1	NM_004176	F	GCCCAGGTGACTCAGCTATT	245
		R	GTCAGAGAGGCCCACCACTT	
VEGF	NM_001025366	F	TTGCCTTGCTGCTCTACCTCCA	126
		R	GATGGCAGTAGCTGCGCTGATA	

18SrRNA, 18S ribosomal ribonucleic acid; *cMYC*, MYC proto-oncogene (bHLH transcription factor); *CYP27A1*, cytochrome P450 27A1; *CYP7B1*, cytochrome P450 7B1; *ERα*, estrogen receptor *α*; *ERβ*, estrogen receptor *β*; *HIF1α,* hypoxia-inducible factor-1*α*; *LXR*, liver X receptor; *SREBP1*, sterol regulatory element binding protein-1, *VEGF*, vascular endothelial growth factor; *F*, forward; *R*, reverse; *bp*, base pair.

liver X receptor (*LXR*), sterol regulatory element binding protein-1 (*SREBP1*), and vascular endothelial growth factor (*VEGF*) were quantified with real-time quantitative PCR using gene-specific primers (**Table 2**) in the FastStart DNA Master SYBR Green I and the LightCycler system (Roche Diagnostics, Mannheim, Germany). PCR amplification began with a 10 min preincubation step at 95°C, followed by 40 cycles of denaturation at 95°C for 10 sec, annealing at 62°C for 10 sec, and elongation at 72°C. The relative concentration of the PCR products derived from the target gene was calculated using the LightCycler System software. A standard curve for each run was constructed by plotting the crossover point against the log concentration. The concentration of target molecules in each sample was then calculated automatically by reference to this curve (r=-1.00), and the specificity of each PCR product was assessed by melting curve analysis. The mRNA expression of each gene was standardized to the expression of 18SrRNA.

Histological analysis

Immunohistochemical (IHC) stain was carried out using an auto-stain system with the exclusive reagents (Discovery XT system; Ventana Roche Diagnostics K.K., Basel, Switzerland). Antibodies of ERβ (Abcam, Cambridge, MA, USA) and CYP27A1 (Abcam) diluted as 1:100 with dilute solution (Ventana) and the prediluted forms of CD68 (Ventana) and CD163 (Ventana) were used for the primary antibodies. Deparaffinized 5-μm thick specimens were incubated with the primary and secondary antibodies for 32 min at 37°C, respectively. Thereafter, detection was achieved using the Ventana ultraView Universal DAB detection kit (Ventana). The nucleus and cytoplasm were then stained using Hematoxylin II Counterstain reagent (Ventana) and the bluing reagent (Ventana). Specific immunoreaction of the primary antibodies was confirmed by incubation without each antibody. Hematoxylin and eosin (H&E) staining of the lung tissue was also carried out by a routine procedure.

Cell culture experiment

The H23 cell line, which was an adenocarcinoma isolated from a patient with NSCLC, was purchased from ATCC (Manassas, VA, USA). The cell culture experiment has been described in a previous study [26]. Cells were cultured on a 24-well plate density of 2.5×10^4 cells per well with RPMI 1640 medium (Gibco; Thermo Fisher Scientific, Waltham, MA, USA), which contains 10% fetal bovine serum (FBS; Gibco), at 37°C in a humidified incubator containing 5% CO_2 and 95% air. After 24 hours, the medium was changed to phenol red-free RPMI 1640 medium (Gibco) containing 10% charcoal-stripped FBS. After undergoing culture for a further 24 hours, the cells were exposed to various concentrations (1 nM-1 μM) of 27HC (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) or exposed to 10 nM E2 (Sigma-Aldrich) in the phenol red-free RPMI-1640 medium with 10% dialyzed FBS for 48 hours. The microscope cell image in the exact area of the center of each well were captured by a charged-coupled device camera before and after the treatments. The number of cells was counted, and the proliferation ratio in the same well after the treatment, compared to that before treatment, was calculated for each well. In addition, the cells were exposed to 1 μM 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]-pyrimidin-3-yl]phenol (PHTPP; Sigma-Aldrich), a specific-antagonist of ERβ, with 1 μM 27HC or 10 nM E2 for 48 hours. Cell proliferation was estimated using the same method. 27-Hydroxycholesterol, E2, and PHTPP were dissolved with 0.1% ethanol as the final concentration. The treated ethanol concentration had no detectable influence on cell growth. The same concentration was also added to the control wells.

