Inhibitory effect of PPARγ on NR0B1 in tumorigenesis of lung adenocarcinoma

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Abstract. NR0B1, an orphan nuclear receptor, is expressed in side population cells and its knockdown reduces tumorigenic and anti-apoptotic potential in lung adenocarcinoma. Peroxisome proliferator-activated receptor γ (PPARγ) is another member of the nuclear receptor family which induces apoptosis in lung cancer. The interaction of NR0B1 with PPARγ was examined. The transactivation ability of PPARγ was inhibited by NR0B1 in lung adenocarcinoma, and the N-terminal region of NR0B1 containing LxxLL motifs mediated its inhibition. Co-immunoprecipitation experiments revealed that this N-terminal region of NR0B1 was essential for the physical interaction with PPARγ. Aldehyde dehydrogenase (ALDH) activity and ALDH3A1 expression, which are correlated with tumorigenic potential of lung adenocarcinoma, increased when NR0B1 expression was induced, but its increase was inhibited by PPARγ overexpression. ALDH activity increased by treatment with PPARγ inhibitor, and the increase was further enhanced when the expression of NR0B1 was induced. Furthermore, the high NR0B1 and low PPARγ expression was a negative prognostic factor in Pathological-Stage IA clinical cases. These results indicate the reciprocal relationship between NR0B1 and PPARγ on the malignant grade of lung adenocarcinoma.

Introduction

Lung cancer is one of the leading causes of cancer-related mortality worldwide (1), and adenocarcinoma is the most common histological subtype. In Japan, the mortality rate of lung cancer is the highest of all types of cancer since 1998 (2). The 5-year survival rate of lung cancer surgically treated is currently 69.6% in all stages, and 86.8% even in stage IA (3). Therefore, more effective strategies of therapy are necessary.

NR0B1, also known as dosage-sensitive sex reversal, adrenal hypoplasia critical region, on X-chromosome gene 1 (DAX-1), is a member of the orphan nuclear receptor family (4,5), and its mutations result in adrenal hypoplasia congenita (6). NR0B1 is physiologically expressed in the adrenal cortex, ovary, Sertoli cells, pituitary gonadotropes, ventromedial hypothalamic nucleus cells, and others (7), and is correlated with gonadal development, sex determination, and steroidogenesis (8). NR0B1 is a negative regulator of steroid production (9-12), which represses the transcription of other nuclear receptors via heterodimerization, including androgen, estrogen, and progesterone receptors (13-15). Such repression is mediated via the N-terminal repeat domain of NR0B1, containing three LxxLL motifs (4,5,16).

Cancers consist of heterogeneous cell populations derived from a single clone. It has previously been demonstrated that cells with tumorigenic potential are limited to a small population, known as cancer stem cells (CSCs). In flow-cytometric analysis, CSCs are stained faintly by Hoechst 33342, and are enriched in a side population, where dimly stained cells are collected (17-19). NR0B1 is one of the highly expressed genes in the side population of lung adenocarcinoma (20). NR0B1 expression is detected in a number of cancers, including endometrial carcinoma, prostate carcinoma, Ewing’s sarcoma, and lung cancer (21-25). We previously reported that NR0B1 is correlated with the malignant potential of lung adenocarcinoma through invasion, colony formation, and tumorigenic activities (25).

Peroxisome proliferator-activated receptor γ (PPARγ) is a member of the nuclear receptor superfamily of ligand-activated transcriptional factors and is implicated in adipogenesis (26,27). PPARγ binds to the peroxisome proliferator responsive element (PPRE) (28), and is expressed in a number of tissues, including type II pneumocytes, in humans (29-31). PPARγ ligands, such as troglitazone (TGZ) and 15-deoxy-delta12-14-prostaglandin J2 (15d-PGJ2), inhibit the growth of human lung adenocarcinoma through ligand-induced differentiation, growth arrest, and induction of apoptosis (32-35). PPARγ is reported to interact with NR0B1 via its DNA binding and hinge domains (36). The domain of NR0B1 which is essential for the...
interaction with PPARγ, however, has yet to be determined. In contrast to NR0B1, PPARγ possesses an antagonistic function against lung adenocarcinoma. Therefore, there is a possibility that NR0B1 and PPARγ possess an opposite effect on tumors. In the present study, we examined the inhibitory effect of PPARγ on NR0B1 in lung adenocarcinoma.

Materials and methods

Plasmids, cells, and chemicals. The human NR0B1 cDNA was cloned into pIRESpuro and pIRE3G vectors (Clontech, Palo Alto, CA), and pCMV7.1-3xFLAG vector (Sigma, St. Louis, MO), respectively. The deletion mutant of NR0B1 (del-NR0B1), lacking the N-terminal region of NR0B1, was PCR-amplified and cloned into pCMV7.1-3xFLAG vector. The human PPARγ cDNA was also cloned into pIRESpuro and pHM6 HA (Roche Diagnostics, Mannheim, Germany) vectors. The luciferase construct containing three repeats of PPRE (PPRE-Luc) was obtained from OriGene (Rockville, MD). HEK-293T and human lung adenocarcinoma cell lines A549 and PC9 were purchased from the American Type Culture Collection (Rockville, MD). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma) supplemented with 10% fetal calf serum (FCS; Nippon Bio-Supp. Center, Tokyo, Japan). GW9662 (Calbiochem, Gibbstown, NJ) was dissolved in dimethylsulfoxide (DMSO) and used as a PPARγ specific antagonist.

Luciferase assay. PPRE-Luc was cotransfected with various amounts of expression plasmid (pIRESpuro) containing NR0B1, del-NR0B1, and PPARγ to A549 and PC9 cells, using TransFast Transfection Reagent (Promega, Madison, WI). Empty vector was added to maintain a consistent amount of DNA used for transfection. After 48 h, cells were harvested, and the luciferase activity was measured as previously reported (37). Cells transfected with empty vector alone were used as control and the relative luciferase activity was represented as folds of control.

Immunoprecipitation assay. The Flag-tagged wt-NR0B1 or del-NR0B1 was coexpressed with HA-tagged PPARγ in HEK-293T cells. The nuclear extract was mixed with either anti-FLAG M2 conjugated beads or anti-HA conjugated beads (Dynabeads, Invitrogen, Carlsbad, CA). Immunocomplexes were analyzed by immunoblot analysis with anti-Flag antibody (Sigma).

Induced expression of NR0B1 with Tet-Express. Inducible expression of NR0B1 was performed with Tet-Express Inducible Expression Systems (Clontech) according to the manufacturer’s instructions. