Histol Histopathol (2018) 33: 497-505

http://www.hh.um.es

Histology and Histopathology

From Cell Biology to Tissue Engineering

Expression of liver X receptors in normal and refractory carcinoma tissues of the human lung and pancreas

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Summary. Liver X receptors (LXRs) participate not only in maintaining cholesterol homeostasis but also in controlling cellular growth in many types of normal and tumor cells. We previously reported that LXRα was aberrantly expressed in human oral squamous cell carcinoma (HOSCC) tissues and cell lines, and that LXR stimulation led to significant reduction of proliferation of HOSCC cells via accelerating cholesterol efflux. Since LXRs and downstream proteins involved in cholesterol metabolism could be also applied as therapeutic targets in small cell lung carcinoma (SCLC) and pancreatic ductal adenocarcinoma (PDAC), we herein analyzed the distribution of LXR proteins in these refractory cancers as well as in normal human lung and pancreatic tissues. LXRβ was observed in ciliated epithelial cells, bronchial gland epithelia, type II alveolar epithelia and alveolar macrophages of the lung, and was less expressed in bronchial basal cells and type I alveolar epithelia. In addition, LXRβ was detected in epithelium of the pancreatic duct and acinar cells of the pancreas, and was weakly expressed in pancreatic islet cells. By contrast, LXRα expression was restricted to alveolar macrophages, and was not evident in any types of epithelial cells in the lung and pancreas. We also demonstrated that LXRβ but not LXRα was abundantly expressed in nine cases of SCLC and twenty cases of PDAC tissues. These findings provide basic information for evaluating the efficacy of LXR-targeted treatment in SCLC and PDAC

Key words: Liver X receptor, Nuclear receptor, Small cell lung carcinoma, Pancreatic ductal adenocarcioma, Epithelial cell

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DOI: 10.14670/HH-11-949

Introduction

Liver X receptor α (LXRα; also known as NR1H3) and LXRβ (also known as NR1H2), members of the nuclear receptor superfamily, are activated by cholesterol derivatives such as oxysterols (Janowski et al., 1996; Calkin and Tontonoz, 2012). LXRs transcriptionally regulate the expression of various genes by forming heterodimers with the retinoid X receptor and binding to LXR response elements (Apfel et al., 1994; Willy et al., 1995). LXRα is known to be strongly expressed in metabolically active organs and cells such as the liver, intestine, kidney, skin, adrenal glands, adipose and macrophages, whereas LXRβ is thought to be ubiquitously distributed throughout the body (Lu et al., 2001; Annicotte et al., 2004; Sakamoto et al., 2007). However, there is little information about the expression or distribution of LXR proteins especially in the lung and pancreas (Gabbi et al., 2008; Higham et al., 2013; Candelaria et al., 2014).

LXRs play an important role not only in maintaining cholesterol homeostasis (Repa et al., 2000; Zelcer and Tontonoz, 2006; Zelcer et al., 2009), but also in regulating cellular proliferation in various types of cells. For instance, LXR activation decreases cellular growth of normal cells, including vascular smooth muscle cells,

uterine endometrial cells, pancreatic β cells, hepatocytes, keratinocytes, and lymphocytes (Jamroz-Wiśniewska et al., 2007; Gabbi et al., 2009; Kim et al., 2009; Bovenga et al., 2015). In addition, LXR agonists reduce the proliferation of a variety of tumors (e.g., prostate, breast, ovarian, and colorectal cancer) *in vitro* and *in vivo* (Bovenga et al., 2015; Lin and Gustafsson, 2015). Nevertheless, the mechanism by which LXRs regulate cellular proliferation has not been established.

We have recently shown that LXRα protein is abundantly expressed in human oral squamous cell carcinoma (HOSCC) tissues and cell lines (Kaneko et al., 2015). We have also demonstrated that LXR activation reduces cellular growth of three different kinds of HOSCC cell lines at concentrations that hardly affected the human skin-derived keratinocyte cell line HaCaT. More importantly, our study highlights that ATP-binding cassette transporter A1 (ABCA1)-accelerated cholesterol efflux primarily contributes to the anti-proliferative effects of LXRs in HOSCC cells. On the other hand, LXRα activation suppresses the growth of mouse skin tumors by decreasing intracellular cholesterol via induction of ABCA1 expression and degradation of low-density lipoprotein receptor (LDLR) (Gabitova et al., 2015). Taken together, these findings suggest that there is a close link between LXR-mediated cholesterol metabolism and growth inhibition.