Western blotting analysis

Seven μg of total protein extracted from the H23 cell line was separated by electrophoresis on a 5-20% gradient sodium dodecyl sulfate-polyacrylamide gel (ATTO Corporation, Tokyo, Japan) following boil for 10 min with a dithiothreitol-containing sample buffer (EzApply; ATTO Corporation), and thereafter, transferred to polyvinylidene fluoride membranes (Immobilon P; Merck KGaA). Blot was incubated with the primary antibodies against ERβ (1:1000; Abcam, Cambridge, UK), and β-actin (1:5000; Sigma-Aldrich Chemical Co., St. Louis, MO, USA), and were further incubated with the respective secondary antibodies (Amersham; GE healthcare, Chicago, IL, USA). The bands were visualized with an enhanced chemiluminescence detection system (Amersham).

Statistical analyses

Data are presented as the mean ± the standard error. Statistical differences between the tumor and paired nontumor regions was carried out by using the paired Student's *t*-test or by using nonparametric Wilcoxson analysis. Multiple comparisons were evaluated by using one-way analysis of variance post hoc Tukey multiple comparison test. A value of $P<0.05$ was significant. Statistics were analyzed using Prism software (version 9; GraphPad Software Inc., San Diego, CA, USA).

Results

27HC concentration in the nontumor and tumor regions of lung cancer tissue

Figure 1A shows the 27HC concentration in the tumor region and in its counterpart nontumor region, classified by adenocarcinoma and SCC cancer types, in lung tissue collected from patients with NSCLC. In both cancer types, the 27HC concentration was significantly higher in the tumor region than in the counterpart nontumor region (**Figure 1A**). In the comparisons of the cancer grades, the 27HC concentration in the tumor region was significantly higher in Grade III than in the other grades, whereas the grades were not different in the nontumor region (**Figure 1B**). Furthermore, the relative 27HC concentration in the tumor region to that of the counterpart nontumor region was higher for Grade III in both cancer types and was particularly marked in SCC (**Figure 1C**). There was no significant relationship between 27HC content in the tumor region and BMI ($r^2=0.02947$, $P=0.4449$). In comparison by smoking history, 27HC contents in tumor region was higher in the smoking group (2.55 ± 1.36 ng/mg, n=13) than in the no smoking group (0.76 ± 0.43, n=3), and was the highest in the unknown group (4.17 ± 2.36, n=7), but no statistical difference due to large difference in number of patients per group.

