GCT TCA ACA AAA ACC CCA AAA GGA-3′ resulted in the amplification of a product containing XhoI and HindIII restriction enzyme cleavage sites. After enzymatic digestion the PCR product was subcloned into the pGL3 basic vector (Promega) and the insert identity was confirmed by sequencing.

2.2. Cell culture, transfection and reporter gene assays

MCF-7 cells were seeded at 3×10^5/ml in six-well plates and maintained at no higher than 70% confluence in DMEM (Trace Scientific Ltd., Melbourne, Australia) supplemented with 10% (v/v) fetal-calf serum (Trace Scientific), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 200 mM L-glutamine (Life Technologies, Inc., Auckland, New Zealand). Cells were transfected using the Nucleofector electroporation apparatus (Amaxa) as directed by the manufacturer. Briefly, 1×10^6 cells were trypsinised, washed and resuspended in 100 μl Solution V with 2 μg DNA and transfected using program E-014, with the LKB1prom vector as well as 10 ng of a renilla expression vector as a transfection control. Cells were plated in 24-well plates and incubated overnight. Prior to treatments, cells were serum-starved for 24 h in phenol-red free medium containing 0.1% BSA. After serum starvation, cells were treated with water-soluble 17β-estradiol (Sigma) at the concentrations indicated. Luciferase reporter assays were carried out using the Dual-Glo Luciferase Assay System (Promega) as described by the manufacturer.

2.3. Western blot analysis

Cells were washed in ice-cold PBS and lysed in ice-cold buffer as previously described [7]. Fifty micrograms of protein was denatured in buffer containing dithiothreitol, run on 8% polyacrylamide gels, and transferred to nitrocellulose for Western blotting. Western blotting was performed to assay phosphorylation of AMPK using antibodies to phosphopeptides based on the amino acid sequence surrounding Thr172 of the α-subunit of human AMPK (Cell Signaling, Beverly, MA). The level of phosphorylation was normalized to the level of total AMPK (Cell Signaling). A specific LKB1 antibody (Cell Signaling) was used to assess LKB1 protein levels. Proteins were visualized with an Alexa Fluor 680 goat anti-rabbit secondary antibody (Molecular Probes, Inc., Eugene, OR), and band intensities were quantified using the Odyssey infrared imaging system (Licor Biosciences, Lincoln, NE).

2.4. RT and real-time PCR

The RNeasy Mini kit (Qiagen) was used to extract total RNA and reverse-transcription was performed using AMV RT and random primers (Promega) as directed by the manufacturer. Briefly, 1.0 μg RNA was incubated with 0.5 μg random primers at 70 °C for 5 min, and RT reaction was incubated at 37 °C for 1 h. Quantification of human LKB1 and L32 transcript was performed on the RotorGene (Corbett) using primers hLKB1-F: 5′-GCC GGG ACT GAC GTG TAG A-3′, hLKB1-R: 5′-CCC AAA AGG AAG GGA AAA ACC-3′, hL32-F: 5′-CAG GGT TCG TAG AAG ATT CAA GGG-3′, hL32-R: 5′-CTT GGA GGA AAC ATT GTG AGC GAT C-3′. Cycling conditions were one cycle at 95 °C for 5 min, followed by a variable number of cycles of 95 °C for 10 s, 59 °C for 15 s, and 72 °C for 20 s. Experimental samples were quantified by comparison with standards of known concentrations. All samples were normalised to L32 transcript levels.

2.5. Chromatin immunoprecipitation

ChIP was performed to examine protein binding to the LKB1 promoter after cells were treated with experimental agents for 45 min. Sample preparation was performed as previously described [7]. Briefly, serum-starved cells were grown to 50% confluency and treated for 45 min at 37 °C for study of binding of transcriptional regulators to the LKB1 promoter. Cells were then cross-linked using 1% formaldehyde for 5 min at room temperature and collected in PBS containing protease inhibitors. Cells were lysed and sonicated at 20% max power 6 times for 30 s pulses using a Sonics sonifier. After sonication, one tenth of the total sample was removed for input. ChIP was performed using the ChIP-IT express kit (Active Motif) as directed by the manufacturer. Briefly, 5 μg of DNA was immunoprecipitated overnight at 4 °C with 5.0 μg antibody (ERα and IgG; Santa Cruz Biotechnology). Protein/DNA complexes were eluted from the beads and treated with proteinase K solution at 37 °C for 1 h. A number of putative ERα, AP-1 and Sp1 binding sites were identified in the region 2.5 kb upstream of the LKB1 promoter transcription start site using several online tools such as *AliBaba2.1* (http://www.gene-regulation.com/pub/programs/alibaba2/index.html), *PROMO* (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) and *Prediction of Nuclear Hormone Receptor Response Elements* (http://asp.ii.uib.no:8090/cgi-bin/NHR-scan/nhr_scan.cgi). Real-time PCR was performed on the purified DNA as described above using primers designed −2287 to −2020 (LKB1-ChIP-F: 5′-CTG CCT TCT TCC TGT TTT GC-3′; LKB1-ChIP-R: 5′-TTC TCC TCC TCC TCC TC-3′) for ERα binding to the LKB1 promoter. Images presented are representative of three separate experiments.

2.6. Breast cancer cases

This research was approved by Ethical Committee of Tohoku University (Approval number 2010-509). Eighteen cases of treatment naive primary breast cancer cases were retrieved from pathology files at Department of Pathology, Tohoku University School of Medicine, Sendai, Japan. Portions of tumour tissues were carefully dissected at the operation theatre following macroscopic evaluation of resected specimens and immediately frozen in liquid nitrogen with OCT compound and further stored at −80 °C for Laser Capture Microdissection (LCM) analysis and subsequent Real-Time PCR (RT-PCR) assay. Portions of the specimens were also immediately fixed in 10% neutral formalin for 18–36 h at room temperature and embedded in paraffin. 4 μM thick tumour sample tissue specimens were prepared by the specimens embedded into OCT compound using cryostat and stained with hematoxylin for detailed morphological analysis under light microscopy for laser dissection of each components. Tumour cells were carefully laser dissected and then collected under light microscopy. The dissected tumour cells components were then submitted for RNA extraction and RT-PCR assay with methods as described above. For IHC or immunohistochemistry, paraffin blocks were cut to 4 μM sections and deparaffinized. The sections were then submitted for antigen retrieval with microwave in citrate buffer (pH 6.0) for 20 min; following the block with normal goat serum for 30 min at 4 °C, the sections were incubated with a polyclonal anti-LKB1 antibody overnight (1:100 dilution, Cell signaling, USA). Envision staining system (DAKO Cp Ltd., Denmark) was used for subsequent staining and LKB1 immunoreactivity was visualized with 3,30-diaminobenzidine (Dojin Chemical Co. Ltd., Osaka, Japan). Reacted sections were then counterstained with hematoxylin.

In order to semiquantitate LKB1 immunoreactivity, relative immunointensity (+, ++) and ratio of immunoreactivity among carcinoma cells were added to classify the status of LKB-1 immunoreactivity into the following three categories. Tumours with no staining or ≤10% of cells with (+) staining were tentatively scored as 0, tumours with >10% of cells with (+) staining or ≤20%

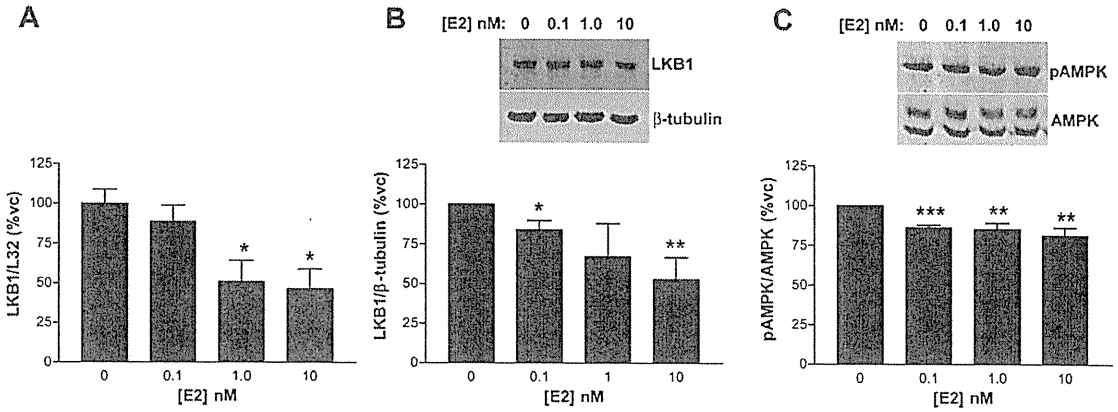

Fig. 1. Estradiol inhibits LKB1 expression and activity. Estradiol treatment of MCF-7 cells resulted in a dose-dependent decrease of LKB1 transcript (A) and protein (B) expression. (C) Estradiol treatment of MCF-7 cells resulted in a decrease in phosphorylation of AMPK. Graphs presented represent mean ± SEM. Single, double, and triple asterisks indicate statistically significant differences: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.005$. vc, vehicle control.

of cells with (++) staining as 1, and tumours with >20% of cells with (++) staining as 2.

ERα immunostaining status and other clinical parameters were retrieved from the charts of the patients.

2.7. Statistical analyses

For *in vitro* analysis, all experiments were performed at least three times and the data are reported as mean ± SEM. Statistical analyses were performed by two-tailed Student's *t* test. Kruskal Wallis non-parametric analyses were used to test correlations between LKB1 immunostaining score and different clinical parameters, Spearman non-parametric correlation for the analysis of the correlation between LKB1 and ERα. Immunostaining score. Single, double, and triple asterisks indicate statistically significant differences: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.005$. GraphPad Prism Version 3.00 was used.

3. Results

3.1. Estradiol decreases the expression of LKB1 in MCF-7 cells

MCF-7 cells are the most common breast cancer cell line employed to model ER-positive tumour cells. Their proliferation is largely dependent on the presence of E2, without which these cells cease to divide. The effect of estradiol on LKB1 expression was examined in MCF-7 cells after serum starvation. Treatment of MCF-7 cells with estradiol resulted in a dose-dependent decrease of LKB1 transcript and protein expression (Fig. 1A and B, respectively). This was accompanied by a similar decrease in phosphorylation of AMPK at Thr172 (Fig. 1C).

3.2. Estradiol decreases ERα binding to the LKB1 promoter

Chromatin immunoprecipitation assays performed using MCF-7 cells demonstrate that ERα binds to the LKB1 promoter. Interestingly, ERα binding to the LKB1 promoter is reduced when MCF-7 cells are treated with 10 nM E2 for 45 min (Fig. 2).