Small cell lung cancer (SCLC) and pancreatic ductal adenocarcinoma (PDAC) represent the most aggressive cancers with highly invasive and metastatic abilities, and are characterized by a high incidence of recurrence and the lowest survival rate (Ryan et al., 2014; Semenova et al., 2015). In addition, chemotherapies and radiation therapy remain the backbone of treatment for these cancers, and to date no effective therapeutic option is available (William and Glisson, 2011; Garrido-Laguna and Hidalgo al., 2015). Thus, novel strategies to cure these refractory cancers are urgently required. Cancer metabolism, which is extremely different from normal cells (Vander Heiden et al., 2009; Tennant et al., 2010), has emerged as a potential target to treat refractory cancers (Ryan et al., 2014). Therefore, as a first step to elucidate whether LXRs and their downstream molecules could be therapeutic targets in SCLC and PDAC, we examined the expression of LXRs in these refractory cancers along with that in normal human lung and pancreatic tissues.

Materials and methods

Isolation of human LXRα and LXRβ cDNA and construction of their expression vectors

Total RNA was isolated from the human small cell lung cancer cell line TKB16 using TRIzol reagent (Invitrogen, NY, USA), RT-PCR was performed using an RT-PCR kit (Perkin-Elmer, NJ, USA) according to manufacturer's instruction. Human LXRα and LXRβ cDNA fragments, in which XhoI and BamHI sites were created, were amplified using PrimeSTAR GXL DNA polymerase (Takara, Tokyo, Japan) with RT products as templates. The forward and reverse PCR primers were as follows: LXRα, 5'-TAGCTCGAGGCCACCATGTC CTTGTGGCTGGGGGCCCCTGTG-3' and 5'-TGTGG ATCCTAGGAGGCAGCCACCAGGCCTCAGCCATC-3'; and LXRβ, 5'-TAGCTCGAGGCCACCATGTCCT CTCCTACCACGAGTTCCCTG-3' and 5'-TGTGGAT CCCTCACTCGTGGACGTCCCAGATCTC-3'. The PCR products were subcloned into the expression vector pcDNA3.1 (-) (Invitrogen). Their sequences were evaluated by DNA sequencing.

Specificity of antibodies

Specificity of mouse monoclonal antibody (mAb) against LXRα (PP-PPZ0412-00; Perseus Proteomics, Tokyo, Japan) (Watanabe et al., 2005; Sakamoto et al., 2007; Kaneko et al., 2015) and goat polyclonal antibody (pAb) against LXRβ (SC-34343; Santa Cruz Biotechnology, CA, USA) (Kaneko et al., 2015) was verified by Western blot analysis. In brief, COS-7 cells were grown on 60-mm culture plates and transfected with 10 μg of empty vector or LXRα and LXRβ expression vectors using Lipofectamine 3000 (Thermo Fisher Scientific, MA, USA). Total cell lysates isolated 48 h after transfection were resolved by one-dimensional SDS-PAGE, and electrophoretically transferred onto a poly vinylidene difluoride membrane (Imobilon-P; Merck, NJ, USA). The membrane was saturated with PBS containing 5% skimmed milk and 0.1% Tween 20, and incubated overnight at 4°C with primary antibodies in PBS containing 1% skimmed milk. After rinsing in PBS containing 0.1% Tween 20, the membrane was incubated for 1 h at room temperature with horseradish peroxidase-conjugated anti-mouse (GE healthcare, IL, USA) and anti-goat (Santa Cruz Biotechnology) IgG in PBS containing 1% skim milk. It was then rinsed again, and finally reacted using EzWestLumi plus (ATTO, Tokyo, Japan). The blots were stripped and immunoprobed with a mouse mAb against β-actin (Sigma-Aldrich, MO, USA).

Human tissues

SCLC and PDAC tissues were respectively obtained from nine and twenty adult patients, respectively, whose primary tumor was resected by surgical operation at Fukushima Medical University. These tissues were fixed with 10% neutral formalin and paraffin-embedded, followed by Hematoxylin-Eosin (HE) or immunohisto-chemical staining. Surgical margin tissues with no cancer involvement in all cases were used as normal lung and pancreatic tissues.

All experimental protocols were approved by the Ethical Committee of Fukushima Medical University and carried out in accordance with relevant guidelines and regulations.

Immunohistochemistry

Paraffin-embedded tissue sections were deparaffinised in xylene and rehydrated through graded ethanol. Antigen retrieval was subsequently performed by autoclaving for 15 min at 121°C in sodium citrate buffer at pH 6.0 (for LXRα), or by microwave heating for 10 min (for LXRβ). After blocking with 5% skimmed milk (Morinaga Milk Industry, Tokyo, Japan), the sections were incubated overnight at 4°C with primary antibodies. After washing with PBS, secondary antibody reaction was performed by using Histofine Simple Stain MAX-PO (MULTI) Kit (Nichirei, Tokyo, Japan) with DAB as a chromogen according to the manufacturer's instructions.