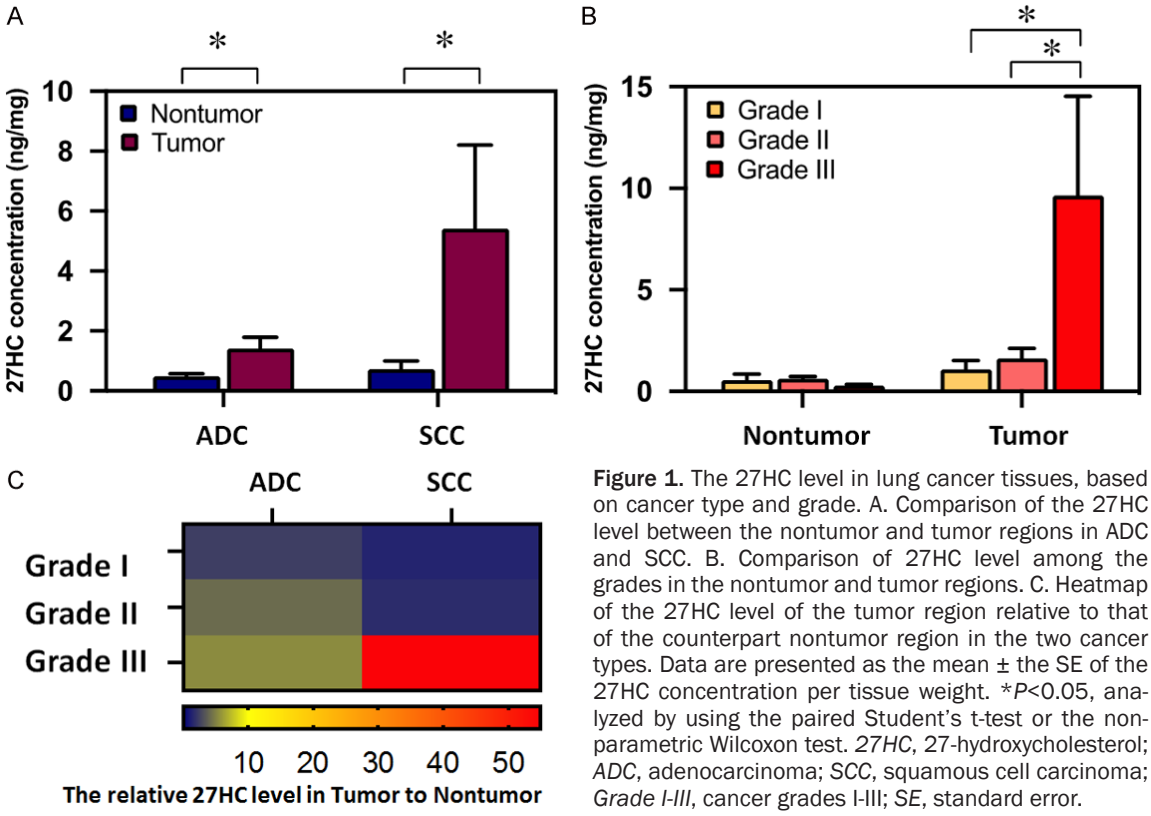

Figure 1. The 27HC level in lung cancer tissues, based on cancer type and grade. A. Comparison of the 27HC level between the nontumor and tumor regions in ADC and SCC. B. Comparison of 27HC level among the grades in the nontumor and tumor regions. C. Heatmap of the 27HC level of the tumor region relative to that of the counterpart nontumor region in the two cancer types. Data are presented as the mean ± the SE of the 27HC concentration per tissue weight. *P<0.05, analyzed by using the paired Student's t-test or the nonparametric Wilcoxon test. *27HC*, 27-hydroxycholesterol; *ADC*, adenocarcinoma; *SCC*, squamous cell carcinoma; *Grade I-III*, cancer grades I-III; *SE*, standard error.

The mRNA expression in the nontumor and tumor regions of lung cancer tissue

Figure 2 shows the mRNA expressions of *ERβ,* 27HC-related metabolic enzymes (*CYP27A1* and *CYP7B1*), cancer proliferation factors (*cMYC*, *HIF1α*, and *VEGF*), and cholesterol/oxysterol-related gene regulators (*LXR* and *SREBP1*) in the tumor and nontumor regions by NSCLC cancer type (**Figure 2**). In both cancer types, the mRNA expressions of *ERβ, CYP27A1, HIF1α*, and *LXR* were significantly higher in the tumor region than in the nontumor region. In addition, SCC had significantly higher mRNA expressions of *cMYC*, *VEGF,* and *SREBP1* in the tumor tissue. However, the *CYP27A1* mRNA expression was not different between the tumor and nontumor regions in both cancer types. *ERα* was not detectable in either region of both cell types (data not shown).

Immunohistochemical staining for CYP27A1 in lung cancer

Figure 3 shows histological images of H&E stain and IHC stains for ERβ, CYP27A1, CD68 in the nontumor and tumor regions of lung tissues obtained from patients with adenocarcinoma (**Figure 3A** and **3B**) and SCC (**Figure 3C** and **3D**). IHC staining revealed that ERβ protein was strongly expressed in the cancer cells of adenocarcinoma (**Figure 3Bb**) and SCC (**Figure 3Db**) but it was not expressed in the nontumor region in either cancer type (**Figure 3Ab** and **3Cb**). Compared to the tumor region of adenocarcinoma (**Figure 3Bb**), the tumor region of SCC had abundant expression of ERβ protein with a high cell proliferation (**Figure 3Db**).

CD68 is a surface marker of alveolar macrophages and expressed in the alveolar nonparenchymal cells of the nontumor regions (**Figure 3Ad** and **3Cd**) and in the infiltrated inflammatory cells of the tumor regions (**Figure 3Bd** and **3Dd**) in adenocarcinoma and in SCC. CYP27A1 protein was similarly expressed in the alveolar nonparenchymal cells of the nontumor regions (**Figure 3Ac** and **3Cc**), as well as in infiltrated inflammatory cells of the tumor region (**Figure 3Bc**), but not in cancer cells (**Figure 3Bc** and **3Dc**) in adenocarcinoma and in SCC. This finding supports that CYP27A1-positive nonparenchymal cells in the alveoli should be alveolar macrophages.

