A further dose-escalation study in 29 patients has been completed recently [46]. The optimum dose was determined to be 40 mg of STX 64 per patient per day in tablet form.

5. Dual sulfatase/aromatase inhibitors

Since aromatase is needed for the synthesis of estrogens that are then converted into estrogen sulfates by estrogen sulfotransferase, hormone-dependent breast cancer may be more effectively treated by dual inhibition of aromatase and steroid sulfatase. A new design strategy was explored that involves introducing the aromatase inhibitory pharmacophore into a template that has been designed primarily for sulfatase inhibition [47]. A series of compounds that can inhibit both aromatase and sulfatase have been developed based on the structure of estrone 3-sulfamate, a typical estrone sulfatase inhibitor [48]. In contrast, a series of single agent dual aromatase-sulfatase inhibitors that are sulfamate derivatives of nonsteroidal AIs, including letrozole and anastrozole, have been successfully developed [49–51]. The design of these dual aromatase–sulfatase inhibitors shares a common strategy; that is, to engender the sulfatase inhibitory pharmacophore into an established aromatase inhibitor with minimal structural change incurred to the original scaffold in order to retain and maximize aromatase inhibition. At the same time, possible negative pharmacological interactions between several aromatase and sulfatase inhibitors given in concert could be avoided. It is also reasoned that resistance to drugs targeting two different enzymes is not likely to develop simultaneously. Thus, *D*ual *A*romatase-*S*ulfatase *I*nhibitors (*DASIs*) have been developed engendering the steroid sulfatase inhibitory pharmacophore into established aromatase inhibitors with minimal structural changes otherwise. At this stage, DASIs are available based on the triazoles letrozole (Fig. 3, **6** [52], anastrozole (Fig. 3, **7**) [53], and YM511 (Fig. 3, **8**) [51], in addition to alternative AIs characterized by their biphenyl templates [54].

STX 681 (Fig. 3, **9**) is a YM511-based DASI that has been shown to have in vivo activity. Using a xenograft nude mouse model, Foster et al. demonstrated that STX 681 completely inhibited the growth of MCF-7_{AROM} and MCF-7_{STS} tumors [55]. The authors conclude that targeting both the aromatase enzyme and the sulfatase enzyme at the same time has the potential to become a novel treatment strategy of hormone-dependent breast cancer (HDBC).

6. Sulfatase inhibitors: ongoing research and future aspects

Given the potency of this new class of sulfamate-based steroid sulfatase inhibitors, the large volume of preclinical data available on the use of steroidal and non-steroidal STS inhibitors in a variety of hormone-dependent cancer models and, given the encouraging results obtained in two phase I studies completed with BN83495 (STX 64) it will be important to carry out clinical trials to assess its efficacy in different clinical settings as well as in non-cancer disease indications. While clinical studies are planned to investigate the effect of BN83495 in women with ER-positive early breast cancer, the compound is currently in further clinical development for advanced endometrial cancer (phase II) as well as in phase I evaluation for castrate-resistant prostate cancer in North America. Additional trials will examine whether combining BN83495 with an AI or LHRH antagonist will improve response rates.

As the biological role of steroid sulfatase is also implicated in several disorders of the skin (acne, psoriasis, hirsuitism) and in memory function, BN83495 may find use in such non-cancer diseases [6].

7. Conclusions

Inhibition of steroid sulfatase is one promising new approach to develop alternative treatment strategies for hormone-sensitive breast cancer. In contrast to aromatase inhibition alone, suppressing plasma and tissue estrogen synthesis, sulfatase inhibition causes both estrogen and androgen depletion simultaneously. Early clinical findings suggest that breast cancer patients with progressive disease while on therapy with aromatase inhibitors, may experience a new response when treated with a steroid sulfatase inhibitor as monotherapy. Most interesting, upregulation of steroid sulfatase has recently been confirmed in breast cancer patients treated with an aromatase inhibitor, suggesting steroid sulfatase to be possibly involved in adaptation to estrogen deprivation and/or endocrine resistance. Phase I-II trials involving sulfatase inhibitors are now initiated to study the influence of these compounds on intra-tumor steroid levels and enzyme activity. Moreover, compounds inhibiting aromatase and sulfatase activity at the same time (DASIs) have been developed.

While sulfatase inhibition certainly is one of the most promising new treatment strategies for hormone-sensitive breast cancer, its role in daily praxis is currently unclear. Ongoing trials will investigate the potential of these drugs either as monotherapy or in combination with established drugs. Finally, the identification of biological relevant tumor markers that might serve as predictive factors (like steroid sulfatase activity in human cancer tissue, normal tissue, hair etc.) is urgently requested to allow the use of these drugs in groups of patients with a high chance for clinical responses.