Microscopy and quantification of immunohistochemical signals

All samples were examined and photographed by using flexible upright fluorescence microscope (BX61; Olympus, Tokyo, Japan) and DP controller software (Olympus). For quantification of immunoreactive signals for LXRβ in SCLC and PDAC tissues, at least 500 cells per section were examined in three randomly-selected high-power fields, and the percentage of positive nuclear staining was calculated.

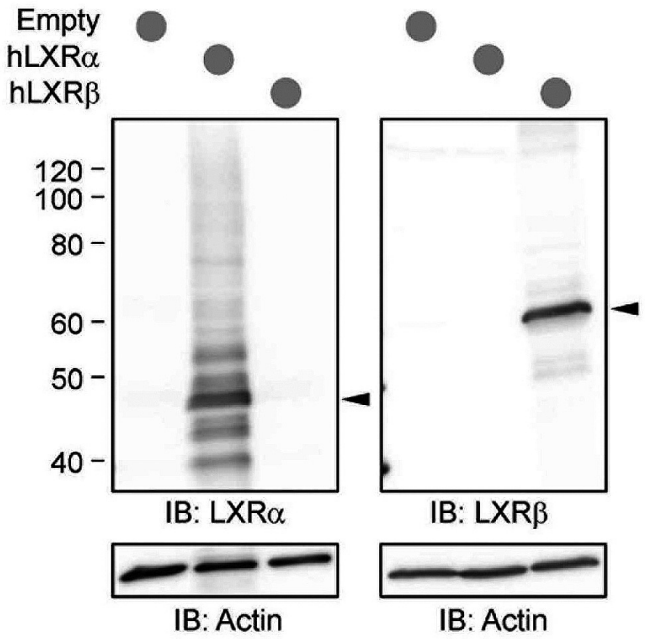

Fig. 1. Anti-LXRα and anti-LXRβ antibodies selectively recognize the corresponding LXRs. COS-7 cells were transfected with either empty or hLXRα and hLXRβ expression vectors, and their total cell lysates were separated by SDS-PAGE and immunoblotted (IB) with the indicated antibodies, followed by chemiluminescence detection. The mobility of molecular mass markers (kilodaltons) is indicated on the left. Arrowheads show the strongest specific signals.

Results

Specificity of anti-LXRα and anti-LXRβ antibodies

To check the specificity of antibodies used, COS-7 cells were transiently transfected with either empty vector or individual expression vectors containing human LXRα and LXRβ cDNAs, and their lysates were subjected to gel electrophoresis and immunoblotting. As shown in Fig. 1, the anti-LXRα Ab reacted with LXRα but not with LXRβ. Similarly, the anti-LXRβ Ab selectively detected LXRβ. Additional specific bands were observed in the immunoblots using these Abs, suggesting that they might also recognize posttranslationally modified LXRs and/or isoforms of the respective LXRs.

Expression of LXR proteins in normal human lung

We next determined, by immunohistochemistry, the distribution of LXRα and LXRβ throughout the respiratory tract of human lung tissue. In the bronchial area, LXRβ appeared to be observed in the nuclei of ciliated epithelial cells and bronchial gland epithelia (Fig. 2a,c,d,f). In addition, weak LXRβ signal was at least in part detected in bronchial basal cells, bronchial smooth muscle cells and vascular endothelial cells. In marked contrast, LXRα exhibited no specific nuclear signals in the bronchial epithelia (Fig. 2b,e). Furthermore, both LXRα and LXRβ were expressed in macrophages of the bronchial lumen.

In the alveolar area, LXRβ was strongly expressed in the nuclei of alveolar macrophages and type II alveolar epithelial cells, and weakly detected in type I alveolar epithelial cells (Fig. 2h). On the other hand, LXRα was only observed in alveolar macrophages but not in alveolar epithelial cells (Fig. 2g).

Table 1. Positive expression rate of LXRβ and clinical data in SCLC samples.

Case	LXRβ positive (%)	Age	Sex	Location	TNM stage
1	92.2	62	M	RM	T2aN0M0
2	81.8	67	M	RU	T1bN0M0
3	78.7	72	M	RL	T1aN0M0
4	79.4	66	M	RL	T1bN0M0
5	90.8	78	M	N/A	N/A
6	83.1	69	F	RL	T1bN0M0
7	72.7	68	M	RU	N/A
8	91.8	69	M	LU	T1bN0M0
9	95.7	73	M	RL	T1bN0M0
mean ± SD	85.1±7.3	69.3±4.3			

RM, right middle lobe; RU, right upper lobe; RL, right lower lobe; LU, left upper lobe; N/A, not available