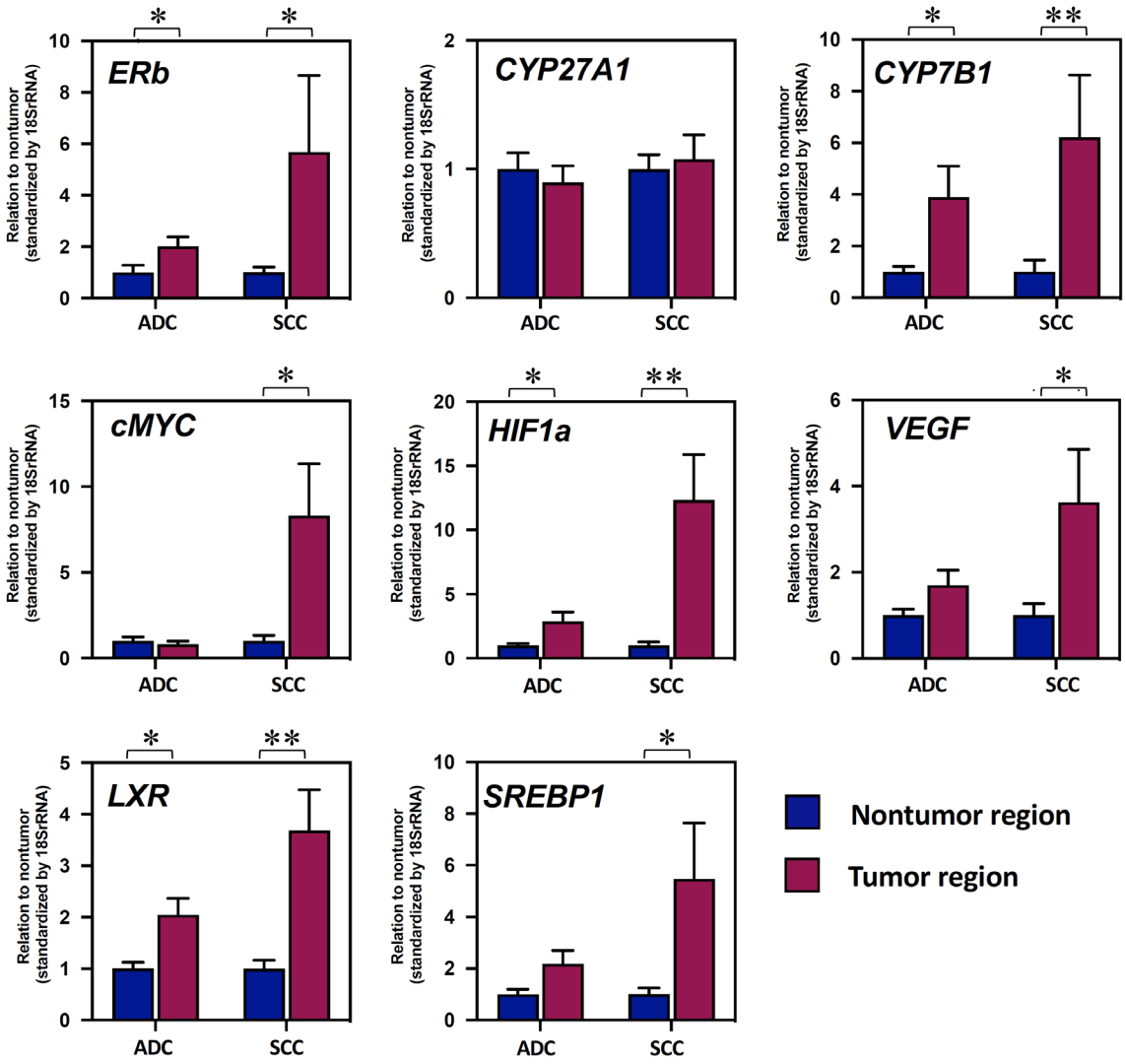

Figure 2. The mRNA expressions in the nontumor and tumor regions of lung cancer tissue collected from the patients with ADC or SCC. The mRNA expression level is expressed relative to that of the respective nontumor region, after standardization by 18SrRNA. The data are presented as the mean ± the SE. **P*<0.05, ***P*<0.01, †*P*<0.001, and ‡*P*<0.0001, based on the paired Student's *t*-test or the nonparametric Wilcoxon test between the nontumor and the counterpart tumor regions. *18SrRNA*, 18S ribosomal ribonucleic acid; *ADC,* adenocarcinoma; *cMYC*, *MYC* proto-oncogene (bHLH transcription factor); *CYP27A1*, cytochrome P450 27A1; *CYP7B1*, cytochrome P450 7B1; *ERβ*, estrogen receptor β; *HIF-1α*, hypoxia-inducible factor-1α; *LXR,* liver X receptor; *SCC*, squamous cell carcinoma; *SREBP-1,* sterol regulatory element binding protein-1; *VEGF*, vascular endothelial growth factor; *SE*, standard error.