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Review

Aromatase in human lung carcinoma

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ABSTRACT

Lung cancer is the leading cause of cancer mortality in both women and men worldwide but gender differences exist in their clinical and biological manifestations. In particular, among life time non-smoker, female are far more likely to develop lung carcinoma than male. Recent studies demonstrated that estrogens are synthesized *in situ* in both male and female lung cancers through aromatase, suggesting that sex steroid may contribute to the pathogenesis and development of lung carcinoma. In addition, human lung carcinomas have been recently demonstrated to be frequently associated with expression of estrogen receptors in both male and female patients and a lower expression of aromatase was reported to be associated with better prognosis. Preclinical studies further demonstrated that aromatase inhibitor (AI) suppressed the lung tumor growth both *in vitro* and *in vivo*. These findings all suggest a potential role of intratumoral aromatase in biological behavior of non-small cell lung cancer (NSCLC), the most common form of human lung malignancy. Therefore, AIs may become viable therapeutic options for disease management in NSCLC patients but further studies are definitely required to obtain a better understanding of the potential roles of intratumoral aromatase expression as a predictive biomarker for clinical outcome in these NSCLC patients.

Contents

1. Introduction

Lung cancer remains the leading cause of cancer mortality worldwide. It is true that tobacco smoking still remains its prime cause among both men and women [1,2] but among non-smoking-associated lung cancer patients, it is also true that women are more likely than men to develop lung carcinomas, especially adenocarcinomas [3,4]. In addition, both estrogen receptors (ERs) and aromatase were reported to be present in human lung tumors [5–16]. These findings all suggest a possible role of estrogens in biological behavior of human lung cancer as summarized in Tables 1 and 2. In particular, a lower expression of intratumoral aromatase was reported to be associated with better prognosis of NSCLC patients especially among postmenopausal female [13,17]. Therefore in this brief review, we focused on possible roles of intratumoral aromatase and will propose aromatase inhibitors as potential future therapeutic option in lung cancer.

2. Estrogenic effects on human lung cancer

Results of previously reported studies all suggested that estrogens could play an important role in lung cancer development in some cases [5,18–25]. Estrogens mainly exert its effects via two different distinctive estrogen receptor (ER) subtypes identified as ERα and ERβ [26,27]. Estrogens stimulate cell proliferation in non-small cell lung carcinoma (NSCLC) cell lines to a far greater extent than in non-neoplastic lung fibroblasts [18]. Estrogens also promoted the transcription of estrogen-responsive genes in NSCLC cells expressing endogenous ERs [19]. These genomic actions were demonstrated to be induced only by ligands specific to ERβ and not

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Table 1
Summary of previous studies on ERs expression in lung carcinoma tissues.

References	Cases	Female/male	Histology	ER posi. %	ERα posi. %	ERβ posi. %	Principle findings
[36]	64	26/38	Ad Sq Pd Lc Bac	10091100100100	------	------	ERs were expressed only in cancerous tissue and not in normal pulmonary tissue of the human lung.
[37]	52	16/36	Ad Sq Lc Bac SCLC	06000	------	------	ERs were expressed more abundantly in lung tumors from women than from men.
[6]	30	8/22	Ad Sq	--	00	8530	ERβ, but not ERα, could play important roles in human lung.
[7]	45	27/18	Ad Bac	8056	--	--	ER expression in lung adenocarcinomas was dependent upon the antibody clones that were used
[8]	32	15/17	Ad Sq	--	67	--	
[9]	278	214/64	AdBacAdsqLcSCLCSq	------	000000	5956100565085	Nuclear ERβ was expressed more frequently in men than women with lung adenocarcinomas and was associated with better clinical outcome
[10]	132	56/76	Ad Sq Lc	---	737550	466850	ERα expression and absence of ERβ expression were associated with a poorer prognosis in NSCLC patients.
[11]	301	127/174	Ad Sq Adsq	---	---	5041–	ERβ overexpression was a positive prognostic marker among stage II and III NSCLC patients.
[12]	104	33/71	Nsc Nsc	--	355*	84	ERβ in a lung tumor was a positive prognostic factor for men with NSCLC.
[13]	59	26/33	Ad Sq	--	6427	9573	Estrogen could be locally produced in NSCLC mainly by aromatase.
[14]	447	187/260	Ad Ad	--	084*	74	Prognostic significance of nuclear ERβ was limited to NSCLC patients with an EGFR mutation.
[15]	317	167/150	Ad Sq	--	0–480–33	22–9816–98	ERα expression was associated with EGFR mutations in lung adenocarcinomas.
[16]	105	38/67	Ad Sq Adsq	---	100	736866	ERβ and aromatase expression was highly concordant in NSCLC patients.

Table 2
Summary of previous studies on aromatase expression in lung carcinoma tissues.