3.3. LKB1 promoter activity is decreased in the presence of estradiol

In order to assess the effect of estradiol on LKB1 promoter activity, a reporter construct was transfected into MCF-7. Consistent with effects on endogenous expression of LKB1 in MCF-7 cells, results demonstrate that estradiol caused a dose-dependent decrease in the activity of the LKB1 promoter in MCF-7 cells (Fig. 3).

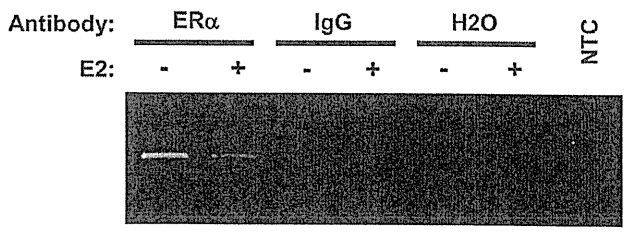

Fig. 2. ERα binds to the LKB1 promoter in the absence of estradiol. ChIP analysis demonstrated that ERα binding to the LKB1 promoter was reduced when MCF-7 cells were treated with 10 nM E2. The result is representative of three separate experiments.

3.4. Correlation between LKB1 and ER status

The results are summarized in Tables 1 and 2. There was no significant correlation between LKB1 IHC score/mRNA and other clinicopathological parameters examined in these cases (Tables 1 and 2, respectively). In addition, there were no significant correlations between LKB1 IHC score and ER Allred score as demonstrated by Spearman non-parametric test ($r = -0.186$, $P = 0.460$).

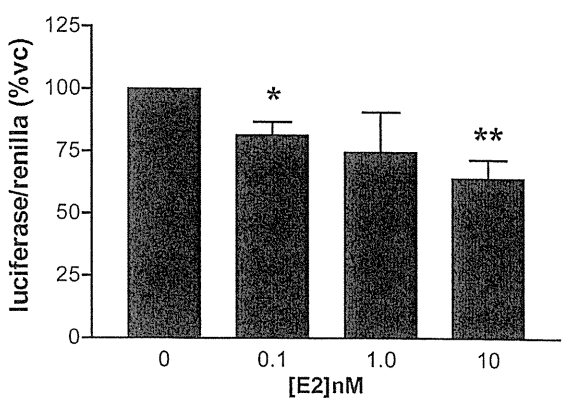

Fig. 3. Estradiol inhibits LKB1 promoter activity. A reporter construct containing 3003 bp of the LKB1 promoter was transfected into MCF-7. Results demonstrate that promoter activity was significantly decreased with increasing doses of estradiol in MCF-7 cells. Graphs presented represent mean ± SEM. Single, double, and triple asterisks indicate statistically significant differences: $^{*}p < 0.05$; $^{**}p < 0.01$. vc, vehicle control.

Table 1
LKB1 IHC in primary breast cancer tissues.

Parameters	*n*	Cases of different LKB1 scores			*P* value†
		0	1	2	
Age					
≤50	6	4	1	1	0.205
>50	12	3	7	2	
Nuclear grade					
I	8	3	3	2	0.068
II	5	4	1	0	
III	5	3	2	0	
Nottingham grade					
I	0	0	0	0	0.317
II	12	7	3	2	
III	2	0	2	0	
ER state[a]					
Negative	2	0	2	0	0.543
Positive	16	7	6	3	
PgR state[b]					
Negative	6	2	4	0	0.761
Positive	12	5	4	3	
HER-2 state[c]					
Negative	14	6	5	3	0.954
Positive	4	1	3	0	

† Kruskal Wallis non-parametric test was used for comparing LKB1 immunostaining score among different groups.
[a] The specimens with ER allred score ≤ 2 were classified into ER negative group, those with ER allred score > 2 were classified into ER positive group.
[b] The specimens with PgR allred score ≤ 2 were classified into PgR negative group, those with PgR allred score > 2 were classified into PgR positive group.
[c] The specimens with HER-2 scored 0 and 1 were classified into HER-2 negative group, those with ER scored 2 and 3 were classified into ER positive group.

4. Discussion

The regulation of LKB1 in various tissues has previously been examined (reviewed in [8,9]). The majority of these studies have focussed on the regulation of LKB1 phosphorylation by PKCζ and its resultant action on AMPK, leaving few indices as to the transcriptional regulation of the *STK11* gene. Results presented herein are therefore the first to describe the transcriptional regulation of LKB1 by estradiol and to identify ERα as a direct modulator of LKB1 promoter activity.

ChIP analysis in MCF7 cells showed binding of ERα to the *STK11* promoter, however consistent with the effects of 17β-estradiol on LKB1 expression, ERα binding to the *STK11* promoter was reduced in the presence of 17β-estradiol. It remains to be determined which site of the *STK11* promoter is involved in the ligand-independent binding of ERα to DNA and whether binding occurs in a similar manner in untransformed cells. Considering recent evidence identifying LKB1 as a tumour suppressor by virtue of its direct interaction with p53 [2,10,11], our results provide an additional mechanism by which estradiol can promote cell cycle progression in cells with a wild-type *TP53* gene.

LKB1 protein expression and pAMPK are decreased in primary breast tumours compared to normal breast epithelium [12,13]. Interestingly, we examined LKB1 mRNA and IHC score in primary tumours obtained from 18 Japanese patients with breast cancer. Results of this evaluation revealed no significant correlations of the LKB1 status with ER status in these patients. This is consistent with previous findings demonstrating that LKB1 and pAMPK IHC had no association with ER status [2,13]. Considering the effect of estradiol to inhibit LKB1 expression and activity in MCF-7 cells, the present study is the first to offer a mechanism whereby LKB1 expression is low in both ER-positive and ER-negative primary tumours, and why there is a discrepancy between untreated cell lines and primary tumours.

In parallel with this process, the inhibition of the LKB1/AMPK pathway will likely also result in the stimulation of *de novo* lipogenesis within the breast cancer cells, which is also an important factor contributing to breast cancer cell proliferation [14], and will prevent AMPK from inhibiting cancer cell proliferation through direct phosphorylation of TSC2 and mTORC1 (mammalian target of rapamycin complex 1), thereby preventing it from effectively shutting down protein synthesis and counteracting the stimulatory effects of Akt [15]. Furthermore, LKB1 has also been shown to negatively regulate aromatase [7], the enzyme responsible for converting androgens to estrogens. Consistent with these findings, it has been shown that metformin, a known LKB1-dependent stimulator of AMPK, inhibits proliferation of breast cancer cells in culture [16,17], aromatase expression in breast stroma [18] and inhibits spontaneous tumours from developing in PTEN deficient mice [19–21].

Table 2
LKB1 mRNA expression in primary breast cancer tissues.

Parameters	*n*	Relative LKB1 mRNA level (%, Mean ± SE)	*P* value†
Age			
≤50	6	1.876 ± 0.496	0.407
>50	12	3.672 ± 1.449	
Nuclear grade			
I	8	3.395 ± 1.699	0.657
II	5	1.604 ± 0.402	
III	5	4.029 ± 2.411	
Nottingham grade			
I	0	–	0.211
II	12	2.791 ± 1.147	
III	2	7.372 ± 6.054	
ER state[a]			
Negative	2	2.721 ± 1.039	0.904
Positive	16	3.118 ± 1.108	
PgR state[b]			
Negative	6	3.935 ± 1.957	0.553
Positive	12	2.643 ± 1.154	
HER-2 state[c]			
Negative	14	2.560 ± 0.988	0.345
Positive	4	4.871 ± 2.929	

†ANOVA was used for comparing relative LKB1 mRNA expression among multigroups; independent Student's *t* test was used for comparing relative LKB1 mRNA expression between two groups.
[a] The specimens with ER allred score ≤ 2 were classified into ER negative group, those with ER allred score > 2 were classified into ER positive group.
[b] The specimens with PgR allred score ≤ 2 were classified into PgR negative group, those with PgR allred score > 2 were classified into PgR positive group.
[c] The specimens with HER-2 scored 0 and 1 were classified into HER-2 negative group, those with ER scored 2 and 3 were classified into ER positive group.

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Original Article

Retinoid receptors in human esophageal squamous cell carcinoma: Retinoid X receptor as a potent prognostic factor

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Retinoids regulate cell proliferation and differentiation in normal and neoplastic tissue. These effects are mainly mediated by two types of nuclear retinoid receptors, retinoic acid receptors (RAR) and retinoid X receptors (RXR). RXR have been demonstrated to play important roles in esophageal carcinoma, but the expression of RXRβ and RXRγ has not been examined in esophagus. Therefore, we examined the immunoreactivity of all subtypes of RAR and RXR in 53 non-neoplastic esophageal epithelium and 74 esophageal squamous cell carcinoma tissues. In non-neoplastic epithelium RARβ immunoreactivity was marked in the basal layer and weak in the suprabasal layer, but immunoreactivity of other retinoid receptors was detected in both of layers. In addition, the status of RARβ and RXRβ immunoreactivity inversely correlated with that of lymph node metastasis ($P = 0.0477$ and $P = 0.0034$, respectively); decreased RXRβ immunoreactivity of carcinoma cells was positively associated with adverse clinical outcome of the patients ($P = 0.0187$). These findings all indicate the important roles of retinoid receptors, especially, RXR in the esophageal squamous cell carcinoma.

Key words: esophageal cancer, immunohistochemistry, metastasis, retinoid receptors

Clinical outcomes of patients with esophageal carcinoma still remain poor, despite the recent advances of therapeutic techniques and perioperative management. Retinoids are known to inhibit cell proliferation in a wide range of normal and neoplastic tissues *in vitro*.[1–6] Retinoids suppress or reverse the process of epithelial carcinogenesis and prevent the development of invasive cancers, including squamous cell carcinoma, arising in the skin, lung and oral cavity in animal models.[7] In addition, the incidence of cancer in a group of patients with severe esophageal squamous dysplasia treated by the synthetic retinoid N-4-(ethoxycarbophenyl) retinamide was reported to be much lower than that of a group treated with placebo.[8]

The effects of retinoids are mainly mediated via two different classes of nuclear retinoid receptors, retinoic acid receptors (RAR)[9–11] and retinoid X receptors (RXR),[12,13] both of which belong to the steroid/thyroid hormone receptor superfamily. Retinoid receptors are known to function as heterodimers of RAR and RXR, or as RXR homodimers, and to activate transcription in a ligand-dependent manner by binding to retinoic acid responsive elements (RARE) located in the promoter region of various target genes.[14] Both RAR and RXR are composed of three subtypes, α, β and γ. Patterns of these retinoid receptor subtypes status or combination are considered to regulate the expression of distinct target genes and the actions of retinoids in various tissues at both physiological and pathological status.[15]

Results of previous studies have demonstrated that the loss of RARβ expression and upregulation of both RARα and RXRα expression are detected in esophageal squamous cell carcinoma by *in situ* hybridization or immunohistochemistry.[4,16–19] In addition, esophageal squamous carcinoma cell lines that did not express RARβ were resistant to retinoic acid treatment and could form colonies in soft agar.[4,20] These findings suggest that retinoids may play important roles in esophageal squamous cell carcinoma. However, the status of RXRβ and RXRγ has not been examined in esophageal squamous cell carcinoma tissue, and the biological and clinical significance of

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retinoids has remained unclear. Therefore, in this study, we examined the expression of all six retinoid receptor subtypes in 74 cases of esophageal squamous cell carcinoma using immunohistochemistry and correlated these findings with various clinicopathological parameters of the patients.