The effects of 27HC and E2 treatments on cell proliferation in H23 cell lines

In the RT-PCR and Western blotting analyses, the expressions of ERβ mRNA, but not ERα, and of ERβ protein were confirmed in H23 cell lines (**Figure 4A** and **4B**). Cell proliferation of the ERβ-expressing H23 cells was significantly increased and was increased more than two-fold with 10 nM E2 treatment as the positive control, compared to the proliferation in the untreated negative control condition after a 48-hour incubation (**Figure 4C**). In treatment at doses between 1 nM and 1 μM, 27HC significantly increased the proliferation of H23 cells at 100 nM and 1 μM, similar to the increase with 10 nM E2 treatment. The significantly increased cell proliferations induced by 1 μM 27HC and 10 nM E2 treatment were significantly suppressed by cotreatment of the specific ERβ agonist PHTPP (**Figure 4D**).

Discussion

To examine the hypothesis that the cholesterol intermediate 27HC may be abundantly con-

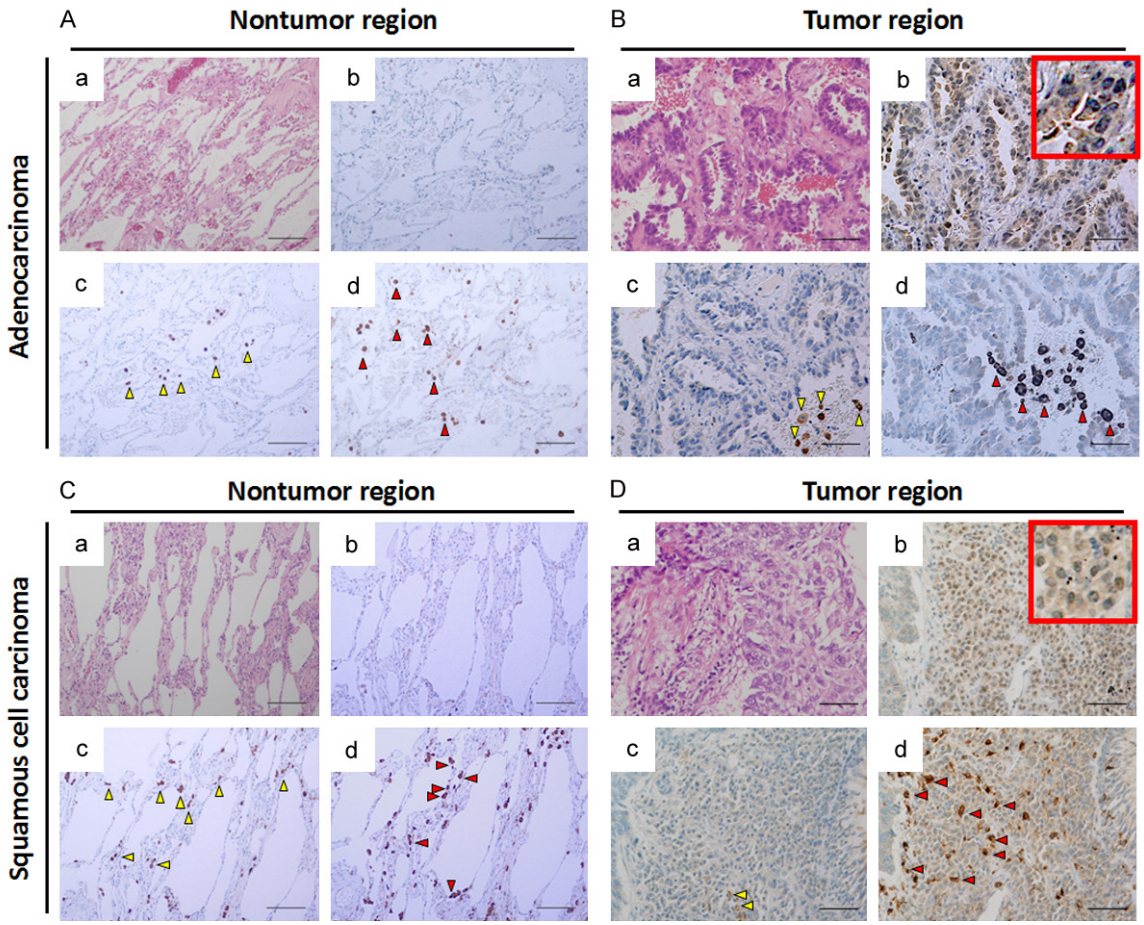

Figure 3. H&E and IHC stains of the (A and C) nontumor and (B and D) tumor regions in adenocarcinoma (upper row) and squamous cell carcinoma (lower row). The subpanels represent (a) H&E stain, (b) ERβ, (c) CYP27A1, and (d) CD68. The pictures (Bb and Db) in small window with red-outline are 3-fold focus image with positive stain of ERβ in plasma cell and nucleus. The arrow heads with yellow (c) and red (d) are positive cells with CYP27A1 and CD68. The adenocarcinoma slides are from a 59-year-old woman with Grade I cancer, and the squamous cell carcinoma slides are from a 74-year-old man with Grade III cancer. The black bars in the nontumor and tumor regions represent 100 μm (objective, ×10) and 200 μm (objective, ×20), respectively.