References	No. of cases	Female/male	Methodology	Histology	Aromatase posi. % 'or' score	Principle findings
[5]	NS	NS	IHC	Nsc	NS	Aromatase was significantly expressed in lung carcinoma tissues.
[75]	53	33/20	IHC	AdSqAdsqBac	90 88 100 75	Aromatase was present and biologically active in human NSCLCs.
[17]	442	NS	IHC	Ad Sq Lc Sc	1.49 ± 0.02 1.56 ± 0.03 1.49 ± 0.06 .99 ± 0.13	Lower levels of aromatase predicted a greater chance of survival in women 65 years and older.
[13]	59	26/33	RT-PCR	Nsc	–	Aromatase expression was significantly associated with intratumoral estradiol concentration.
[73]	10	NS	IHC	Nsc	–	Aromatase was expressed in NSCLC tissue and aromatase inhibitor reduced tumor growth in NSCLC xenograft.
[54]	78	NS	IHC	Ad Aq	60 70	Aromatase expression in NSCLC was independent of any clinical and pathological parameter except tumor stage.
[16]	105	38/67	IHC	Ad Sq Adsq Lc	86 82 100 0	Aromatase expression was significantly associated with ERβ expression in NSCLC patients.
[64]	9	3/6	RT-PCR/IHC	Ad Sq	100 0	Aromatase was expressed only in carcinoma cells but not in stromal cells.

Posi. %, percentage of positive cases; Nsc, non-small cell carcinoma (histological type unknown); Ad, adenocarcinoma; Sq, squamous cell carcinoma; Adsq, adenosquamous cell carcinoma; Bac, brochio-alveolar carcinoma; Lc, large cell carcinoma; SCLC, small cell lung carcinoma; Pd, poorly differentiated carcinoma; *, cytoplasmic staining; –, not examined.

by ligands specific to ERα [20]. In addition to genomic actions, estrogenic actions may occur at the cell surface in NSCLC cells involving mainly membrane/cytoplasmic pools of ERs [5,19–23]. In breast carcinoma cells, ERs utilize the membrane epidermal growth factor receptor (EGFR) to rapidly signal through various kinase cascades, i.e. mitogen activated protein kinase (MAPK) and/or protein kinase B (PKB/Akt) [28]. NSCLC cells demonstrated similar transactivation of EGFR on administration of exogenous estrogens which ultimately resulted in MAPK activation [21]. Many other investigators also reported a similar activation of MAPK and/or Akt on 17β-estradiol treatment in NSCLC cells, but without the transactivation of EGFR [5,19,20,22,23]. However, these non-genomic actions in NSCLC cell lines have not been well characterized compared to those in breast carcinoma cell lines and further investigations are required for further clarification [29–34]. Among estrogens, 17β-estradiol treatment has been demonstrated to result in significantly enhanced cell proliferation in various lung carcinoma cell lines [5,19–23]. In addition, 17β-estradiol exposure stimulated the growth of lung carcinoma xenografts [21,24,25]. Whether estrogen exerts its effects in NSCLC primarily through genomic or non-genomic signaling pathway has, however, still remained in dispute. In conclusion at this juncture, NSCLC is reasonably considered a novel estrogen target tissue.

3. Expression of ERs in lung carcinoma cells

Both ERα and ERβ have been reported to be expressed in variety of tissues including ovary, breast, CNS, bone and kidney [35]. Earlier studies on the presence of ERs in lung tumor focused only on the classical ERα, then termed simply as ER. ERβ expression in human lung carcinoma has been examined by various investigators but patterns of both ERα and ERβ in NSCLCs using immunohistochemistry were highly inconsistent varying from "0 to 100% for ERα" and "30 to 100% for ERβ" as summarized in Table 1. Earlier studies demonstrated that ERs were expressed only in lung tumor cells and not in normal lung tissue with a much higher frequency in female patients [36,37]. However, ERs, particularly ERβ, was then demonstrated to be expressed and to be functional both in normal and cancerous lung tissue of both [6,21,38,39]. Previously, only ERα was considered as tumor promoter, whereas ERβ was believed to inhibit tumorigenesis due to the absence of its expression in ovarian, breast, and cervical cancers, when compared to normal tissue [40]. However, results of recent studies demonstrated that ERβ can function as a tumor promoter in the absence of ERα expression [41–45]. Many NSCLC cell lines lacking ERα but expressing ERβ demonstrated similar tumor promoting features [19–21,24,25]. In contrast to the previously reported results of both *in vitro* and *in vivo* studies in NSCLC cell lines, those of several reported immunohistochemical analysis of NSCLC patients all indicated that the status of ERβ immunoreactivity was associated with better clinical outcome especially in male patients [9,11,12]. In addition, the presence of ERα and the absence of ERβ expression in lung tumor tissues were also reported to be associated with poor prognosis in NSCLC patients [10]. Recently this reported prognostic significance of ERβ could be limited to the NSCLC patients with EGFR mutations [14]. In addition, EGFR mutations, which were more frequently reported in Japanese patients with lung adenocarcinoma than those in non-Japanese patients [46], were associated with ERα expression [15] whereas ERβ expression was associated with aromatase expression in NSCLC patients [16]. These reports all suggest a possible functional correlation between ER expression with either aromatase expression or/and EGFR mutations in NSCLC patients. However, it is also important to note that several different criteria and antibodies were employed to define "ERα and/or ERβ" positivity in these studies and a comparison using standard immunohistochemical method has to be explored of status and clinical significance of ERs in NSCLC patients.