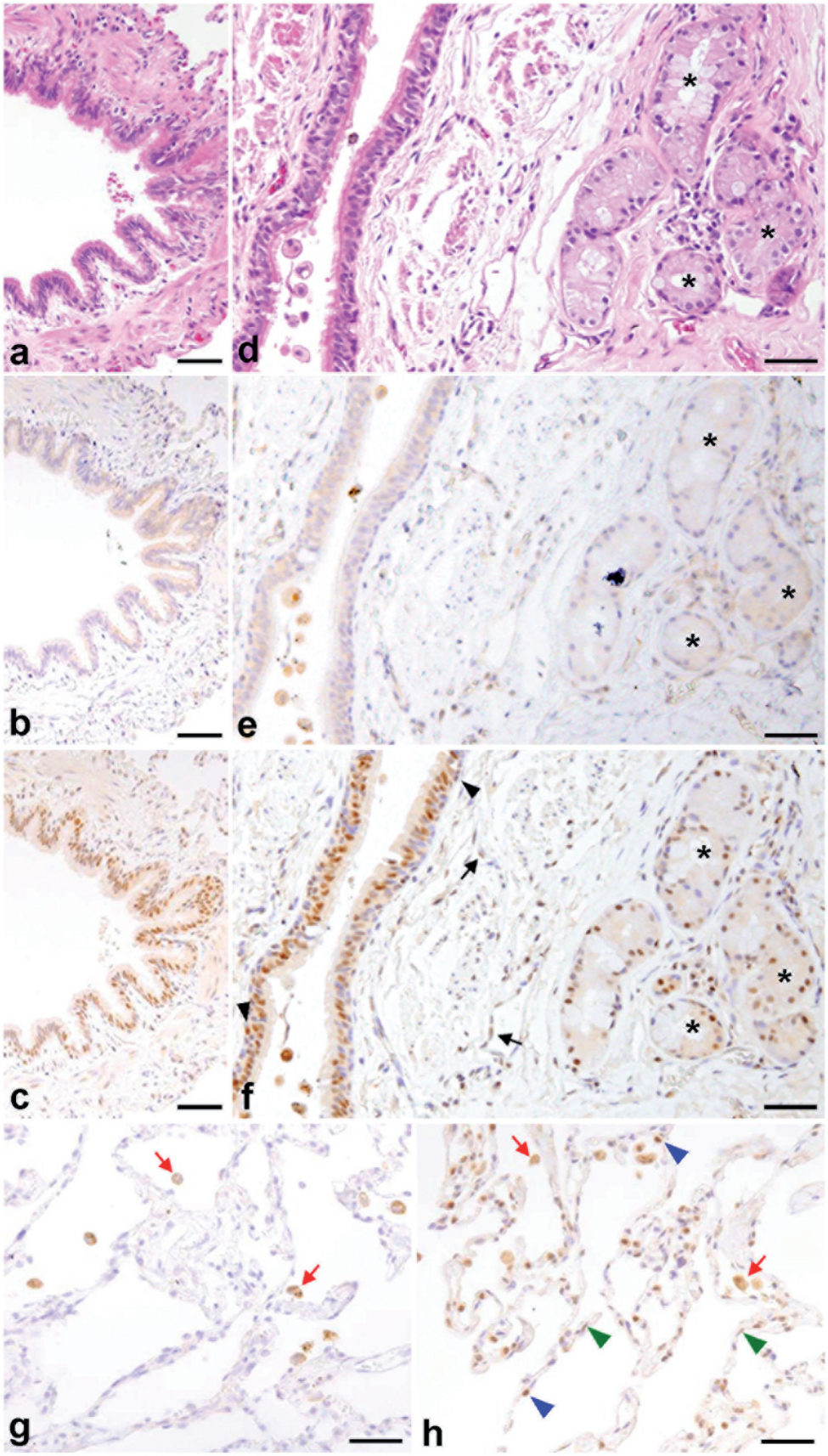

Fig. 2. LXRβ is expressed in ciliated epithelia, bronchial gland epithelia and alveolar epithelia of normal human lung. The normal human lung tissue was subjected to HE staining **(a, d)** and immunohistochemical staining for LXRα **(b, e, g)** and LXRβ **(c, f, h)**, and representative images are shown. Asterisks indicate bronchial glands. Black arrows and arrowheads show weak to moderate nuclear signals of LXRβ in endothelial cells and bronchial basal cells, respectively. Red arrows indicate positive signals for LXRα and LXRβ in alveolar macrophage. Green and blue arrowheads show LXRβ-positive signals in alveolar type I and type II epithelial cells, respectively. Scale bars: 50 μm.

Distribution of LXR proteins in normal human pancreas

We subsequently examined the expression of LXRs in normal human pancreatic tissues. LXRβ protein was greatly observed not only in the majority of pancreatic duct epithelia but also in acinar epithelial cells, though the signal intensity differed among epithelial cells of the acinus (Fig. 3a,c,d,f). LXRβ was also weakly detected in some nuclei of vascular endothelial cells and fibroblasts of the pancreas. Moreover, LXRβ was faintly expressed in nuclei of islet cells, in which the expression levels were much weaker than those in the surrounding acinar cells (Fig. 3g,i). By contrast, LXRα exhibited negative nuclear signals in all types of epithelia of the pancreatic duct, acinus and islets (Fig. 3b,e,h).