tained in lung tumor and associated with the cancer pathogenesis, similar to the role of SERM in promoting cell growth in breast cancer [13, 14] and in hepatoma and colon cancer cell lines [12, 15] the present study evaluated the metabolic and gene factors associated with 27HC and ER between the tumor region and its counterpart nontumor region within the same lung tissue collected from patients with NSCLC who were diagnosed with adenocarcinoma or SCC. The influence of 27HC treatment on the cell proliferation in the ERβ-positive lung cell line H23 was examined. The 27HC concentration and *ERβ* mRNA expression were significantly higher in the tumor region than in the nontumor region of the lung tissue in both NSCLC cancer types. In addition, the 27HC concentration in the tumor region was significantly higher in Grade III than it was in the other grades. In particular, the higher 27HC concentration in high-grade tumors was markedly increased in SCC. These results suggested that 27HC is associated with the cell proliferation activity of NSCLC through ERβ. This finding is supported by the results that 27HC significantly and in a dose-dependent manner promoted the proliferation of H23 cells, and this effect was canceled by a specific ERβ antagonist, PHTPP. The present results corroborated those of a previous study [22] reporting the promotive effects of 27HC in other lung cancer cell lines expressing ERβ and support the idea that 27HC exacerbates lung cancer pathogenesis via the ER.