4. Estrogen synthesis in lung carcinoma

The upward trend of lung cancer deaths among female non-smokers was confined to elderly women [3]. In breast cancer patients 74% of the newly diagnosed patients were also post-menopausal [47]. Risks of developing estrogen dependent cancer, i.e. breast and endometrial cancer, and conceivably ovarian cancer, for postmenopausal women increase significantly with serum concentrations levels of estrogens [48–50]. In premenopausal women, the ovary is the principle source of circulating estrogens [51,52]. However, it is also true that a large proportion of estrogens in women (approximately 75% before menopause, and close to 100% after menopause) are produced in peripheral hormone-target tissues through aromatase from abundantly present circulating precursor adrenal androgens [53]. Aromatase was reported to be expressed frequently in both male and female patients with human lung carcinoma, Table 2. Aromatase expression was also significantly associated with intra-tumoral estrogen concentration in NSCLCs [13]. A lower expression of aromatase was associated with better prognosis of NSCLC patients especially among post-menopausal female [17]. In addition, aromatase expression was significantly associated with ERβ expression [16] and tumor stage [54] in two different reported studies. These findings all indicated the possible importance of elevated *in situ* estrogen concentrations through aromatase in NSCLC patients. However, other enzymes, i.e. 17β-hydroxysteroid dehydrogenase (17β-HSD) isozyme, steroid sulfatase (STS) and estrogen sulfotransferase (EST), also play pivotal roles in intra-tumoral estrogen production [55,56]. 17β-HSD type1 was expressed more frequently than 17β-HSD type2, which suggested further dependency of NSCLC on *in situ* estrogen production [13]. There have been no studies available on possible roles of STS and EST in NSCLC patients. In addition, among NSCLC patients intratumoral concentration of estradiol was significantly higher in men than postmenopausal women [13] and males frequently co-express ERs and aromatase [16]. Therefore, ER pathway would be expected to be targeted in males with NSCLC as well, especially if local estrogen production is present via aromatase but further investigations are required for clarification.

5. Regulation of aromatase in lung cancer

Among estrogen-dependent tumors, breast cancer tissues and endometrial cancer tissue expresses aromatase primarily in stromal cells adjacent to tumors cells and to a much lower degree in carcinoma cells [57–59,61,63]. However, among breast cancer tissues some studies demonstrated a more intense aromatase staining in the malignant epithelia [60,62]. In contrast, aromatase was detected predominantly in parenchymal/carcinoma cells in human lung carcinoma tissues [64]. Aromatase expression was much lower in NSCLC cell lines than breast carcinoma cell lines [62,64]. However, the level of aromatase expression in NSCLC tissues, where aromatase was detected in about 60–70% of NSCLC cases, was much higher than NSCLC cell lines [64], as summarized in Table 2. In human breast carcinomas, aromatase activity was predominantly confined to stromal cells which was reported to be regulated by various factors derived from breast carcinoma cells, such as prostaglandin E_2 (PGE_2) and cytokines including interleukin (IL)-1, IL-6, IL-11 and tumor necrosis factor (TNF)-α [65–68]. However, results of recent *in vitro* studies in both breast and endometrial carcinoma cell lines did demonstrate an increment in aromatase activity in carcinoma cells following the stimulus form stromal cells in co-culture system [62,63]. In addition, recent studies using the

Table 3
Summary of previous studies on use of estrogen down modulators in lung carcinomas.

References	Animal/cells	Methodology	Treatment	Drug	Principle findings
[18]	Mice/H23	Tumor xenograft	ER blocker	ICI 182,780	ER blocker inhibited the tumor growth.
[19]	273T	Gene array	ER blocker	ICI 182,780	ER blocker inhibited the E2 responsive gene expression.
[5]	NIH-H23	Cell prolifertion	Anti-estrogen EGFR inhibitor	Faslodex Gefitinib	Combination therapy with both EGFR-TKI and anti-estrogen was far more effective.
[21]	A549 273T Mice/201T	Cell proliferation Apoptosis assay Tumor xenograft	ER blocker EGFR inhibitor	ICI 182,780 Gefitinib	Combination therapy with both EGFR-TKI and ER blocker was far more effective than either blocker alone against NSCLCs.
[75]	A549/H23 Mice/A549	Cell proliferation Tumor xenograft	Aromatase inhibitor	Anastrozole	Aromatase inhibitor suppressed the tumor growth both *in vitro* and *in vivo*.
[23]	Mice/A549	Tumor xenograft	Anti-estrogen EGFR inhibitor	Faslodex Erlotinib	Combination therapy with both EGFR-TKI and anti-estrogen was far more effective than either blocker alone against NSCLCs.
[13]	ERα over. A549 ERβ over. A549	Cell proliferation	ER blocker Anti-estrogen Anti-estrogen Aromatase inhibitor	ICI 182,780 Tamoixifene Raloxifene Letrozole	Cell proliferation caused by testosterone was significantly inhibited by the addition of the letrozole in both A549 + ERα and A549 + ERβ cells.
[73]	H23 Mice/H23	Cell proliferation Tumor xenograft	ER blocker DNA damage/Apoptosis Aromatase inhibitor ER blocker EGFR inhibitor	ICI 182,780 Cisplatin Exemestane ICI 182,780 vandetanib	Combination therapy with both cisplatin and aromatase inhibitor was far more effective than either blocker alone. Combination therapy with both EGFR-TKI and ER blocker was far more effective than either blocker alone.
[70]	H23/A549	Cell number	Aromatase inhibitor	Exemestane	Exemestane treatment alone reduced cell number of NSCLC cell lines.
[71]	A549 H1650	Cell proliferation	Anti-estrogen EGFR inhibitor	Tamoixifene Gefitinib	Combination therapy with both EGFR-TKI and anti-estrogen was far more effective than either blocker alone.
[64]	LK87	Cell proliferation	ER blocker AR blocker Aromatase inhibitor	ICI 182,780 Flutamide Letrozole	Combined treatment with testosterone and androgen receptor blocker caused enhanced proliferative effect which was abrogatedby treatment with either ER blocker or aromatase inhibitor.

