Original Article

Increased Farnesoid X Receptor Expression in Non-small Cell Lung Cancer

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Abstract: **Objective:** Farnesoid X activated receptor (FXR) is one of the nuclear hormone receptors which is activated by farnesol and related molecules, and is specifically expressed in tissues in which bile acids function. Although hepatocellular carcinoma cells (HepG2) are reported to express FXR, there is no study examining its expression in other types of cancer. In this study, we studied the expression of FXR mRNA and protein in non-small cell lung cancers (NSCLC). **Methods:** The expression of FXR messenger RNA (mRNA) and protein in NSCLC was examined by reverse transcription-polymerase chain reaction (RT-PCR), Western blot analysis, and immunohistochemistry. **Results:** FXR mRNA was expressed in 6 of 8 NSCLC tissues by RT-PCR. Western blot analysis exhibited the presence of FXR protein in 8 of 9 NSCLC tissues. Immunohistochemistry showed immunoreactive FXR in 35 of 37 NSCLC tissues. The intensity and frequency of FXR expression was strongest in adenocarcinoma followed by squamous cell carcinoma and large cell carcinoma, respectively.
Conclusion: Our results suggest that FXR may play a role in the pathogenesis of NSCLC.

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Key words: Farnesoid X activated receptor, Farnesol, Non-small cell lung cancer, Immunohistochemistry

1. Introduction

Lung cancer remains the leading cause of death by cancer among males and is second among females in Japan, mainly because of the difficulty of early diagnosis, and recurrence [1]. Despite recent advances in radiotherapy and chemotherapy, the survival of patients with lung cancer has not improved significantly. Current interest is, therefore, focused on chemoprevention of lung cancer. In particular, research regarding the chemopreventive effects of nonsteroidal antiinflammatory drugs with regard to many human cancers including those of the colon, breast, pancreas, and liver has been largely comfirmed [2)~4)]. Recently, orphan nuclear receptors defined as peroxisome proliferator activated receptors (PPARs) have been reported to play a role in the pathogenesis of several types of cancers [5)~8)]. In particular, activation of PPAR-γ has been shown to have antiproliferative effects on several tumor-derived cell lines [5)~9)].

Farnesoid X activated receptor (FXR) is also a member of the steroid/nuclear superfamily of genes. These genes encode intracellular receptors that regulate transcription by the active binding of lipophilic signal molecules, dimerization of a receptor complex, and binding to specific hexamer halfsite consensus sequences in the promoter regions of target genes [10]. FXR appears to be localized in tissues important to steroidogenesis (liver, kidney, adrenal, and the intestine) [11]. FXR can bind and be activated by cholesterol precusor farnesol and variety of endogeneous bile acids, indicating that FXR may play a critical role in cholesterol and bile acid homeostasis [11].

However, no other medical significance of FXR has been demonstrated. In particular, there is no study concerning the relationship between FXR expression and malignant tumors apart from hepatocellular carcinoma (HepG 2) [12]. We examined the expression of FXR messenger RNA (mRNA) and its protein in human lung cancer tissues by reverse transcription-polymerase chain reaction (RT-PCR), Western blot analysis, and immunohistochemistry.

2. Materials and Methods

Antibody. Antibody against FXR was from Santa Cruz

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(Santa Cruz Biotechnology, Inc., CA, USA).

Patient Samples. Thirty seven primary non-small cell lung cancer specimens including 21 adenocarcinomas, 11 squamous cell carcinomas, and 5 large cell carcinomas were obtained from surgically removed lung tissues from the Kyoto First Red Cross Hospital. The mean age of the patients was 70.4 years (range, 50～82 years). Six normal lung tissue specimens were derived from patients with metastatic colon cancer. Consent for the study was obtained from each patient. A subset of the tumor samples was frozen in liquid nitrogen and stored at −80℃ for analysis of mRNA and Western blotting. All cancers were primary lung tumors and were classified by the same pathologist.

Immunohistochemistry. Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissues using citrate microwave antigen retrieval as described previously [13]. The sections were deparafinized and endogenous peroxidase activity was exhausted by incubating in 0.3% peroxide in methanol for 45 min. The sections were preincubated with 0.2% BSA in PBS for 20 min and with diluted rabbit serum (1: 66.7) for 20 min followed by incubation with anti-FXR antibody (2 μg/ml) or purified normal goat IgG (2 μg/ml) in a humid chamber. After overnight incubation at 4℃, the sections were washed with PBS and further incubated with biotinylated rabbit anti-goat IgG (Vector Laboratories, Inc. Burlingame, CA) for 30 min. Then after washing with PBS, the sections were incubated with avidin and biotinylated horseradish peroxidase complex for 45 min. Finally, the sections were washed with PBS for 10 min and color was developed by immersing the sections in a solution of 0.05%wt/vol 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma, St. Louis, MO), and 0.01% hydrogen peroxide in 0.05 M Tris, pH 7.4, for 3～5 min. The sections were counterstained with hematoxylin.