Expression of LXR proteins in SCLC and PDAC tissues

We then evaluated the expression of LXRs in SCLC and PDAC tissues. In all nine cases where the SCLC tissues were examined, LXRβ was strongly expressed in the nuclei of most cancer cells (Fig. 4a,c). However, LXRα showed negative nuclear specific signals or faint cytoplasmic immunoreactivity in SCLC tissues (Fig. 4b).

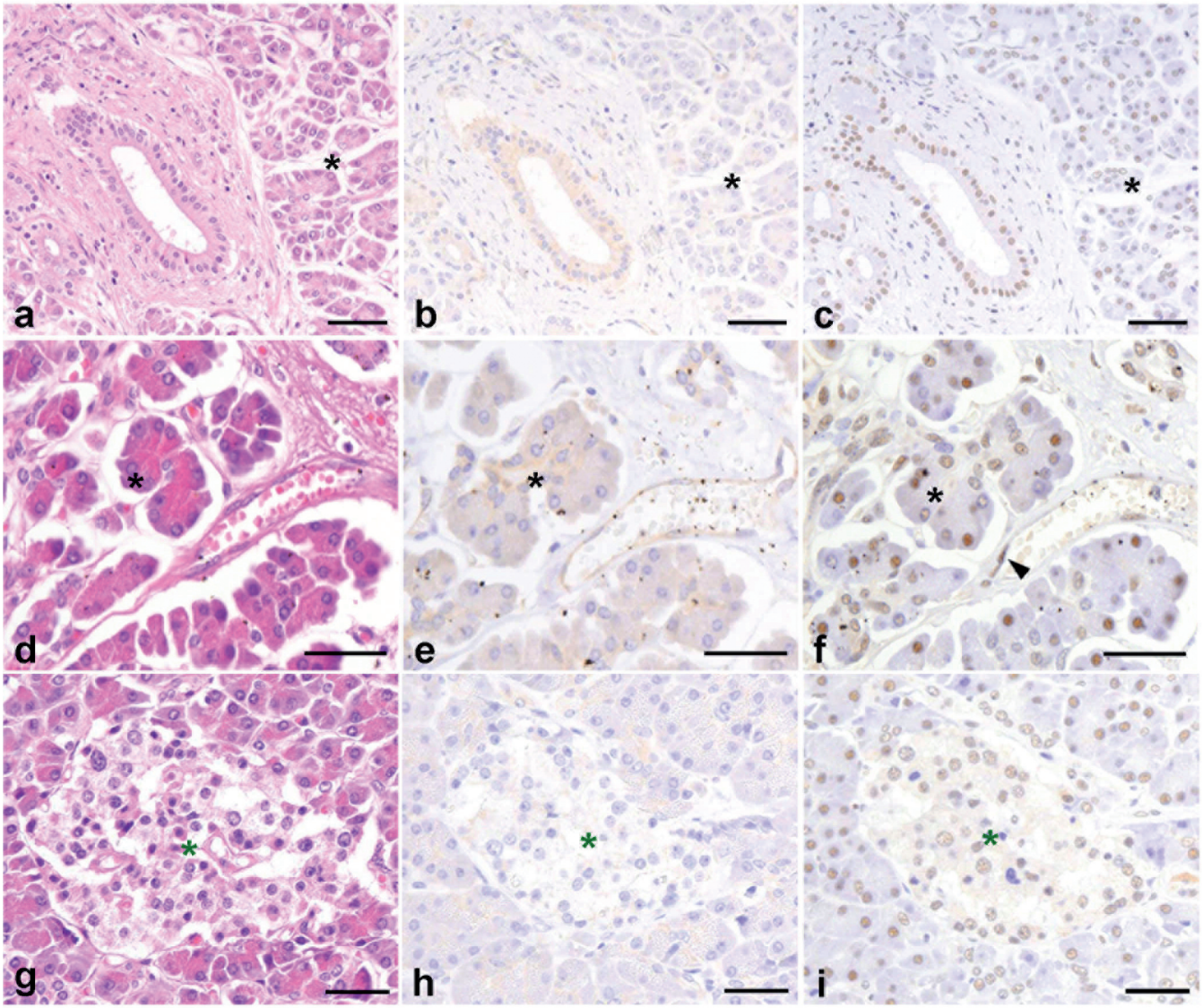

Fig. 3. LXRβ is detected in ductal and acinar epithelial cells of normal human pancreatic tissue. The normal human pancreatic tissue was subjected to HE staining **(a, d, g)** and immunohistochemical staining for LXRα **(b, e, h)** and LXRβ **(c, f, i),** and representative images are shown. Black and green asterisks indicate pancreatic acinus and islets of Langerhans, respectively. Arrowhead shows the weak to moderate LXRβ-positive signal in vascular endothelial cells. Scale bars: 50 μm.