co-culture methodology to simulate *in vivo* stromal–carcinoma cell interactions in NSCLCs, stromal stimulus were reported to result in increased aromatase expression in NSCLC cells [64]. These stromal derived factors were identified as cytokines, i.e. interleukin-6 and oncostatin M [64]. Several investigators demonstrated that soluble factors, derived from lung carcinoma cells induced differentiation and cell proliferation of fibroblastic stromal cells [69]. Therefore, aromatase-inducible cytokines secreted from stromal cells of human lung carcinoma tissues may also be under the control of an interaction with carcinoma cells in the lung cancer microenvironment. However, further investigations are needed to clarify the mechanisms between various cytokines produced as a result of carcinoma–stromal interactions and aromatase induction in NSCLC cells.

6. Potential application of aromatase inhibitor therapy in lung cancer patients

The importance of estrogens in NSCLC is crucial and provides a strong rationale to evaluate anti-tumor activities of estrogen down modulators in lung cancer. However, abrogation of estrogen signaling resulted in either upregulation or activation of epidermal growth factor receptor (EGFR) protein suggesting that the EGFR pathway becomes activated when estrogen is depleted in NSCLC cells [21,70,71]. Similarly ERβ expression was increased on treatment with EGFR tyrosine kinase inhibitor (EGFR-TKI) in NSCLC cells [21,71]. This bi-directional crosstalk between EGFR signaling and estrogen signaling in NSCLCs suggested that combining or simultaneous therapies to target both the pathways is most reasonably considered the most beneficial antitumor effects in the patients with NSCLC. Many reports, both *in vitro* and *in vivo*, have demonstrated that combination therapy with EGFR-TKI along with estrogen down modulators resulted in enhanced anti-tumor activity than either treatment alone in NSCLCs, Table 3. In addition, results of a clinical study on 22 postmenopausal female NSCLC patients demonstrated that combination treatment with both ER blocker, fulvestarnt, and EFGR-TKI, geftinib, was well tolerated [72]. Phase II clinical trial for combination therapy with erotinib, an EGFR-TKI, and fulvestant, an ER blocker, versus erlotinib alone in NSCLC patients are also underway (ClinicalTrails.gov Identifier; NCT00100854 and NCT00592007). Exemestane, an irreversible steroidal inactivator, either alone [70] or in combination with cisplatin [73], a standard chemotherapy in NSCLC patients [74], demonstrated significant anti-tumor effects in two separate studies. Both letrozole and anastrozole, reversible steroidal inactivators, demonstrated similar anti-tumor activity in NSCLCs [13,64,75]. In breast cancer patients aromatase inhibitor generally results in significantly increased response rates and greater duration of response than selective ER modulator (SERM), i.e. tamoxifen [76]. In addition, patients assigned to exemestane displayed a trend of lower incidence of subsequent primary lung cancer compared to those maintained on tamoxifen [77]. These results all suggest that aromatase inhibitors could be more viable therapeutic option than SERMs for NSCLC patients in the future. As an initial step, phase II randomized trial of fluvestrant and anastrozole as consolidation therapy in postmenopausal women with advanced NSCLC is to be scheduled (ClinicalTrial.gov Identifier; NCT00932152). Both male and female NSCLC patients express ERs and aromatase, and cell lines derived from both sexes respond to estrogens, anti-estrogens, and aromatase inhibitors therefore therapeutic treatments with aromatase inhibitors would benefit all patients, not just women.