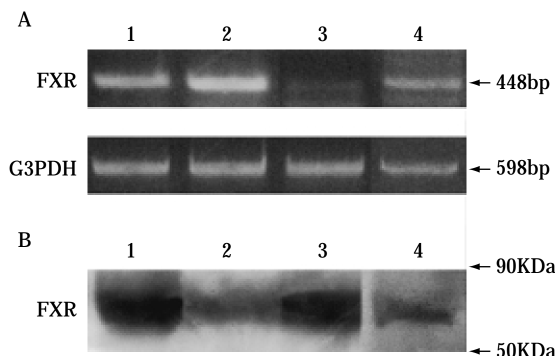
Immunohistochemical Analysis. For each of the tissue specimens from NSCLC, the extent and intensity of staining with anti-FXR antibody in cancer cells were graded on a scale of 0～4 by two blinded observers on two separate occasions using coded slides as previously described [13]. Grade 4 indicates maximally intense staining, whereas 0 indicates no staining.

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Total RNA was isolated from 4 adenocarcinomas, 3 squamous cell carcinomas, a large cell carcinoma from resected lung tissues, and 3 normal lung tissues by the acid guanidium thiocyanate-phenol-chloroform method. We performed an RT-PCR procedure to determine the FXR mRNA expression as previously described. Total RNA was used as a template for DNA synthesis using a Superscript preamplification system (Gibco, Rockville, MD, USA) according to the manufacturer's instructions. In PCR reactions the temperatures for denaturation were 94℃ for 40 s, extension at 72℃ for 50 s, annealing at 52℃ for 50 s, and the number of amplification cycles was 35. The PCR products were analyzed on a 2% agarose/TAE gel.

Oligonucleotide primers of FXR were designed to amplify eight partial cDNA sequences covering the full-length of the transcriptional element. The synthetic oligonucleotides were obtained from Nippon Flour Mills Co., Ltd. (Kanazawa, Japan). We used glyceraldehyde-3-polyachrylamide-dehydrogenase (G3PDH) mRNA as a control. The primers used were as follows: (a) FXR: sense 5'-CGTGACTTGCGN CAAGTGACC-3', antisense 5'- CCANGACATCAGCATCT CAGCG-3'[11], (b) G3PDH: sense 5'-CCACCCATGGCAA ATTCCATGGCA-3', antisense 5'-TCTAGAGGGCAGGTC AGGTCCACC-3'.

Western Blot Analysis. Protein was isolated from 5 adenocarcinomas, 3 squamous cell carcinomas, a large cell

Fig. 1. Representative results of mRNA and Western blotting expression of FXR in lung cancer tissues. A) FXR mRNA by RT-PCR, B) Western blot analysis for FXR protein in representative tissues from lung cancer tissues.
1. Adenocarcinoma, 2. Squamous cell carcinoma, 3. Large cell carcinoma. 4. HepG2 (as a positive control).



carcinoma from surgically removed lung tissues, and 5 normal lung tissues. Lysates were made by standard method. Briefly, samples were equalized to 20 μg/lane and run on a 7.5% polyachylamide gel. Proteins were blotted on to immobion polyvinylidene difluoride membrane（ATTO, Tokyo, Japan）. The filter was blocked in PBS, 0.05% Tween 20, and 5% nonfat milk at room temperature for 90 min. All antibodies were diluted in the same buffer. Antibodies were used at a dilution of 1: 100（anti-FXR）, and incubated at room temperature for 60 min. The secondary antibodies were used at a dilution of 1: 1000 and incubated at room temperature for 60 min. The stripping procedures were performed using standard methods.