MATERIALS AND METHODS

Patients and tissue samples

In this study 74 surgical pathology specimens of esophageal squamous cell carcinomas were retrieved from surgical pathology files of the Department of Pathology, Tohoku University Hospital, Sendai, Japan. These specimens were obtained from patients who underwent esophagotomy from 1994 to 1999. All the patients examined received neither irradiation nor chemotherapy prior to surgery. Potentially curative resection was defined as the absence of distant metastasis, the removal of all gross tumors, and the histologically confirmed absence of tumor tissue at the surgical margins of the resected specimens. Each patient underwent cervico-thoraco-abdominal (three field) lymph node dissection.[21,22] The mean follow-up time for patients was 71 months (range 8–121 months). Non-neoplastic squamous epithelium was also available for examination in all 74 cases. The specimens had been all routinely processed (10% formalin-fixed and paraffin-embedded). The Ethics Committee at Tohoku University School of Medicine approved the research protocol for this study.

Antibodies

Polyclonal antibodies for RARα (sc-551), RARγ (sc-550) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), monoclonal antibody for RARβ was purchased from Lab Vision Corporation (NeoMarkers, Fremont, CA, USA). Polyclonal antibodies for RXRα, RXRβ and RXRγ were raised against synthetic peptides containing the following mouse RXR amino acid residues: RXRα 92–109; RXRβ 78–93; RXRγ 35–54. The characterization of the RXR antibodies was confirmed by immunoblotting and immunoprecipitation as described previously, and use of these antibodies for immunohistochemistry has been reported previously.[23] Monoclonal antibodies for Ki-67 (MIB1) and p53 were purchased from Immunotech (Marseille, France) and Chemicon (Temecula, CA, USA), respectively. The optimal dilution and pretreatment methods for immunostaining are summarized in Table 1.

Table 1 Summary of primary antibodies

Antibodies	Dilution	Antigen retrieval†
RARα (polyclonal)	1:500	Autoclave
RARβ (monoclonal)	1:1 (predilution)	Autoclave
RARγ (polyclonal)	1:500	Autoclave
RXRα (polyclonal)	1:3500	Autoclave
RXRβ (polyclonal)	1:1500	Autoclave
RXRγ (polyclonal)	1:1500	Autoclave
Ki-67 (monoclonal)	1:50	Autoclave
P53 (monoclonal)	1:50	Autoclave

†Autoclave for 5 min at 121°C in 0.01 mol/L sodium citrate buffer (pH 6.0).
RAR, retinoic acid receptor; RXR, retinoid X receptor.

Immunohistochemistry

Immunohistochemical analysis was performed as follows.[24] Serial 3 μm thick sections were prepared. Tissue sections were deparaffinized in xylene and dehydrated in a gradient of ethanol. An antigen retrieval method was then employed. The slides were heated in an autoclave at 121°C for 5 min in citric acid buffer (2 mM citric acid and 9 mM trisodium citrate dehydrate, pH 6.0). Sections were then incubated with 10% normal goat serum for the polyclonal antibody, or normal rabbit serum for the monoclonal antibody to reduce nonspecific background immunostaining. Tissue sections were incubated for 12 h at 4°C with primary antibodies, except for RARβ, which was incubated for 30 min at room temperature. The dilutions of primary antibodies used are summarized in Table 1. Thereafter intrinsic peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 30 min at room temperature. The sections were then incubated with biotinylated goat antirabbit IgG (Histofine Kit; Nichirei, Tokyo, Japan) and with horseradish peroxidase-conjugated streptavidin (Nichirei). Reacted sections were developed with 3,3′-diaminobenzidine (DAB) and counterstained with hematoxylin. As a negative control, sections were incubated with normal rabbit IgG instead of primary antibodies. No specific immunoreactivity was detected in these tissue sections.

Scoring of immunoreactivity

All immunolabeled cells were evaluated as positive, regardless of the immunointensity. For evaluation of retinoid receptors, Ki-67 and p53, scoring in proliferative lesions were evaluated independently by two of the authors (FF and TS) in high-power field (x400) using light microscopy. In each lesion at least 500 cells were counted and the percentage of immunoreactivity (i.e. labeling index; LI), was determined.[25,26] Cases that were found to have p53 LI ≥ 50% were considered p53-positive esophageal carcinomas according to a previous report.[27] In non-neoplastic epithelium, two different layers of epithelium (basal and suprabasal) were histologically identified and evaluated separately. In each region 200–500 cells were counted, and LI was subsequently obtained.

Statistical analyses

Values for LI for retinoid receptors were summarized as a mean ± SD. An association between the LI of retinoid receptors and clinicopathological parameters between the values in the absence or presence of Hx630 was evaluated using a one-way ANOVA and Bonferroni's test. The correlation analysis among different parameters with continuous variables was assessed with a correlation coefficient (r) and regression equation. Overall survival curves were generated according to the Kaplan–Meier method, and statistical significance was calculated using the log–rank test. Univariate analyses were evaluated by Cox proportional hazards regression model. A P value less than 0.05 was considered significant.

RESULTS

Non-neoplastic epithelium of the esophagus

Immunoreactivity of all six retinoid receptor subtypes was detected in the nuclei of non-neoplastic squamous epithelium (Fig. 1a–f). In basal layer of non-neoplastic squamous epithelium of the esophagus, immunoreactivity of RARβ, RXRβ and RXRγ was abundant (LI > 50) (RARβLI = 56.9, RXRβLI = 53.1 and RXRγLI = 65.4), while that of RARα, RARγ and RXRα was present (RARα LI = 22.7, RARγ LI = 39.3 and RXRα LI = 43.7) in a less abundance. In suprabasal layer, immunoreactivity of RARγ and RXRα, β, γ was abundantly present (RARγ LI = 56.5, RXRα LI = 59.3, RXRβ LI = 59.2 and RXRγ LI = 58.4), and that of RARα was detected (RARα LI = 31.4) in a less abundance. RARβ immunoreactivity was negligible in the suprabasal layer (LI = 6.6). RARβ LI was significantly decreased in suprabasal layer compared with basal layer ($P < 0.0001$) (Fig. 2).

Esophageal squamous cell carcinoma

Immunoreactivity of RARα, β, γ and RXRα, β, γ was detected in the nuclei of carcinoma cells (Fig. 1g–l). Immunoreactivity of RARγ and RXRα, β, γ was abundant (LI > 50; RARγ LI = 55.9; RXRα LI = 53.3; RXRβ LI = 64.4; and RXRγ LI = 54.1), and that of RARα was detected in less abundance(LI = 41.0), RARβ LI was significantly lower (LI = 15.1) in these cells (Fig. 3). Among the immunoreactivities of retinoid receptor subtypes examined, statistically significant correlations were detected between RARα LI and RXRγ LI ($P = 0.0438$), RARβ LI and RARγ LI ($P = 0.0275$), RARβ LI and RXRα LI ($P = 0.0304$), RARβ LI and RXRβ LI ($P = 0.0084$), RARγ LI and RXRα LI ($P = 0.0391$), RARγ LI and RXRβ LI ($P = 0.0120$), RXRα LI and RXRβ LI ($P < 0.0001$) and RXRα LI and RXRγ LI ($P = 0.0057$) (Table 2).

Association of RAR and RXR immunoreactivities with clinicopathological features and clinical outcome of the patients

Association between the status of retinoid receptor immunoreactivity and the clinicopathological features of patients is summarized in Tables 3 and 4. There was a significant inverse correlation between the status of RARβ immunoreactivity and lymph node metastasis in the patients ($P = 0.0477$), but no significant association was detected between RARα or RARγ immunoreactivity and the clinicopathological features of the patients examined in this study (Table 3).

As shown in Table 4, RXRα immunoreactivity was inversely associated with the depth of invasion ($P = 0.0184$), and tumor, node, metastasis (TNM) stage ($P = 0.0329$). RXRβ immunoreactivity was inversely associated with patients' lymph node metastasis status ($P = 0.0034$) and the expression of p53 ($P = 0.0315$). In addition, RXRβ immunoreactivity was also inversely associated with depth of invasion ($P = 0.0388$), and therefore TNM stage ($P = 0.0137$). RXRγ immunoreactivity was low in T1b stage carcinomas, but no other significant association was detected in this study.

Table 5 summarizes the results of the univariate analysis of the clinical outcome of esophageal carcinoma patients according to the status of immunoreactivity of retinoid receptors. RXRβ immunoreactivity was significantly associated with a better prognosis ($P = 0.0187$), but no significant association was detected in other retinoid receptor subtypes. TNM stage also turned out to be a significant prognostic factor for overall survival in this study ($P = 0.0087$). As shown in Fig. 4, RXRβ immunoreactivity was significantly associated with improved clinical outcome in 74 esophageal squamous cell carcinomas ($P = 0.0368$).