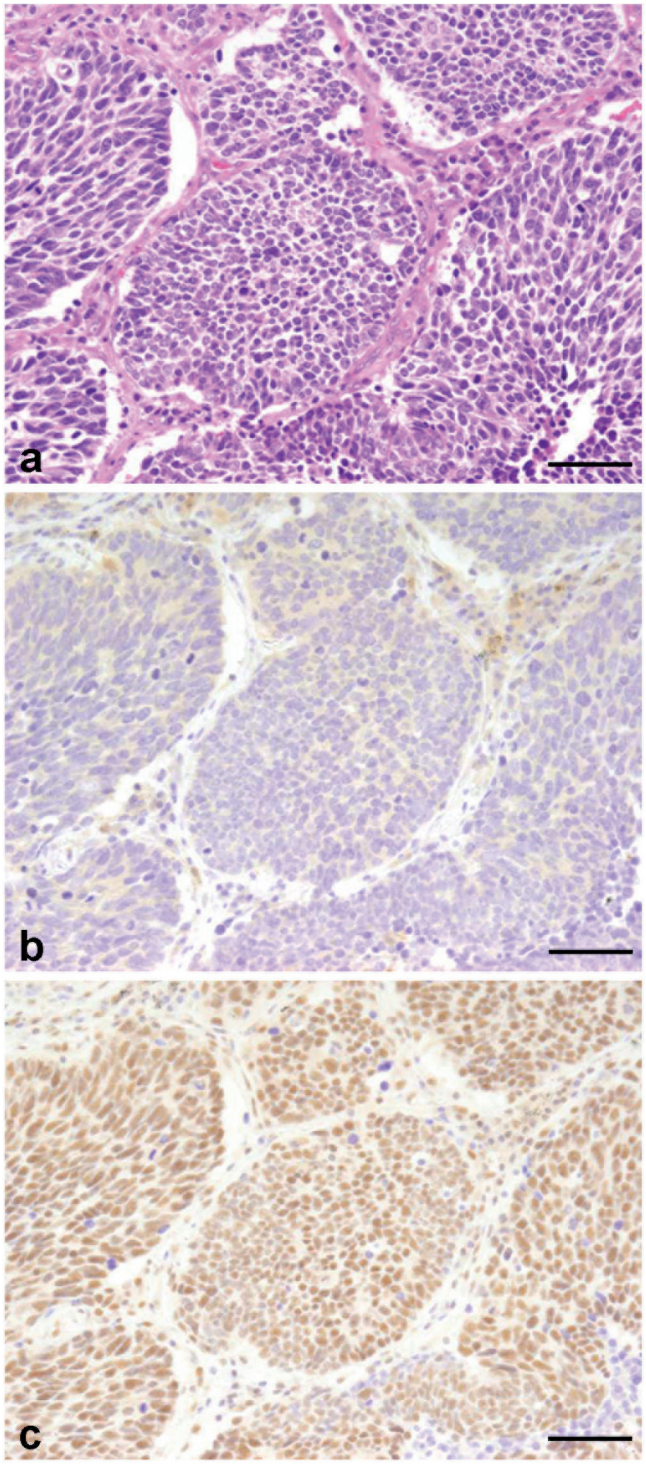

Fig. 4. LXRβ is strongly expressed in SCLC tissues. SCLC tissues were subjected to HE staining **(a)** and to immunohistochemical staining for LXRα **(b)** and LXRβ **(c)**, and representative images are shown. Scale bars: 50 μm.