3. Results

FXR Expression in Primary NSCLC. Expression of FXR was examined in NSCLC tissues and normal lung tissues. As shown in Fig. 1A, mRNA was detectable in 6 of 8 NSCLC tissues by RT-PCR. Western blotting revealed immunoreactive FXR in 8 of 9 NSCLC tissues（Fig. 1B）. To evaluate the localization of FXR in NSCLC tissues and normal lung tissues, immunohistochemical examination of FXR expression was performed on 37 paraffin-embedded tumors obtained from patients with NSCLC. As shown in Table 1, positive FXR expression was noted in 21 adenocarcinomas, 10 of 11 squamous cell carcinomas, and 4 of 5

Table 1. Characteristics of the patients

Patient no.	Histologic type	Sex	Age	Differentiation	T-stage	N-stage	M-stage	B.I.	cancer cells
1	SCC	F	70	moderate	2	1	0	500	2.5
2	SCC	M	80	well	2	0	0	800	2
3	SCC	M	71	poor	2	0	0	1600	4
4	SCC	M	70	well	2	2	0	3000	3
5	SCC	M	68	poor	1	1	0	1680	2
6	SCC	M	75	poor	2	2	0	1200	3
7	SCC	M	50	well	3	2	0	1600	2.5
8	SCC	F	64	well	2	1	0	440	3
9	SCC	M	78	poor	2	1	0	3300	2.5
10	SCC	M	74	moderate	3	1	0	1100	0
11	SCC	M	82	moderate	1	0	0	1000	1
12	AD	F	67	well	1	0	0	500	4
13	AD	M	69	poor	3	1	0	800	4
14	AD	M	66	well	3	1	0	300	4
15	AD	F	74	well	2	0	0	1080	3.5
16	AD	M	78	poor	2	0	0	1800	4
17	AD	M	52	moderate	2	1	0	900	2
18	AD	F	71	moderate	1	2	0	1000	4
19	AD	F	66	moderate	1	1	0	208	4
20	AD	M	75	moderate	2	0	0	1000	2.5
21	AD	M	72	well	1	0	0	1200	4
22	AD	M	69	well	1	0	0	400	2
23	AD	M	77	poor	3	2	0	1650	4
24	AD	F	73	well	1	0	0	0	3.5
25	AD	M	66	moderate	2	0	0	600	4
26	AD	M	79	poor	3	1	0	0	4
27	AD	F	73	moderate	2	0	0	0	3
28	AD	F	57	moderate	1	0	0	0	3.5
29	AD	F	78	well	1	0	0	0	4
30	AD	F	58	well	2	0	0	0	3.5
31	AD	F	67	well	4	0	0	0	4
32	AD	F	74	well	2	2	0	0	4
33	LCC	F	74		3	2	0	600	1
34	LCC	M	66		3	0	0	400	1.5
35	LCC	M	70		3	0	0	700	3
36	LCC	M	74		1	2	0	600	0
37	LCC	M	78		4	0	0	0	2

SCC indicates squamous cell carcinoma; AD, adenocarcinoma; LCC, large cell carcinoma; and B.I., Brinkman Index.

large cell carcinomas. Expression of FXR was localized to tumor cells. The staining pattern was granular and localized to both the nucleus and cytoplasm (Fig. 2). The intensity of the immunoreactive FXR expression was strongest in adenocarcinomas followed by large cell carcinomas and squamous cell carcinomas, respectively. In addition, we evaluated the expression of FXR in normal lung tissues by RT-PCR, Western blotting, and immunohistochemistry. Neither RT-PCR nor Western blot analysis revealed the expression of FXR in normal lung tissues. In immunohistochemistry, normal lung tissues expressed FXR at very low or negligible levels (data not shown).