DISCUSSION

To the best of our knowledge, this is the first report demonstrating immunolocalization of all six retinoid receptor subtypes in patients with esophageal carcinoma and non-neoplastic esophageal epithelium. In the non-neoplastic squamous epithelium of the esophagus, RARβ immunoreactivity was mainly detected in the basal layer, which demonstrated different immunolocalization patterns from other retinoid receptors. Previously, Crowe *et al.*[28] and Schon and Rheinwald[29] independently reported that RARβ mRNA expression was correlated with mRNA levels of K19 expressed in the basal cells of non-keratinizing epithelium, but not in keratinizing epithelium. Therefore, RARβ has been considered to play an important role in squamous differentiation in the esophageal epithelium by retinoids. Considering that RARβ was expressed in normal non-keratinizing

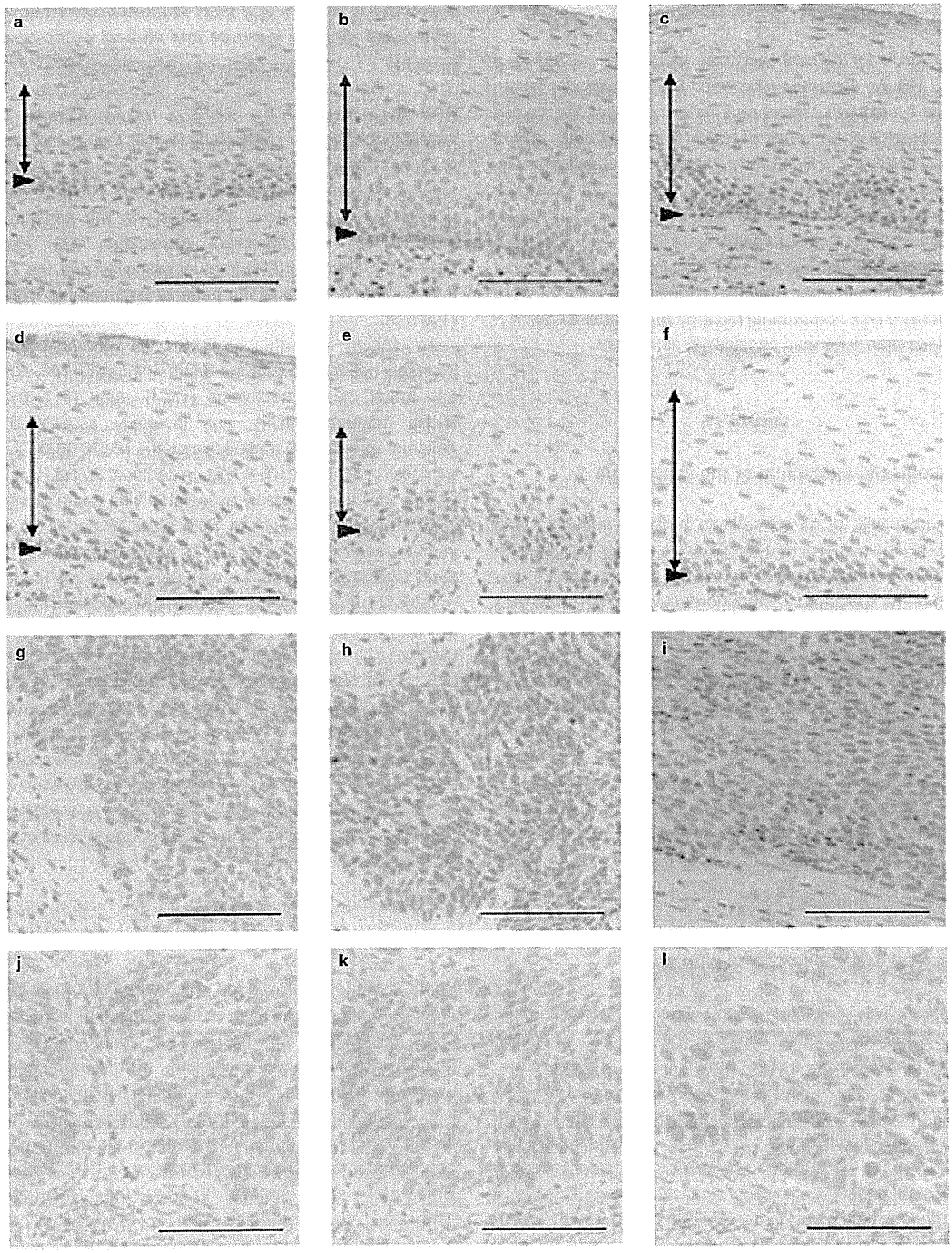

Figure 1 Immunohistochemistry for retinoic acid receptor (RAR) and retinoid X receptor (RXR) in non-neoplastic epithelium and esophageal carcinoma. In non-neoplastic epithelium of the esophagus, RARα (**a**), RARγ (**c**), RXRα (**d**), RXRβ (**e**), RXRγ (**f**) immunoreactivity was detected both in the basal (arrowheads) and suprabasal layers (both arrows), while RARβ (**b**) immunoreactivity was detected mainly in the basal layer. In esophageal carcinoma, carcinoma cells were frequently positive for RARα (**g**), RARγ (**i**), RXRα (**j**), RXRβ (**k**) and RXRγ (**l**), while RARβ immunoreactivity was weak (**h**). The labeling index (LI) of the pictured non-neoplastic epithelium were RARα basal 42, RARα suprabasal 53, RARβ basal 65, RARβ suprabasal 0, RARγ basal 45, RARγ suprabasal 60, RXRα basal 58, RXRα suprabasal 70, RXRβ basal 75, RXRβ suprabasal 61, RXRγ basal 63 and RXRγ suprabasal 79. In the esophageal carcinoma LI pictured were RARα 57, RARβ 46, RARγ 68, RXRα 70, RXRβ 79 and RXRγ 23, respectively.

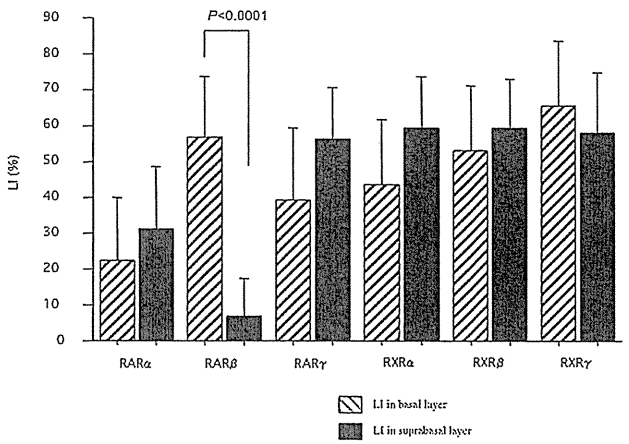

Figure 2 Immunohistochemistry of retinoic acid receptor (RAR) and retinoid X receptor (RXR) in 53 non-neoplastic epithelium of esophagus. RARβ labeling index (LI) was significantly decreased in the suprabasal layer compared with the basal layer ($P < 0.0001$). Data are presented as mean ± SD.

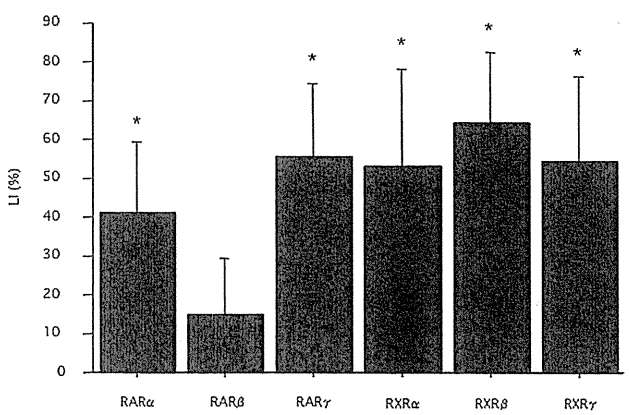

Figure 3 Immunohistochemistry of retinoic acid receptor (RAR) and retinoid X receptor (RXR) in 74 esophageal squamous cell carcinomas. RARβ labeling index (LI) was significantly more decreased than the other retinoid receptors. Data are presented as mean ± SD. *; $P < 0.001$ vs. RARβ LI by ANOVA followed by a Bonferroni's adjustment.

epithelium such as oral[30] and esophagal,[4,16–19] but not in normal keratinizing epithelium, like skin,[31] squamous differentiation by retinoids may be regulated by different retinoid receptor subtypes according to tissue.

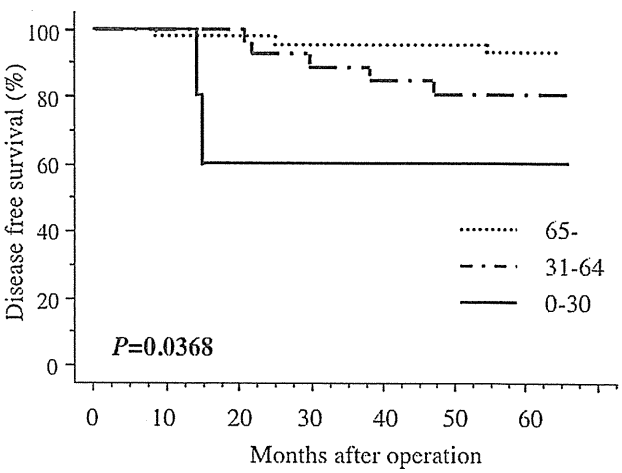

Figure 4 Overall survival of 74 patients with esophageal carcinoma according to retinoid X receptor β (RXRβ) immunoreactivity (Kaplan-Meier method). RXRβ immunoreactivity was significantly associated with better prognosis ($P = 0.0368$). Immunoreactivity was separated into three groups (0–30, 31–64, 65–100%).

In the esophageal squamous cell carcinomas, RXRβ immunoreactivity was inversely associated with the status of lymph node metastasis of patients, which was also significantly associated with better clinical outcomes of the patients. Biological and clinical significance of RXRβ has been examined in a variety of carcinoma tissues. Brabender *et al.* reported that using real-time polymerase chain reaction analysis amounts of RXR mRNA expression were decreased in non-small-cell lung cancer compared with matching normal lung tissue (RXRα, 67%; RXRβ, 55%; RXRγ, 89%) and the patients whose tumors exhibited high RXRβ expression levels had statistically significant better overall survival.[32] In addition, using immunohistochemistry, Alfaro *et al.* demonstrated that diminished RXRβ expression may be related to prostate cancer progression.[33] Ariga *et al.* reported that the RXRβ protein level was significantly lower in ductal carcinoma *in situ* than in intraductal proliferative lesions.[26] Tamoto *et al.* reported that in the esophagus the results of gene expression profile data were correlated with the status of lymph node metastasis in 36 esophageal squamous cell carcinoma tissues using cDNA array.[34] These results demonstrate that RXRβ gene expression is diminished in carcinoma cases with lymph node metastasis. Results of our present

Table 2 Association among immunoreactivity of RAR and RXR in 74 esophageal carcinoma tissues

Immunoreactivity	RAR β	RAR γ	RXR α	RXR β	RXR γ
RAR α	0.9269 (r = –0.011)	0.7429 (r = 0.039)	0,0700 (r = 0.212)	0.0637 (r = 0.217)	**0.0438** (r = 0.253)
RAR β		**0.0275** (r = 0.256)	**0.0304** (r = 0.252)	**0.0084** (r = 0.304)	0.9779 (r = –0.003)
RAR γ			**0.0391** (r = 0.240)	**0.0120** (r = 0.291)	0.5132 (r = –0.077)
RXR α				**<0.0001** (r = 0.514)	**0.0057** (r = 0.319)
RXR β					0.8180 (r = 0.027)

$P < 0.05$ was considered significant, and highlighted in bold.
RAR, retinoic acid receptor; RXR, retinoid X receptor.