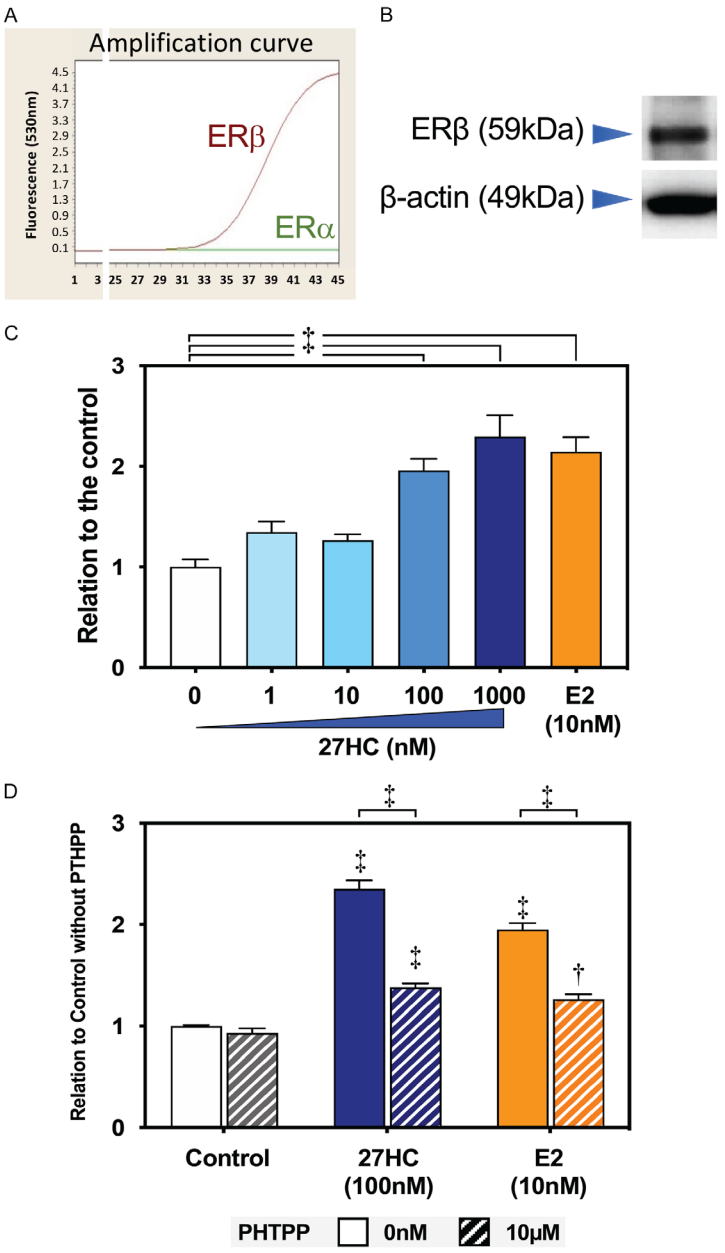

Figure 4. The effect of 27HC treatment on cell proliferation of H23 cells. A. Amplification curve of ERα and ERβ mRNA in H23 cell by the Realtime PCR method. B. The protein expressions of ERβ and β-actin in H23 cell measured by the Western Blotting method. Protein collected from H23 cells cultured with normal medium was used for detections of ERβ and β-actin. C. The proliferation of H23 cells treated with various concentrations of 27HC or with 10 nM E2. The H23 cells were treated with 1 nM to 1 μM 27HC or with 10 nM E2. ‡P<0.0001, compared to the control condition. D. The proliferation of H23 cells treated with 1 μM 27HC or 10 nM E2 with and without 10 μM PHTPP, a specific ERβ antagonist. †P<0.001 and ‡P<0.0001, compared to the respective control condition, and ‡P<0.0001 on columns with diagonal bars, compared to with and without PHTPP treatment. Cell proliferation is expressed as the related ratio versus the control condition. Data are presented as the mean ± the SE (N=8 per condition). Multiple comparison analysis was carried out using one-way ANOVA multiple comparison Dunnett's post hoc test. *27HC,* 27-hydroxycholesterol; *E2,* β-estradiol; *ERα*, estrogen receptor α; *ERβ*, estrogen receptor β, *PHTPP,* 4-[2-phenyl-5,7-bis(trifluoromethyl) pyrazolo[1,5-a]-pyrimidin-3-yl]phenol; *SE*, standard error.

In IHC staining (**Figure 3**), CYP27A1-positive alveolar macrophages, confirmed with the surface marker CD68, were in the nontumor regions and in the infiltrated sites around tumor tissues in SCC and in adenocarcinoma. However, ERβ protein was markedly expressed in the cancer cells of the tumor regions. ERβ expression was abundant in high-density proliferating cancer cells, which reflects a higher *ERβ* mRNA expression in the tumor region. These results support the idea that the 27HC excreted from the alveolar macrophages in cholesterol metabolism through CYP27A1 is transported into lung cancer cells to have a role as an ER modulator for the promotion of cell proliferation.

Alveolar macrophage has an important role in the immune response of lung cancer; however, its cytostatic activity differs, based on the cancer cell type [27]. Pouniotis *et al.* [27] demonstrated that a defect in phagocytic function; a change in phenotype with decreased surface expressions of CD54, CD83, and CD206; and reduced production of proinflammatory cytokines of tumor necrosis factor-α, interleukin (IL)-1 and IL-6 on alveolar macrophages isolated from lung tissue of SCC, as well as other types of lung cancer, including small cell carcinoma and large cell undifferentiated carcinoma. However, these alterations were not found in alveolar macrophages isolated from adenocarcinoma. These results suggest that, among NSCLC types, the ability of alveolar macrophage to generate antitumor immune response is compromised in SCC, compared to adenocarcinoma. The production of 27HC from alveolar macrophage may likewise differ between NSCLC cancer types. However, more detailed investigations are required on this point.

In addition to the activation of alveolar macrophage, ERβ expression in cancer cells should be an important factor in the role of 27HC as a SERM. The present cell culture experiments showed that the proliferation of H23 cells, which were isolated from adenocarcinoma, was significantly increased by 27HC treatment (**Figure 4C**), and preliminary confirmed that gene and protein of ERβ were highly expressed in H23 cell (**Figure 4A** and **4B**). These results of the cell culture experiments implied that cell proliferation is promoted if ERβ expression and its ligand 27HC both exist, regardless of lung cancer type, which is supported by the present evidence that the 27HC level and ERβ expression in the tumor region were higher in high-grade tumors of both cancer types (**Figure 1**). In this cell culture experiment, the significant promotive effect of 27HC treatment on the proliferation of H23 cells was at doses above 100 nM (**Figure 4C**). On the other hand, 27HC contents in the tumor region were approximately 2 and 7 ng/mg (5.0 and 17.4 nmol/g) as averages in adenocarcinoma and squamous cell carcinoma, respectively (**Figure 1A**). In conversion using water mass, 27HC level in the tumor regions in each cancer type could be estimated as 5.0 and 17.4 μM, and so, the cell culture results support that 27HC levels in the tumor region of both cancer types were sufficient to promote the growth of lung cancer cell.

In association with the higher 27HC content in the tumor region, the mRNA expression of *CYP7B1*, which encodes CYP7B1, the catabolic enzyme of 27HC, was significantly higher in the tumor region than in the nontumor region in both cancer types (**Figure 2**). CYP7B1, which is ubiquitously expressed in nearly all human tissues, is involved in the metabolism of androgens such as dehydroepiandrosterone, 5-androstene-3β,17β-diol, 5α-androstane-3β,17β-diol, and 5α-androstane-3α,17β-diol to their corresponding 7α-hydroxy steroids [28-30]. However, the significant increase in *CYP7B1* mRNA expression in the tumor region is likely caused by the increased 27HC level in the tumor region. The significant promotion of *CYP7B1* mRNA and protein expressions has similarly been observed in prostate cancer expressing ERβ, which suggests its potential importance in the development and progression of prostate cancer [31]. Likewise, CYP7B1 may be a possible key factor on regulation of the disease progression in lung cancer, and this point needs to be clarified in future study.