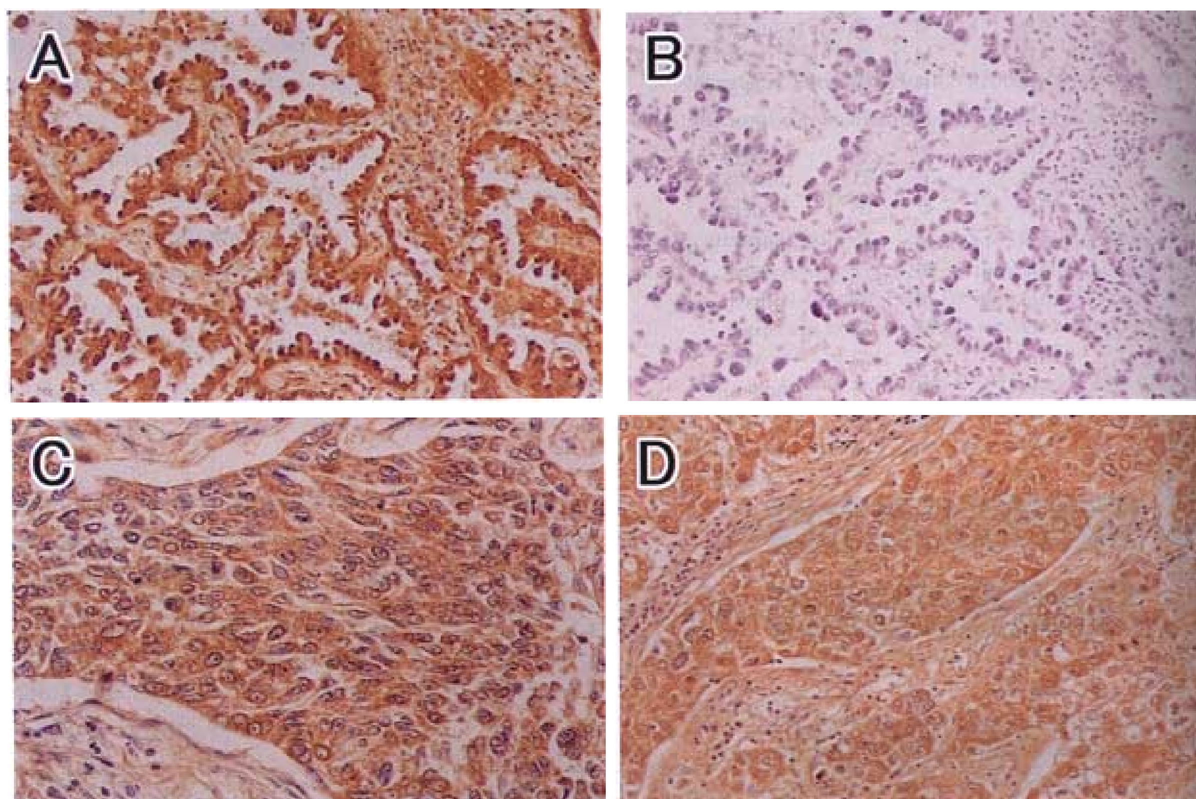
4. Discussion

We demonstrated that FXR mRNA and protein is detected in NSCLC tissues. On the other hand, as in previous studies[10)11)], FXR was expressed at very low or negligible levels in normal lung tissues.

O'Brien et al.[14)] suggested that activation of FXR-mediated events may play a tumor promotion role in peroxisome proliferator-induced rodent hepatocarcinogenesis. Another study has demonstrated that deoxycholic acid (DCA) is shown to increase mitotic events of colonic mucosa[15)] and preneoplastic lesions in rats fed with cholic acid were found to be less susceptible to apoptotic stimuli [16)]. In addition, an increased serum level of DCA and reduced bile acid-induced apoptosis of colonic mucosa goblet cells has also been reported in human colorectal cancers[17)18)]. On the other hand, ursodeoxycholic acid (UDCA) has been shown to decrease tumor incidence in a bile acid-facilitated rat model of colon cancer [19)]. Primary bile acid and secondary bile acid DCA activate FXR 11), and Howard and colleagues have shown that UDCA could inhibit DCA and chenodeoxycholic acid (CDCA) to activate FXR [20)]. Taken together, FXR may play a role in tumor formation. Our results also may agree with the hypothesis in regard to NSCLC.

Farnesol, one of FXR potent agonists, is essential compound required for cell proliferation and differentiation. Farnesol has been shown to arrest the proliferation of a number of cell lines and to induce apoptosis of several tumor-derived cell lines [21)~23)]. However, the mechanisms

Fig. 2. Representative results of FXR immunostaining in non-small lung cancers. Three distinct pathological lung cancers expressing FXR. Intense immunoreactivity can be seen in tumor cells. A) adenocarcinoma, B) adenocarcinoma immunostained with anti-FXR antibody adsorbed with FXR peptide, C) squamous cell carcinoma, D) large cell carcinoma



in which farnesol inhibits cell proliferation and preferentially induces apoptosis of neoplastic cells remain obscure. Miquel et al.[24] demonstrated that farnesol induced inhibition of phosphatidylcholine biosynthesis at the last step of the CDP-choline pathway controled by choline phosphotransferase in human lung adenocarcinoma A549 cells. Another likely mode of action of farnesol is by inhibition of protein prenyltransferases. Inhibition of farnesyltransferase has been shown to cause tumor regression in animal models[25]. Taking our results into consideration, however, farnesol's inhibitory effect on malignant cells may be explained, at least in part, by the activation of FXR. Further elucidation about FXR expression and tumorigenesis will be needed.

In summary, this is apparently the first report demonstrating the expression of FXR in NSCLC tissues. Based on the results of this study, FXR may become a novel target for preventing or treating NSCLC.

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