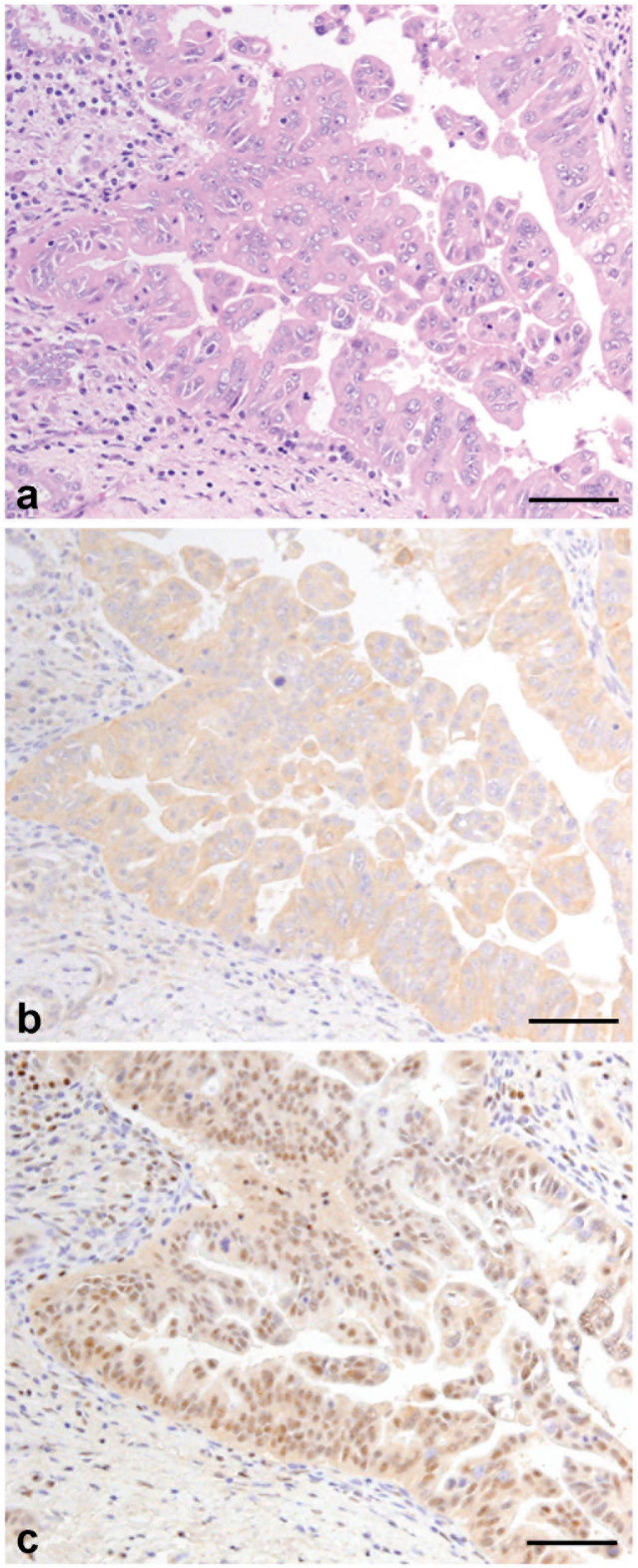

Fig. 5. LXRβ is aberrantly observed in PDAC tissues. PDAC tissues were subjected to HE staining **(a)** and to immunohistochemical staining for LXRα **(b)** and LXRβ **(c)**, and representative images are shown. Scale bars: 100 μm.

Table 2. Positive expression rate of LXRβ and clinical data in PDAC samples.

Case	LXRβ positive (%)	Age	Sex	Location	TNM stage	Tumor size
1	95.6	49	F	Pbt	T2N1M0	TS2
2	95.4	62	F	Ph	T3N1M0	TS2
3	85.2	47	F	Ph	T2N0M0	TS2
4	78.8	64	M	Ph	T3N1M0	N/A
5	94.9	49	F	Ph	T3N1M0	TS3
6	66.8	81	M	Ph	T2N0M0	TS1
7	89.7	76	F	Pb	T2N1M0	TS3
8	84.9	78	F	Ph	N/A	TS2
9	95.6	73	M	Ph	T2N0M0	N/A
10	95.4	68	M	Pb	T2N3M0	TS1
11	87.5	65	M	Ph	T2N0M0	TS2
12	81.6	71	F	Ph	T2N0M0	TS2
13	87.0	56	M	Ph	T3N2M0	TS2
14	91.7	71	M	Ph	T2N0M0	TS2
15	90.0	75	M	Pt	T3N0M0	TS2
16	80.4	74	M	Pb	N/A	TS4
17	98.5	65	F	Pt	T3N0M0	TS2
18	95.1	60	F	Ph	T2N0M0	TS2
19	84.6	73	M	Ph	T3N1M0	TS2
20	94.2	79	M	Ph	T3N2M0	TS3
mean ± SD	88.6±7.6	66.1±10.4				

Pbt, pancreatic body and tail; Ph, pancreatic head; Pb, pancreatic body; Pt, pancreatic tail; N/A, not available; Tumor size: TS1, TS1≤20 mm; TS2, 20 mm<TS2≤40 mm; TS3, 40 mm<TS3≤60 mm; TS4, TS4≥60 mm

We therefore determined the percentage of LXRβ-positive expression in nine cases of SCLC tissues. As shown in Table 1, the positive ratio of LXRβ in SCLC cases ranged from 73% to 96%, and the mean ± SD was 85.1±7.3%.