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Short communication

LKB1 expression is inhibited by estradiol-17β in MCF-7 cells

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Estrogen receptor

ABSTRACT

The liver kinase B1 (LKB1) is encoded by the *STK11* gene and acts as a tumour suppressor and a regulator of energy homeostasis. LKB1 expression is reduced in primary breast tumours compared to normal breast epithelium. Although its expression in primary tumours does not appear to correlate with estrogen receptor (ER) status, it is differentially expressed in breast cancer cell lines where ER-negative cells have lower LKB1 expression than ER-positive cells. The present study aimed to examine the effects of estradiol on LKB1 expression and activity in the ER-positive breast cancer cell line MCF-7. Results demonstrate that estradiol causes a dose-dependent decrease in LKB1 transcript and protein expression and consistent with this, a significant decrease in the phosphorylation of the LKB1 target AMPK ($P \leq 0.05$). In order to assess whether effects of estradiol were due to effects on ERα binding to the *STK11* promoter, ChIP was performed. Results demonstrate that ERα binds to the *STK11* promoter in a ligand-independent manner and that this interaction is decreased in the presence of estradiol. Moreover, *STK11* promoter activity is significantly decreased in the presence of estradiol ($P \leq 0.05$). LKB1 transcript and IHC score were assessed in primary tumours of 18 patients and demonstrated no significant correlation with ER status ($n = 18$). Our results thereby provide a mechanism whereby LKB1 is decreased in ER-positive breast tumours.

1. Introduction

The observation that liver kinase B1 (LKB1), encoded by the *STK11* gene, can cause G1 cell growth arrest when over-expressed in breast cancer cells led to an increased interest in the newly identified tumour suppressor [1]. Interestingly, breast cancer cell lines have differential expression of LKB1 depending on their estrogen receptor (ER) status. The human breast cancer cell line MCF-7, which is ER-positive, expresses LKB1, whereas ER-negative cell lines, such as MDA-MB-435 and MDA-MB-231, have a reduced LKB1 expression [2]. AMP-activated protein kinase (AMPK) is now recognized as a master regulator of energy homeostasis and is tightly regulated by endocrine signals, including leptin, adiponectin, estradiol and phytoestrogens [3–5]. LKB1 activates AMPK by directly phosphorylating its α-catalytic subunit at Thr172. The activation of AMPK in liver and adipocytes results in decreased lipogenesis and increased fatty acid oxidation. Interestingly, a high rate of lipogenesis is essential for the proliferation of many tumour cells including breast cancer cells [6], suggesting that LKB1/AMPK must be down-regulated in breast cancer cells to allow this process to go forward. The present study aimed to examine the effect of estradiol on LKB1 expression and activity in the human breast cancer cell line MCF-7 and to relate these findings to expression in clinical samples.

2. Materials and methods

2.1. Plasmids

The LKB1prom reporter construct was generated by amplifying a 3998 bp fragment of the *STK11* promoter located −3002 to +996 using primers LKB1prom-F: 5′-ACT TTG GAA ATT CAG TGT GTA GGG CA-3′ and LKB1prom-R: 5′-CAA CAA AAA CCC CAA AAG GA-3′ from BAC clone #RP11-50C6 (BAC PAC Resources, Children's Hospital Oakland Research Institute). Further PCR using primers LKB1prom-XhoI-F: 5′-CGG GAA TCT CGA GAC TTT GGA AAT TCA GTG TGT AGG GCA-3′ and LKB1prom-HindIII-R: 5′-AAA GCG CAA

Abbreviations: LKB1, liver kinase B1; STK11, serine-threonine kinase 11; ER, estrogen receptor; AMPK, AMP-activated protein kinase; ChIP, chromatin immunoprecipitation.

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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 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%

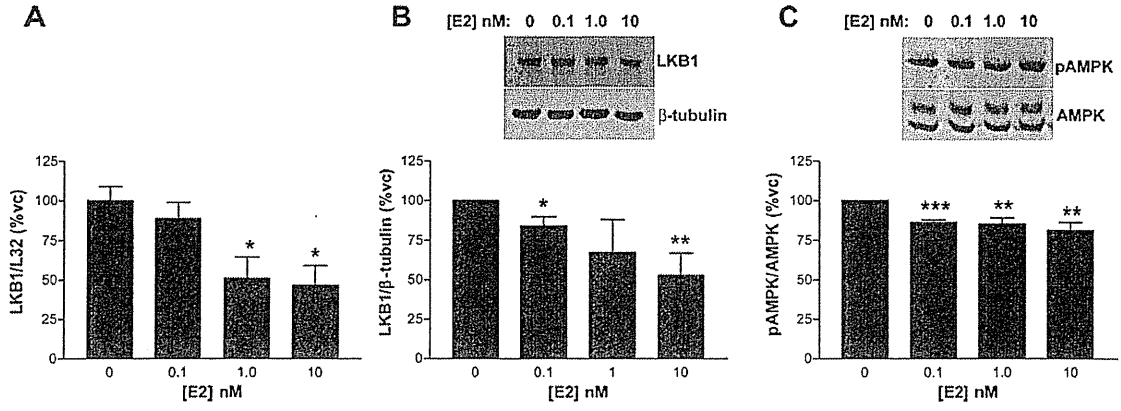

Fig. 1. Estradiol inhibits LKB1 expression and activity. Estradiol treatment of MCF-7 cells resulted in a dose-dependent decrease of LKB1 transcript (A) and protein (B) expression. (C) Estradiol treatment of MCF-7 cells resulted in a decrease in phosphorylation of AMPK. Graphs presented represent mean ± 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).