Table 3 Summary of *P*-values for correlation between LI of RAR and the clinicopathological parameters in 74 esophageal carcinoma patients

Characteristics	*n*	RAR α	*P*	RAR β	*P*	RAR γ	*P*
Age							
≤60 years	19	42.8 (±16.0)	0.6269	14.4 (±15.4)	0.7967	56.9 (±18.4)	0.7661
≥61 years	55	40.4 (±19.5)		15.4 (±13.8)		55.5 (±18.6)	
Sex							
Men	62	39.9 (±19.4)	0.2499	14.3 (±13.3)	0.2204	57.1 (±17.8)	0.1772
Women	12	46.7 (±12.9)		19.8 (±17.7)		49.3 (±21.1)	
Histological grade							
Well	11	43.3 (±18.1)	0.553	13.0 (±12.9)	0.2703	47.4 (±20.9)	0.05
Moderate	48	39.3 (±18.4)		14.0 (±13.7)		59.6 (±16.4)	
Poor	15	44.8 (±20.0)		20.4 (±16.1)		49.9 (±20.3)	
Depth of tumor							
T1a	15	42.4 (±15.5)	0.9228	22.2 (±13.7)	0.0927	61.8 (±13.0)	0.1705
T1b	43	41.0 (±20.5)		13.6 (±14.8)		56.2 (±18.7)	
T2	16	39.7 (±16.6)		12.7 (±11.1)		49.4 (±20.9)	
TNM stage							
I	47	41.3 (±20.7)	0.8709	17.6 (±15.7)	0.0529	56.8 (±18.8)	0.577
II	27	40.5 (±14.6)		11.0 (±9.9)		54.3 (±18.0)	
Lymph node metastasis							
Positive	19	36.0 (±17.8)	0.1765	9.6 (±8.0)	**0.0477**	51.7 (±19.7)	0.2621
Negative	55	42.7 (±18.7)		17.1 (±15.3)		57.3 (±17.9)	
p53†							
Positive	37	40.6 (±15.4)	0.8672	14.4 (±13.1)	0.6542	53.8 (±19.4)	0.3446
Negative	37	41.4 (±21.5)		15.9 (±15.2)		57.9 (±17.5)	
Ki-67			0.0931 (r = 0.197)		0.4375 (r = –0.092)		0.3973 (r = –0.100)

Data are presented as mean ± SD.
$P < 0.05$ was considered significant, and highlighted in bold.
†Positive cases were those stained over 50%.[26]
RAR, retinoic acid receptor; TNM, tumor, node, metastasis; *r*, correlation coefficient.

study were consistent with those of these previous reports. Therefore, RXRβ is considered to play an important role in the inhibition of esophageal carcinoma development and proliferation.

Previous *in vivo* studies demonstrated the presence of RAR/RXR heterodimers such as RARα/RXRα,[35] RARγ/RXRα,[36] RARβ/RXRα,[37] RARγ/RXRβ,[38] and RARβ/RXRβ[38] in several cancer cell lines. In neuroblastoma cells, RARγ/RXRβ was the predominant heterodimer in the absence of 9-*cis* retinoic acid, whereas the balance shifted in favor of RARβ/RXRβ in the presence of ligands.[38] There are no reports of the biological functions of retinoid receptor dimerization in human esophageal carcinoma, but results of our present study demonstrated a significant association of immunoreactivity between RARα/RXRγ, RARβ/RARγ, RARβ/RXRα, RARβ/RXRβ, RARγ/RXRα, RARγ/RXRβ, RXRα/RXRβ and RXRα/RXRγ. Therefore these dimerizations may play important roles in the mediation of retinoid actions in the esophageal carcinoma. Results of our present study also demonstrated that RARβ and RXRβ immunoreactivity were inversely associated with the status of lymph node metastasis in patients. Therefore, the RARβ/RXRβ heterodimer may play an important role in the development of esophageal carcinoma.

In the present study, there are significant correlations among not only RAR/RXR but also RXR/RXR. These data suggest that RXR homodimers are present in cancerous tissues and play a role in esophageal carcinoma. There are no reported studies regarding RXR homodimers and the actions of the RXR agonist in esophagus. However if RXR

Table 4 Summary of *P*-values for correlation between LI of RXR and the clinicopathological parameters in 74 esophageal carcinoma patients

Characteristics	*n*	RXR α	*P*	RXR β	*P*	RXR γ	*P*
Age							
≤60 years	19	60.6 (±23.2)	0.1361	62.7 (±21.1)	0.6516	57.5 (±22.8)	0.448
≥61 years	55	50.8 (±24.7)		64.9 (±17.4)		52.9 (±22.0)	
Sex							
Men	62	52.9 (±23.9)	0.7383	64.6 (±19.8)	0.7563	54.3 (±22.0)	0.8963
Women	12	55.5 (±28.9)		62.8 (±19.8)		53.3 (±23.8)	
Histological grade							
Well	11	59.6 (±24.1)	0.5542	64.5 (±20.8)	0.3536	49.8 (±24.0)	0.7134
Moderate	48	51.2 (±23.6)		62.5 (±19.5)		54.2 (±21.6)	
Poor	15	55.5 (±28.5)		70.3 (±11.0)		51.7 (±23.7)	
Depth of tumor							
T1a	15	66.7 (±18.4)	**0.0184**	72.5 (±14.2)	**0.0388**	67.6 (±17.3)	**0.0093**
T1b	43	52.8 (±24.0)		64.6 (±18.1)		48.2 (±22.8)	
T2	16	42.2 (±26.4)		55.9 (±19.4)		57.3 (±18.9)	
TNM stage							
I	47	57.9 (±23.2)	**0.0329**	68.3 (±16.4)	**0.0137**	53.3 (±24.2)	0.6897
II	27	45.3 (±25.3)		57.5 (±19.7)		55.5 (±18.4)	
Lymph node metastasis							
Positive	19	46.9 (±26.6)	0.1873	53.9 (±22.1)	**0.0034**	57.8 (±16.3)	0.3985
Negative	55	55.5 (±23.7)		67.9 (±15.4)		52.8 (±23.9)	
p53†							
Positive	37	53.9 (±25.9)	0.8551	59.8 (±20.9)	**0.0315**	55.1 (±20.3)	0.702
Negative	37	52.8 (±23.5)		68.9 (±14.1)		53.1 (±24.1)	
Ki-67			0.8710 ($r = 0.019$)		0.8499 ($r = -0.022$)		0.7838 ($r = -0.032$)

Data are presented as mean ± SD.
$P < 0.05$ was considered significant and highlighted in bold.
†Positive cases were those stained over 50%.[26]
RXR, retinoid X receptor; TNM, tumor, node, metastasis.

Table 5 Univariate analysis of overall survival in 74 esophageal carcinoma patients examined

Covariate	*P*-value	Relative risk (95% CI)
RAR α LI (0–90)†	0.2167	1.02 (0.989–1.052)
RAR β LI (0–53)†	0.2063	1.037 (0.98–1.096)
RAR γ LI (0–81)†	0.3996	1.013 (0.984–1.042)
RXR α LI (0–91)†	0.1392	1.018 (0.994–1.043)
RXR β LI (0–92)†	**0.0187**	**1.036 (1.006–1.068)**
RXR γ LI (0–89)†	0.5509	0.991 (0.962–1.021)
TNM state (II/I)	**0.0087**	**7.972 (1.691–37.587)**
Ki67 LI (73-9)†	0.8811	1.003 (0.96–1.048)
p53 (Positive/Negative)	0.4309	1.663 (0.469–8.896)
Age (<61/≥61)	0.6219	1.477 (0.313–6.958)

†Data were evaluated as continuous variables in the univariate analyses. All other data were evaluated as dichotomized variables.
CI, confidence interval; RAR, retinoic acid receptor; RXR, retinoid X receptor; TNM, tumor, node, metastasis.

homodimers exist in the esophagus, RXR agonists, which are associated with fewer side-effects than the RAR agonist, could become useful in clinical settings but further investigations are required to clarify the role of heterodimers and homodimers in the human esophageal cancer and its pathology.

In summary, we immunolocalized all six retinoid receptor subtypes in non-neoplastic epithelium and squamous cell carcinoma of esophageal tissues and demonstrated that RXR were widely distributed. Among these receptor subtypes, RXRβ immunoreactivity was inversely associated with the status of lymph node metastasis of patients and was significantly associated with a better clinical outcome. The results of our present study indicate that retinoid receptors play important roles in esophageal squamous cell carcinomas and, especially RXRβ, is a prognostic factor of patients.

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Increased 5α-Reductase Type 2 Expression in Human Breast Carcinoma following Aromatase Inhibitor Therapy: The Correlation with Decreased Tumor Cell Proliferation

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Abstract Tumor cell proliferation and progression of breast cancer are influenced by female sex steroids. However, not all breast cancer patients respond to aromatase inhibitors (AI), and many patients become unresponsive or relapse. Recent studies demonstrate that not only estrogens but also androgens may serve as regulators of estrogen-responsive as well as estrogen-unresponsive human breast cancers. However, the mechanism underlying these androgenic actions has remained relatively unknown. Therefore, in this study, we evaluated the effects of AI upon the expression of enzymes involved in intratumoral androgen production including 17β-hydroxysteroid dehydrogenase type 5 (17βHSD5), 5α-reductase types 1 and 2 (5αRed1 and 5αRed2) as well as androgen receptor (AR) levels and correlated the findings with therapeutic responses including Ki67 labeling index (Ki67). Eighty-two postmenopausal invasive ductal carcinoma patients were enrolled in CAAN study from November 2001 to April 2004. Pre- and post-treatment specimens of 29 cases were available for this study. The status of 17βHSD5, 5αRed1, 5αRed2, and Ki67 in pre- and post-treatment specimens were evaluated. The significant increments of 5αRed2 as well as AR were detected in biological response group whose Ki67 LI decreased by more than 40% of the pre-treatment level. This is the first study demonstrating an increment of 5αRed2 and AR in the group of the patients associated with Ki67 decrement following AI treatment. These results suggest that increased 5αRed2 and AR following AI treatment may partly contribute to reduce the tumor cell proliferation through increasing intratumoral androgen concentrations and its receptor.