LXRβ was also greatly observed in the nuclei of most PDAC cells (Fig. 5a,c), as in that of SCLC tissues. The proportion of LXRβ-positive expression in PDAC cases varied from 67% to 99%, and the mean ± SD was 88.6±7.6% (Table 2). On the other hand, LXRα exhibited negative nuclear specific signals or faint cytoplasmic immunoreactivity in PDAC tissues (Fig. 5b).

Discussion

In the present study, we first demonstrated that the expression profile of LXRβ was different from that of LXRα in normal human lung tissues. We showed that LXRβ was predominantly observed not only in ciliated epithelial cells as reported previously (Higham et al., 2013), but also in gland epithelia along the bronchial tract. We also found that LXRβ was expressed in bronchial basal cells much less than in highly-differentiated ciliated epithelia. Taken collectively with our previous finding revealing that LXRβ expression predominated in more differentiated prickle cells compared with that in basal and parabasal cells of the skin and oral mucosa (Kaneko et al., 2015), LXRβ seems to be preferentially expressed in more differentiated epithelial cells of skin, oral and lung tissues. LXRβ was also detected in alveolar epithelial cells as reported previously (Smoak et al., 2008; Higham et al., 2013), although the signal intensity in type II alveolar epithelial cells was stronger than type I cells. In contrast, LXRα expression was restricted to alveolar macrophages, which also expressed LXRβ, and was not observed in any epithelial cells along the respiratory tract. Thus, we conclude that LXRβ represents the major subtype that mediates the LXR signal in normal lung epithelial tissues.

LXRβ was expressed in epithelial cells of normal human pancreatic duct and islet cells as reported previously (Gabbi et al., 2009; Ogihara et al., 2010; Candelaria et al., 2014). Our immunohistochemical study, however, showed that LXRβ protein was intensely detected in pancreatic acinar epithelia, though the positive signals were less frequent compared with those in ductal epithelia. Moreover, the staining intensity of LXRβ in pancreatic duct and acinar epithelial cells was much stronger than that in islet cells. Conversely, no LXRα expression appeared in normal pancreatic epithelial tissues. Hence, these findings strongly suggest that LXRβ predominantly transduces the LXR signals in normal pancreatic epithelial tissues as in normal lung epithelia. Since the distinct distribution of LXRα and LXRβ is also evident in the intestine, brain, skin, and oral mucosa (Fan et al., 2008; Modica et al., 2010; Kruse et al., 2012; Hong and Tontonoz, 2014; Kaneko et al., 2015), our results reinforce the notion that each LXR subtype possesses specific physiological functions.

Another conclusion of the present study is that LXRβ but not LXRα is abundantly expressed in SCLC tissues. The aberrant expression of LXRβ was also apparent in PDAC tissues as reported previously (Candelaria et al., 2014). Since the positive ratio of the LXRβ expression was exclusively high in both SCLC and PDAC tissues analysed (at least about 70% of cancer cells were positive for LXRβ), we were not able to perform statistical analysis between LXRβ expression and any clinic-pathological factors. LXRβ is known to be the major subtype expressed in malignant melanoma and glioblastoma (Pencheva et al., 2014; Villa et al., 2016), whereas LXRα acts as the predominant subtype expressed in HOSCC tissues (Kaneko et al., 2015). Taken together, either LXRα or LXRβ seem to be differently overexpressed in distinct types of cancers. It is noteworthy that LXRβ activation suppresses growth, invasion, angiogenesis, progression and metastasis of murine malignant melanoma cells, resulting in prolonged animal survival (Pencheva et al., 2014). Owing to the fact that LXRβ protein was aberrantly expressed in SCLC and PDAC tissues, it should be verified as to whether LXRβ and its downstream molecules involved in cholesterol metabolism could be therapeutic targets in these refractory cancers. In fact, statins, which suppress cholesterol synthesis, inhibit the proliferation of SCLC cells *in vitro* and *in vivo* (Khanzada et al., 2006). Moreover, LDLR implicated in

cholesterol uptake is reported to be a promising target to limit PDAC progression in murine model (Guillaumond et al., 2015).

In summary, we have provided evidence showing that LXRβ is preferentially expressed in various types of epithelial cells of normal human lung and pancreatic tissues. We have also demonstrated that LXRβ was abundantly expressed in SCLC and PDAC tissues. Interestingly, we previously revealed that synthetic LXR ligands significantly reduced the cellular growth of three different HOSCC cell lines at concentrations that barely influenced a human skin-derived keratinocytes. Taken collectivity with the notion that cancer cells have a greater cholesterol demand than normal cells, LXRβ-targeted treatment could be an encouraging option in SCLC and PDAC.

Acknowledgements. We thank Ayumi Hozumi and Keiko Watari for their technical assistance. This work was supported by JSPS KAKENHI Grant Number JP16K19083.

Conflict of interest. The authors declare no conflicts of interest.

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Accepted November 28, 2017