Concomitant with the significantly increased 27HC level in the tumor region, the mRNA expressions of the oncogene *cMYC*, and the cancer cell growth factors *VEGF* and *HIF1α* in SCC were also significantly higher in the tumor region than in the nontumor region (**Figure 2**). The transcriptional factor *cMYC* is a master regulator that controls many aspects of proliferation and metabolism in diverse types of human cancers [32, 33]. In breast cancer, previous studies [34-36] have reported that the *cMYC* gene expression is upregulated by estrogen-induced ER activation and coactivators through activating estrogen response element and activator protein 1 transcription factors. In addition, 27HC stabilizes cMYC protein via suppressing key negative modulators of MYC protein stability, a serine/threonine phosphatase PP2-A, an aspartate-based serine/threonine phosphatase SCP1, and ubiquitin ligase FBW7 at the transcription level in MCF-7 breast cancer cells [37].

A regulation on cancer metabolism is that cMYC also controls the supply of energy fuels. In addition to the specific reprogramming of energy metabolism in cancer cells such as Warburg's effect [38] and glutaminolysis [39], many amino acids (AAs) are utilized as intermediate precursors for energy fuels to meet increased energetic demands for active cell growth. In breast cancer cells, cMYC has been reported to act as a positive regulator for AA transporters such as system alanine-serine-cysteine transporter 2 (ASCT2; SLC1A5), AA transporter responsible for the activity of system $B^{0,+}$ ($ATB^{0,+}$; SLC6A14), L-type AA transporter 1 (SLC7A5), and system x_c^--transporter-related protein (SLC7A11) [40]. In addition, investigators have also reported that in breast cancer ASCT2 and $ATB^{0,+}$ are the respective target genes of cMYC [41, 42] and ER [43]. We previously reported significantly increased mRNA expressions of these AA transporters in the tumor region, compared to those in the counterpart nontumor region in lung cancer tissue collected from patients with NSCLC [44]. Therefore, 27HC should contribute to the augmented gene transcription of AA transporters induced by cMYC also in the lung cancer cells of patients with NSCLC.

27HC is also an endogenous agonist of a nuclear receptor LXR that regulates the metabolism of the cholesterol derivatives oxysterols. By using a mouse mammary tumor virus-poly-

oma middle T-antigen model, Nelson *et al*. [14] reported that 27HC injection stimulated metastasis to the lung by promoting the epithelial-to-mesenchymal transition by activating LXR, regardless of ER expression in breast cancer cells. The transcriptional factor SREBP1, a primary target gene of LXR, is highly expressed in NSCLC cell lines A549 and H1299 and in human lung cancer tissues [45]. In the NSCLC cell lines, the migration and invasion abilities and proliferation were suppressed by the down-regulation of SREBP1 together with an increase in the epithelial marker protein *E*-cadherin and a decrease in the mesenchymal marker proteins *N*-cadherin and vimentin [45].

In the present study, the mRNA expressions of SREBP1 and LXR were also significantly higher in the tumor region than in the nontumor region. Therefore, the increased 27HC content in lung cancer cells, similar to breast cancer, possibly promotes distant metastasis. However, the metastasis of lung cancer cells in patients of the present study were not fully evaluated because the M category was "0" (i.e., no distant metastasis) or "x" (undetermined). Therefore, in the tumor region, the influence of the increased 27HC content on metastasis was unclear in the present study, and further study is required.

A limitation of this study is the small number of cases used for statistical categorization of the measured parameters by the properties of the diseases and the patients such as histological type, age, and sex. In the future, the accumulation of numerous cases; subdividing them, based on the properties of diseases and the patients; and further elucidating the relationship between each pattern and 27HC will be necessary. For example, this information may allow the prediction of prognosis and the determination of therapeutic effects by studying the concentration of 27HC in blood samples of patients with lung cancer.

In conclusion, the present results supported idea that, similar to the SERM action in breast cancer, 27HC that is excreted from the alveolar macrophages is a factor that promotes the proliferation of NSCLC highly expressing ERs and aggravates their pathological conditions. Therefore, lowering the production of 27HC by alveolar macrophages and interfering with the activation of ERs in lung cancer cells should be an important target for cancer therapy in NSCLC.

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Disclosure of conflict of interest

None.

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