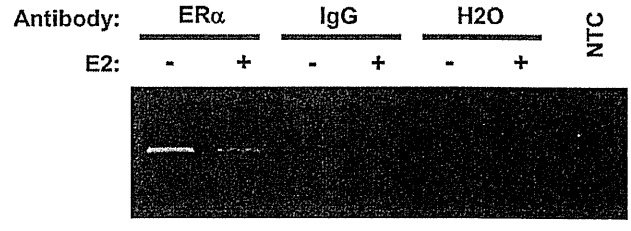

Fig. 2. ERα binds to the LKB1 promoter in the absence of estradiol. ChIP analysis demonstrated that ERα binding to the LKB1 promoter was reduced when MCF-7 cells were treated with 10 nM E2. The result is representative of three separate experiments.

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 ($r = -0.186$, $P = 0.460$).

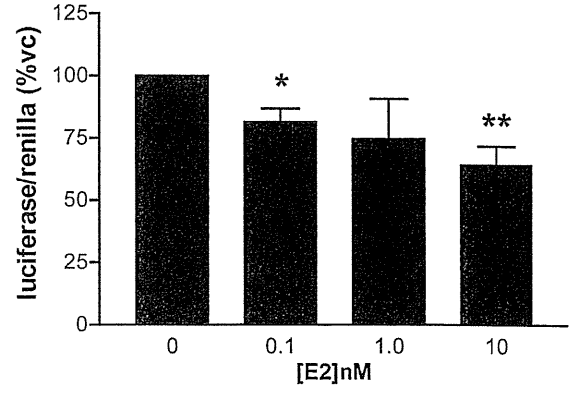

Fig. 3. Estradiol inhibits LKB1 promoter activity. A reporter construct containing 3003 bp of the LKB1 promoter was transfected into MCF-7. Results demonstrate that promoter activity was significantly decreased with increasing doses of estradiol in MCF-7 cells. Graphs presented represent mean ± SEM. 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