Keywords Breast cancer · Androgen · Androgen receptor · 5α-reductase · Aromatase inhibitor · Ki67

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Introduction

Breast cancer is the most common malignancy among women worldwide and the leading cause of cancer-related death in many countries [1, 2]. Hormones, especially sex steroid hormones, play a pivotal role in endocrine-mediated tumorigenesis and have been demonstrated to influence carcinoma cell growth and progression [3, 4]. Among these sex steroids, estrogens, especially estradiol or E2, a biologically potent estrogen, has been demonstrated to play pivotal roles in cell proliferation, development, and invasion of these hormone-dependent breast carcinoma cells [4, 5]. Aromatase inhibitors (AI) have been demonstrated to be more effective and to have fewer side effects in estrogen receptor (ER)-positive breast cancer patients than the

conventional anti-estrogen tamoxifen [6–8]. However, some patients did not respond to this therapy or developed clinical resistance during the course of this therapy [9]. Therefore, it becomes very important to evaluate the mechanisms of these clinical resistances to AI therapy in estrogen receptor positive breast cancer patients. Results of several previous studies demonstrated that androgens exert opposing effects upon the growth and development as well as upon an inhibition of the proliferation of breast carcinoma cells [10, 11], although some controversies existed [12]. In addition, estrogens and androgens have been both reported to be locally produced in breast carcinoma tissue in an intracrine manner [13, 14]. Androgen receptor (AR) is commonly expressed in human breast carcinoma tissues [15]. These data of in situ production of androgen and the presence of AR in breast carcinoma suggest potentially important roles of androgens in breast carcinomas. In particular, androgen producing enzymes, such as 17β-hydroxysteroid dehydrogenase type 5 (17βHSD5; conversion from circulating androstenedione to testosterone) and 5α-reductase types 1 and 2 (5αRed1 and 5αRed2, respectively; reduction of testosterone to 5α-dihydrotestosterone (DHT)) have been reported to be abundantly expressed in breast carcinoma tissues [16]. Especially, in situ production of DHT has been reported in breast cancer tissues [17]. This locally produced DHT then binds with the highest affinity to AR and promotes AR transcriptional activity [16].

We have previously demonstrated an association between the status of intratumoral androgenic enzymes, 5αRed1, and DHT concentration in the breast carcinoma tissue and an inverse correlation between intratumoral DHT concentration and aromatase expression in cell culture experiments [17]. Results of our previous study above indicated that aromatase, whose substrates include testosterone, may act as a negative regulator for in situ production of DHT in breast carcinoma tissue. Therefore, the alterations of these in situ androgen metabolisms following AI treatment can provide very important information toward a better understanding of the changes of local endocrine environment associated with estrogen depletion. Especially, the comparison of the specimens between pre- and post-AI treatment in neoadjuvant therapy may provide important information as to the changes of intratumoral intracrine environment caused by AI. We have recently reported significant increment of the enzymes; estrogen sulfatase and 17β-hydroxysteroid dehydrogenase type 1, the enzymes also involved in intratumoral estrogen production, following AI therapy, which may represent the compensatory response of breast carcinoma tissues to estrogen deprivation state [18]. In addition, Takagi et al. has also recently demonstrated the increment of intratumoral DHT concentration and17β-hydroxysteroid dehydrogenase type 2 (17βHSD2) expressions in breast carcinoma tissues following exemestane treatment and further reported that 17βHSD2 expression was induced by both DHT and exemestane in a dose dependent fashion in their in vitro studies [19]. However, to the best of our knowledge, the alterations of major androgen producing enzymes such as 17βHSD5, 5αRed1, and 5αRed2 before and after AI treatment of breast cancer patients have not been reported at all (Figs. 1 and 2).

Therefore, in this study, we evaluated the alterations of these enzymes including 17βHSD5, 5αRed1 and 5αRed2, and AR expression in breast carcinoma tissue before and after the neoadjuvant AI treatment using the immunohistochemistry (IHC). We then correlated the obtained findings with the alteration of Ki67 of individual patients and the changes of ER, progesterone receptor (PgR), and human epidermal growth factor receptor type 2 (Her2) in breast carcinoma tissues before and after the therapy in order to further understand these changes of intratumoral androgen producing pathways. In particular, we evaluated the clinical and biological significance of intratumoral androgenic enzymes, especially 5αRed2, in association with the decreased Ki67 from estrogen depletion caused by AI therapy.

Materials and Methods

Breast Carcinoma Cases

The specimens available for examinations in this study were pre- and post-treatment samples obtained from Celecoxib Anti-aromatase Neoadjuvant trial (CAAN trial). This was a neoadjuvant clinical trial conducted, from November 2001 to April 2004, at The University of Hong Kong and Queen Mary Hospital, Hong Kong [20]. The study design had been reported previously [20] but, in brief, all 82 patients enrolled in this neoadjuvant study were postmenopausal women with histological proof of invasive ductal breast carcinoma and positive ER/PgR status determined by the IHC analysis [20]. Informed consents had been obtained from all the patients prior to their enrollment into this trial, which had been approved by the local ethics committee. In CAAN trial study, it was conducted to investigate the efficacy and safety of neoadjuvant therapy combining AI with COX-2 inhibitor. According to the protocol of CAAN trial, the patients were randomly assigned to receive exemestane 25 mg daily and celecoxib 400 mg twice daily (group A, n=30), exemestane 25 mg daily (group B, n=24) and letrozole 2.5 mg daily (group C, n=28), respectively. Each patient was treated for 3 months and surgery was performed within 7 days after the treatment. As reported previously, there were no significant

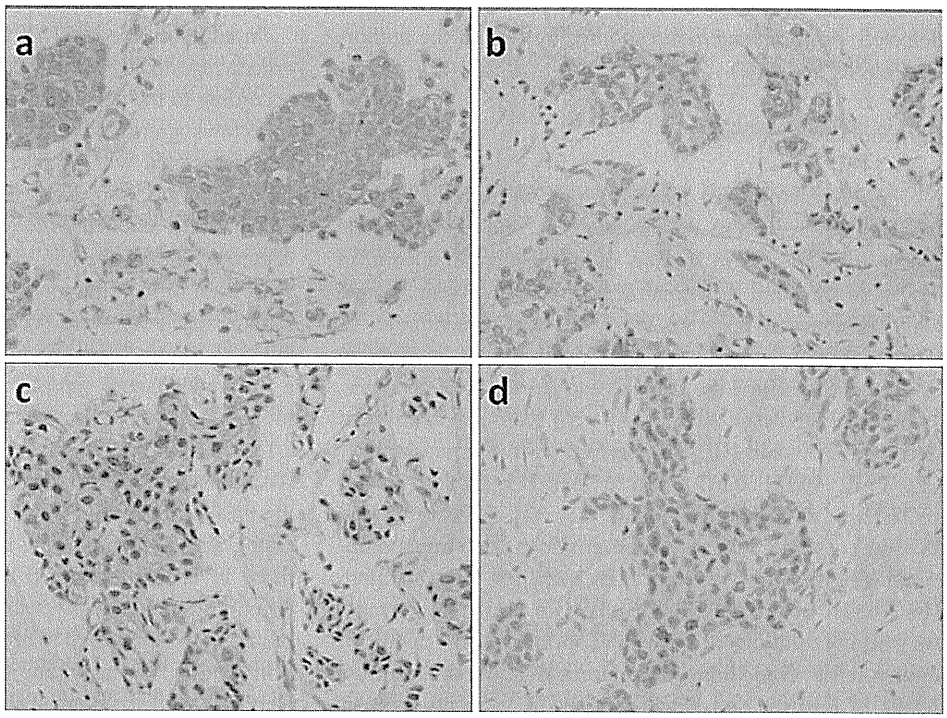

Fig. 1 Representative illustrations of immunohistochemistry: 17βHSD5 (**a**), 5αRed1 (**b**), 5αRed2 (**c**), and AR (**d**) in one case of invasive ductal carcinoma. Immunoreactivity of 17βHSD5, 5αRed1, and 5αRed2 were detected in the cytoplasm of invasive ductal carcinoma cells while those of AR in the nucleus. Original magnification, ×200

differences in term of clinical and pathological responses among these three different treatment groups [20]. Therefore, the responses toward AI therapy were by no means influenced by the concurrent use of celecoxib.

The pre- and post-treatment specimens of 29 patients were available for this pathological response and IHC evaluation study. According to the protocol of CAAN trial, these 29 patients were randomly assigned to receive the treatment as follows (group A, $n=10$; group B, $n=8$; and group C, $n=11$). Their mean age was 74.6 years (range, 51–93 years).

Pathological Response

Tissue sections of the same tumors from pre-treatment core needle biopsies and final surgical specimens were obtained and assessed for the changes in cellularity and degree of fibrosis in hematoxylin–eosin-stained slides. Pathological response was categorized, using the modified criteria described by Miller et al. [21] and assessed as follows: complete when there was no evidence of carcinoma cell at the original tumor site; partial response when histological decrement in cellularity and/or increment in fibrosis was

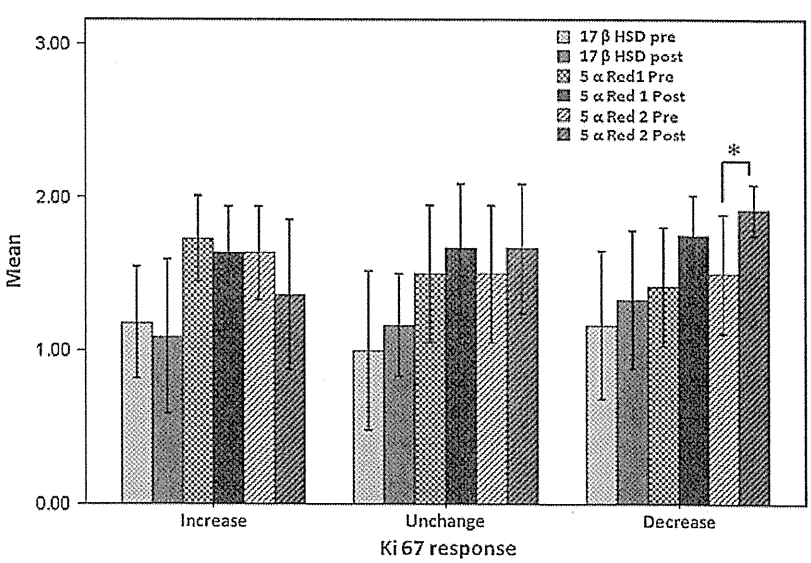

Fig. 2 Demonstration of the mean value of the intratumoral androgenic enzymes before and after the AI treatment grouped by the Ki67 LI response. *Error bar* represents ±2 standard error of measurement (SEM). *$P<0.05$, significant difference between pre- and post-treatment values

detected; or no change/nonresponse, by two of the authors above (NC and MC).

Immunohistochemistry

All immunohistological investigations were performed on the pre-treatment core needle biopsies and final surgical specimens. One 4-μm section of each submitted paraffin blocks of pre- and post-treatment specimens were stained with hematoxylin–eosin to verify an adequate number of invasive breast carcinoma cells and the quality of fixation in order to determine the suitability of further IHC analysis. In brief, serial tissue sections (4-μm) were prepared from selected blocks and IHC was performed to immunolocalize ER, PgR, Her2, Ki67, AR, 17βHSD5, 5αRed1, and 5αRed2, as described previously [17, 22]. A Histofine Kit (Nichirei, Tokyo, Japan), which employs the streptavidin-biotin amplification method, was used for IHC staining. The lists of primary antibodies used in our present study, the working dilutions of individual antibodies, the details of antigen retrieval methods, the sources of antibodies and the details of positive and negative controls were all summarized in Table 1. The antigen–antibody complex was visualized with 3, 3′-diaminobenzidine (DAB) solution (1 mM DAB, 50 mM Tris–HCl buffer (pH 7.6), and 0.006% H_2O_2), and counterstained with hematoxylin.

The immunostained slides were independently evaluated by two of the authors (NC and TS), blinded to clinical outcome of individual patients. 17βHSD5, 5αRed1 and 5αRed2 immunoreactivity were evaluated using a semi-quantitative method as follows: score 2, >50% positive cells; score 1, 1–50% positive cells; and score 0, no immunoreactivity, as previously described by Suzuki et al. [23]. Evaluation of Ki67 was performed by counting of 1,000 carcinoma cells or more from each cases and the percentage of immunoreactivity was subsequently determined as a labeling index (LI) [24].

In addition, the Ki67 LI was then subclassified, using the criteria reported by Miller et al. [21], into three different groups according to the percentage of Ki67 alterations after treatment as follows: group1; increased group, the Ki67 LI in this group was associated with an increment after therapy, group2; no change group, the Ki67 LI demonstrated unchanged or reduction for less than 40% of the pre-treatment level, group3; decreased group, the Ki67 LI demonstrated the reduction for more than 40% of the pre-treatment level. ER, PgR, and AR immunoreactivity were scored by assigning proportion and intensity scores, according to Allred's procedure [25]. In brief, a proportion score represented the estimated proportion of immunopositive tumor cells as follows: 0 (none), 1 (<1/100), 2 (1/100 to 1/10), 3 (1/10–1/3), 4 (1/3 to 2/3), and 5 (>2/3). An intensity score represented the average immunointensity of the positive cells as follows: 0 (none), 1 (weak), 2 (intermediate), and 3 (strong). Any nuclear discernible immunoreactivity in breast carcinoma cells were counted toward both proportion and intensity scores. The proportion and intensity scores were then added to obtain a total score that could range from 0 to 8.The membrane staining pattern was estimated in Her2 IHC and scored on a scale of 0 to 3 [26].

Statistical Analysis

The Kruskal–Wallis test was used to compare the pre-treatment IHC scores of all biological markers according to three groups of AI treatment in individual patients. The Wilcoxon matched-pairs signed ranks test was employed in order to determine the mean differences between pre- and post-treatment IHC scores of individual biological markers in relation to the pathological responses status and the alterations of Ki67 LI. The correlations among intratumoral androgenic enzymes (17βHSD5, 5αRed1, and 5αRed2) before and after AI treatment were analyzed using Spearman's rank nonparametric correlation test. Logistic regression analysis was conducted to determine whether the

Table 1 The list of antibodies employed for immunostaining in this study

Biological markers	Dilution	Pre-treatment method for antigen retrieval	Providers	Positive and negative controls
AR	1:50	Autoclave in citrate buffer	Dako, Denmark	Prostate gland
17βHSD5	1:200	Not required	Sigma	Testis
5αRed1	1:2,000	Not required	–[a]	Liver
5αRed2	1:1,000	Not required	–[a]	Liver
Ki67	1:100	Autoclave in citrate buffer	Dako, Denmark	Breast cancer
ER	Undiluted	Pre-treatment by heat in automated machine	Roche diagnostic, Germany	Breast cancer
PgR	Undiluted	Pre-treatment by heat in automated machine	Roche diagnostic, Germany	Breast cancer
Her2	Undiluted	Pre-treatment by heat in automated machine	Roche diagnostic, Germany	Breast cancer

AR androgen receptor, *17βHSD5* 17β-hydroxysteroid dehydrogenase type 5, *5αRed1* 5αreductase type 1, *5αRed2* 5αreductase type 2, *Ki67* Ki67 protein, *ER* estrogen receptor, *PgR* progesterone receptor, *Her2* human epidermal growth factor receptor type 2

[a] Kindly provided by Dr. D.W. Russell (University of Texas Southwestern Medical Center, Dallas, Texas)

Table 2 The alterations of biological markers before and after the aromatase inhibitors treatment

Biological markers	Pre-treatment value (mean (SEM))	Post-treatment value (mean (SEM))	Mean difference (95% CI)	*p* value
17βHSD5	1.138 (0.128)	1.207 (0.135)	−0.06879 (−0.3165, 0.1786)	0.6221
5αRed1	1.552 (0.106)	1.689 (0.087)	−0.1379 (−0.3811, 0.1052)	0.3394
5αRed2	1.552 (0.106)	1.655 (0.114)	−0.1034 (−0.3971, 0.1902)	0.5771
AR	6.103 (0.295)	6.862 (0.242)	−0.7586 (−1.3210, −0.1959)	0.0127*
ER	7.034 (0.202)	7.586 (0.105)	−0.5517 (−0.9780, −0.1255)	0.015*
PgR	6.965 (0.195)	5.862 (0.321)	1.103 (0.3967, 1.810)	0.0017*
Her 2	1.758 (0.146)	1.586 (0.168)	0.1724 (−0.1163, 0.4661)	0.2958
Ki67	16.352 (1.902)	12.162 (1.754)	4.19 (0.1332, 8.246)	0.0439*

Data showed by mean (standard error of measurement (SEM) of the IHC score of the pre- and post-treatment values; mean difference (pre- and post-treatment values) with 95% confidence interval (lower and upper values) *p* value calculated by Wilcoxon's matched-pairs signed-rank test

17βHSD5 17β-hydroxysteroid dehydrogenase type 5, *5αRed1* 5αreductase type 1, *5αRed2* 5αreductase type 2, *AR* androgen receptor, *ER* estrogen receptor, *PgR* progesterone receptor, *Her2* human epidermal growth factor receptor type 2, *Ki67* Ki67 protein

*p value<0.05

changes in androgenic enzymes, especially 5αRed2, predicted for decreased Ki67 LI or response group. The statistically significance was considered the p value<0.05.

Results

Biopsies from 29 patients who had been treated with exemestane and celecoxib (group A, n=10), exemestane (group B, n=8), or letrozole (group C, n=11), were available for evaluation of pathological response assessment and IHC studies. Pathological responders and nonresponders were 7 (24.1%) and 22 cases (75.9%), respectively.

Immunohistochemistry

The median of pre-treatment individual biological markers were compared but demonstrated no statistical significance (Nonparametric ANOVAs; Data not shown). We then analyzed the changes of IHC scores of all biological markers after the treatment. The statistically significant reduction in PgR expression and Ki67 LI were detected (p=0.0017 and p=0.0439, respectively), as previously reported in letrozole [21], anastrozole [27], and exemestane [22] neoadjuvant treatment but the expression levels of both ER and AR were increased (p=0.015 and p=0.0127, respectively). In addition, the expressions of intratumoral androgenic enzymes were increased but theses increments did not reach statistical significance (Table 2).

An Association of Alterations of Intratumoral Androgenic Enzymes and Ki67 LI

Differences of the individual enzymes and other biological markers between pre- and post-treatment were evaluated according to those categories of Ki67 LI described above.

Table 3 The changes in biological markers after the aromatase inhibitors treatment grouped by the changes of Ki67 labeling index

Biological markers	Ki67 increased (n=11)		Ki67 unchanged (n=6)		Ki67 decreased (n=12)	
	Mean difference (95% CI)	*p* value	Mean difference (95% CI)	*p* value	Mean difference (95% CI)	*p* value
17βHSD5	0.0909 (−0.3798, 0.5616)	0.655	−0.1667 (−0.9568, 0.6234)	0.564	−0.1667 (−0.5335, 0.2002)	0.317
5αRed1	0.0909 (−0.2714, 0.4532)	0.564	−0.1667 (−0.9568, 0.6234)	0.564	−0.3333 (−0.7472, 0.08051)	0.102
5αRed2	0.2727 (−0.3349, 0.8804)	0.276	−0.1667 (−0.9568, 0.6234)	0.564	−0.4167 (−0.7438, −0.08949)	0.025*
AR	−0.8182 (−1.935, 0.2986)	0.164	−0.3333 (−1.604, 0.9378)	0.625	−0.9167 (−1.8730, 0.0396)	0.039*
ER	−0.6364 (−1.257, −0.01537)	0.053	−1.000 (−2.878, 0.8776)	0.197	−0.250 (−0.8003, 0.3003)	0.317
PgR	0.000 (−0.6008, 0.6008)	1.000	1.000 (−0.3277, 2.328)	0.098	2.167 (0.7633, 3.570)	0.005*
Her 2	0.000 (−0.5203, 0.5203)	1.000	0.3333 (−0.2087, 0.8753)	0.157	0.250 (−0.3003, 0.8003)	0.317

Data demonstrated by mean difference (pre- and post-treatment values) with 95% confidence interval (lower and upper values); *p* value calculated by Wilcoxon's matched-pairs signed-rank test

n sample in each group, *17βHSD5* 17β-hydroxysteroid dehydrogenase type 5, *5αRed1* 5αreductase type 1, *5αRed2* 5αreductase type 2, *AR* androgen receptor, *ER* estrogen receptor, *PgR* progesterone receptor, *Her2* human epidermal growth factor receptor type 2. *Ki67* Ki67 protein

*p value<0.05

Immunoreactivity of ER, PgR, Her2, AR, 17βHSD5, 5αRed1, and 5αRed2 in pre-treatment specimens were not significantly different among these three different groups of Ki67 LI changes (nonparametric ANOVAs; data not shown). In group 1 or whose Ki67 LI increased after the therapy and group 2 or whose Ki67 LI unchanged or decreased with less than 40% of the pre-treatment level, no statistically significant difference was detected among any of intratumoral enzymes and biomarkers examined between the specimens before and after the treatment. In group 3 or whose Ki67 LI decreased with more than 40% of the pre-treatment level, the significant increment of 5αRed2 and AR and decrement of PgR expression were demonstrated in this study (p=0.025, p=0.039, and p=0.005, respectively), whereas the expression of other biological markers did not show any statistically significances (Table 3).