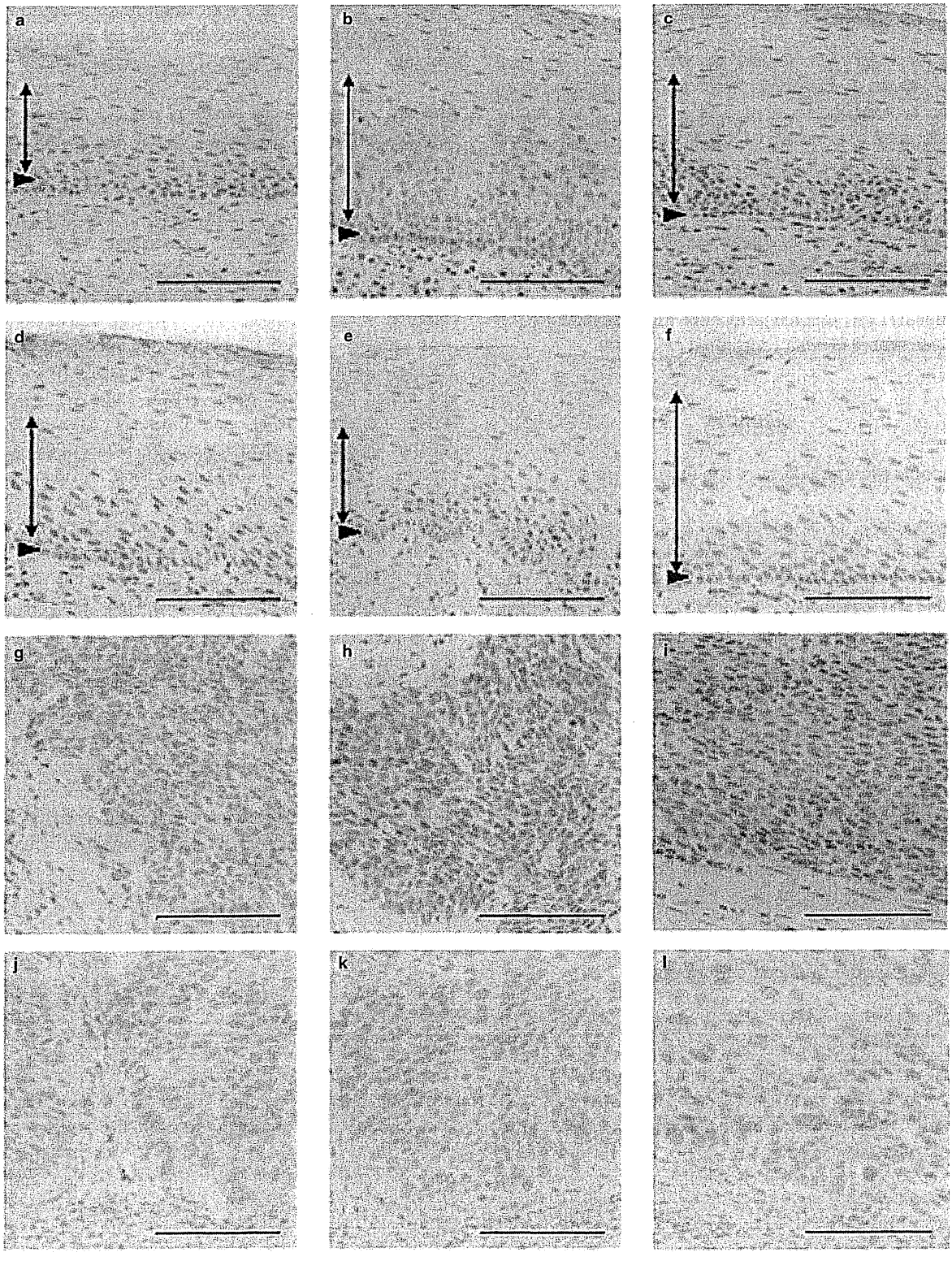

Figure 1 Immunohistochemistry for retinoic acid receptor (RAR) and retinoid X receptor (RXR) in non-neoplastic epithelium and esophageal carcinoma. In non-neoplastic epithelium of the esophagus, RARα (**a**), RARγ (**c**), RXRα (**d**), RXRβ (**e**), RXRγ (**f**) immunoreactivity was detected both in 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.

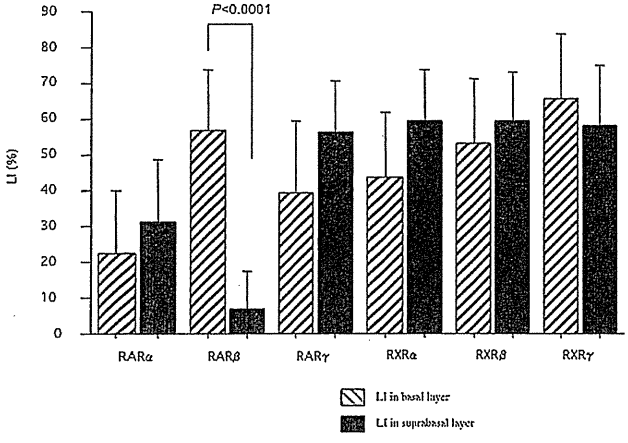

Figure 2 Immunohistochemistry of retinoic acid receptor (RAR) and retinoid X receptor (RXR) in 53 non-neoplastic epithelium of esophagus. RARβ labeling index (LI) was significantly decreased in the suprabasal layer compared with the basal layer ($P < 0.0001$). Data are presented as mean ± SD.

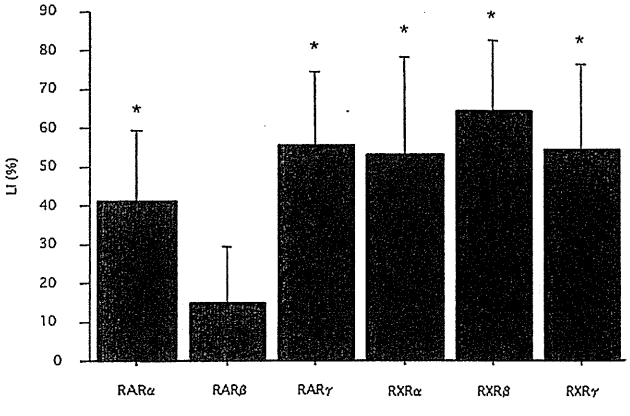

Figure 3 Immunohistochemistry of retinoic acid receptor (RAR) and retinoid X receptor (RXR) in 74 esophageal squamous cell carcinomas. RARβ labeling index (LI) was significantly more decreased than the other retinoid receptors. Data are presented as mean ± SD. *; $P < 0.001$ vs. RARβ LI by ANOVA followed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

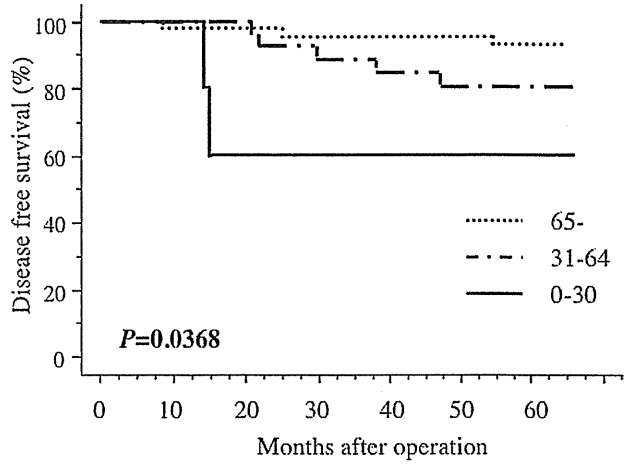

Figure 4 Overall survival of 74 patients with esophageal carcinoma according to retinoid X receptor β (RXRβ) immunoreactivity (Kaplan-Meier method). RXRβ immunoreactivity was significantly associated with better prognosis ($P = 0.0368$). Immunoreactivity was separated into three groups (0–30, 31–64,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