Correlation Among Intratumoral Androgenic Enzymes Before and After AI Treatment

We then examined the correlation between IHC scores of intratumoral enzymes in tumors before and after the treatment according to the categories of Ki67 LI. In pre-treatment group of the patients, androgenic enzymes including 17βHSD5, 5αRed1 and 5αRed2, were significantly correlated with each other (Table 4). Those correlations were, however, changed following AI treatment. In group 1 or whose Ki67 LI increased after the therapy, 17βHSD5 was still correlated with 5αRed2 (p=0.009) as well as 5αRed1 with 5αRed2 (p=0.001) but loss of correlation between 17βHSD5 and 5αRed1 was detected (p=0.067). In group 2 or whose Ki67 LI unchanged or decreased with less than 40% of the pre-treatment level, only the correlation between 5αRed1 and 5αRed2 remained significant. The level of statistical significance was not reached in group 3 or those Ki67 LI decreased by more than 40% of the pre-treatment level (Table 4).

The Relative Importance of Androgenic Enzymes on Ki67 LI Decrement by AI Treatment

We further evaluated the effects of alterations of androgenic enzymes to determine whether these alterations, especially those of 5αRed2, were correlated with the status of response or nonresponse to the AI treatment determined by Ki67 LI changes. The status of each androgenic enzymes in post-treatment was further subclassified into three different groups according to the level of their changes after the treatment as follows: group 1; increased group, the status of the enzymes in this group was associated with an increment compared to the pre-treatment level. Group 2; no change group, the status of the enzymes was the same as that in the pre-treatment level. Group3; decreased group, the status of enzymes was

Table 4 The correlation between biological markers involve in androgen production before and after the treatment with aromatase inhibitors grouped by the changes in Ki 67 labeling index

Biological markers			Post-treatment					
	Before treatment (n=29)		Ki67 increased (n=11)		Ki67 unchanged (n=6)		Ki67 decreased (n=12)	
	5αRed1	5αRed2	5αRed1	5αRed2	5αRed1	5αRed2	5αRed1	5αRed2
17βHSD5	0.59 (0.001)*	0.695 (0.00)*	0.57 (0.067)	0.743 (0.009)*	0.316 (0.541)	0.316 (0.541)	0.243 (0.446)	0.477 (0.117)
5αRed1		0.87 (0.00)*		0.859 (0.001)*		1.000 (0.000)*		0.522 (0.082)

Data demonstrated by the correlation coefficient with (p value) calculated by Spearman's rank correlation

n samples in each group, *17βHSD5* 17β-hydroxysteroid dehydrogenase type 5, *5αRed1* 5αreductase type 1, *5αRed2* 5αreductase type 2, *Ki67* Ki67 protein

*p value<0.05

decreased following the therapy. We could not find any significance among these groups in the logistic regression analysis (Table 5).

Discussion

Numerous studies have been reported on the possible roles of androgens in human breast cancer but it is also true that controversies exist as to clinical or biological significance of androgens, especially in estrogen dependent breast cancer [10–14, 17, 19, 22]. Previously, Sonne–Hansen and Lykkesfeldt reported the presence of a significant aromatase activity in the MCF-7 cells and this activity was also reported to be sufficient for the breast carcinoma cells to aromatize testosterone to estrogen, which resulted in significant cell growth stimulation [14]. In addition, both the steroidal and nonsteroidal aromatase inhibitors were reported to be able to completely abolish the growth-stimulatory effects of testosterone [14]. However, Macedo et al. reported that androgens, such as androstenedione and 5α-DHT, inhibited MCF-7 cell proliferation in a low-estrogen milieu and letrozole treatment did inhibit breast carcinoma cell proliferation by inhibiting the conversion of androgens to estrogen, and subsequently making androgens available to exert their anti-proliferative effects possibly through up-regulation of AR [10].

We also demonstrated statistically significant AR increment following the AI treatment, which is consistent with the results of previous reported studies above, but it is also true that Yamashita et al. did not detect this change during the exemestane treatment [22]. In addition, Suzuki et al. recently reported that intratumoral DHT of human breast carcinoma tissues was mainly determined by the status of 5αRed1 and aromatase [28]. In our present study, we demonstrated the correlation between the effects of AI treatment and the changes of androgenic enzymes expression. The significant correlation was also detected between the decrement in Ki67 LI or biological response of the AI treatment and the increment of 5αRed2 following AI administration in breast carcinoma patients.

Locally produced estrogens play a major role in proliferation of estrogen dependent breast cancer and androgens are considered to predominantly exert anti-proliferative effects via AR [15]. Intratumoral estrogens can be produced from circulating androgens, especially those derived from the zona reticularis of an adrenal cortex, catalyzed by the aromatase enzyme in which the neo-adjuvant AI treatment blocks this enzyme with immense potency and exquisite specificity [6]. We previously demonstrated an increment of the intratumoral enzymes following AI therapy in the compensatory direction toward increasing intratumoral estrogen production [18]. However, the alteration of androgen metabolizing enzymes as a result of the neoadjuvant hormonal breast cancer therapy has not been examined at all.

Local androgen concentration has been well known to be significantly increased in breast cancer by AI treatment, as previously reported in various in vitro studies [10–12, 17, 29]. Takagi et al. recently demonstrated an increment of DHT concentration in breast carcinoma tissue following the exemestane therapy as well as the inhibitory effects of DHT on estradiol-mediated T-47D cells proliferation [19]. These findings all suggested that AI not only suppress aromatase enzyme and cause estrogen depletion in consequence, but also provide additional effects through increasing local DHT concentration, which may result in decreased cell proliferation of tumor cells. These findings were consistent with results of our present study that the statistically significant increment of 5αRed2 enzyme was detected only in the group associated with reduction of Ki67 LI with more than 40% of the pre-treatment level or group 3 (p=0.025) (Table 3). Following AI treatment, an accumulation of in situ androgens in breast cancer tissues may occur and the enzyme 5αRed2 can serve as an important regulator of local actions of androgens because this enzyme converts testosterone into the biologically more active and nonaromatizable DHT [4, 11, 17]. However, further studies such as the analysis of much larger number of neoadjuvant treated patients are required for confirmation of this interesting hypothesis.

The potent and direct inhibitory effects of DHT on human breast cancer cell proliferation were first demonstrated by Poulin et al. in 1988 [30]. Two isoforms of

Table 5 Odds ratio of each androgenic enzymes related to the Ki67 labeling index alterations following the aromatase inhibitors treatment

Biological markers	Post-treatment IHC status	Odds ratio (95% CI)	p value
17βHSD5	Increased	0.368 (0.028, 4.746)	0.443
	Unchanged	Reference	
	Decreased	0.696 (0.045, 10.766)	0.795
5αRed1	Increased	1.644 (0.156, 17.359)	0.679
	Unchanged	Reference	
	Decreased	Cannot be calculated	1.000
5αRed2	Increased	3.739 (0.177, 79.081)	0.397
	Unchanged	Reference	
	Decreased	0.000 (0.000, –)	0.999

Data showed the odds ratio of the Ki67 response with (95% confidence interval) and p value calculated by logistic regression analysis; post-treatment IHC status means the change in the IHC scores after the treatment; the unchanged of IHC scores after treatment were used as reference for the comparison

17βHSD5 17β-hydroxysteroid dehydrogenase type 5, *5αRed1* 5α-reductase type 1, *5αRed2* 5αreductase type 2, *Ki67* Ki67 protein

5αRed have been known to exist, encoded by different genes: SRD5A1 (chromosome 5p15) and SRD5A2 (chromosome 2p23) [31, 32]. The two types of 5αRed share 50% amino acid sequence identity and possess similar substrate specific but have different optimal pH and sensitivity to inhibitors [32]. 5αRed2 is the major form of the enzyme expressed in the human prostate [32] but rarely detected in human breast carcinoma [28]. Both Wiebe et al. [33] and Suzuki et al. [17, 28] demonstrated the expression of 5αRed1 in several types of human breast cancer cell lines using semi-quantitative RT-PCR and in human breast carcinoma tissues using IHC and RT-PCR, respectively. In addition, significant increment of 5αRed1 and 5αRed2 genes expression of human breast carcinoma as compared to normal breast tissue has been illustrated in the semi-quantitative RT-PCR study [34]. However, the regulatory mechanisms of 5αRed2 in human breast carcinoma have remained largely unknown and it awaits further investigations for clarification.

In our present study, we did not, however, detect the significant alterations in the enzymes involved in androgen metabolism in non response groups (groups 1 and 2) (Table 3). This finding suggests that androgen metabolism is not influenced by the AI treatment in these groups of patients with breast cancer or nonresponders. The loss of correlation of intratumoral androgenic enzymes in breast carcinoma tissue; 17βHSD5, 5αRed1, and 5αRed2, after AI treatment (Table 4) as well as the alterations of 5αRed1 and 5αRed2 enzymes (Table 2) were detected, but these changes did not reach statistical significance. This may be due to the relatively small size of the patients examined, especially the rather limited number of available specimens in our present study. In addition, the breast carcinoma cases associated with greater reduction of Ki67 LI tended to be associated with an increased 5αRed2, but this correlation did not reach statistical significance (Table 5).

After menopause, most of the biologically active androgens (as well as estrogens) are synthesized in peripheral intracrine tissues, for example in the breast, from precursors of adrenal origin without release of active androgens in the extracellular space and the circulation [4, 11]. In addition, DHT concentrations were demonstrated to be significantly higher in breast cancer tissues than in plasma [35]. In addition, both 17β-hydrosteroid dehydrogenase and 5α-reductases have been considered to act to increase DHT production by competing with aromatase for substrates in hormone-dependent breast carcinoma [19, 28]. As mentioned above, 5αRed1 is the predominant form of 5α-reductases at least in human breast cancer [17, 28, 32], but the results of our present study clearly demonstrate the importance of 5αRed2, which is rarely expressed in breast cancer but was increased in response group or those associated with more Ki67 decrement. We therefore hypothesized that this rather de novo 5αRed2 increment may be related to the effects of AI other than depleting in situ estrogens, i.e., the potential increment of the endogenous androgens which may exert their anti-proliferative effects via the AR, especially in a low-estrogen milieu, as demonstrated in the breast cancer cell lines study [10] and possibly to an induction in apoptosis signaling pathways. Androgens, androstenedione, and DHT, were reported to have a proapoptotic effect by strongly reducing Bcl-2 expression in MCF-7 cells, and this androgenic inhibitory effect was mediated via the AR [10, 36].

In summary, this is the first study which demonstrates an alteration of the androgen producing enzymes following the AI treatment, especially a de novo increment of 5αRed2 as well as of AR may be considered at least one of the mechanisms to account for the decreased breast carcinoma cell proliferation after AI therapy through an increment of local concentrations of androgens and their actions. However, the regulatory mechanisms of 5αRed2 in human breast carcinoma have remained largely unknown.

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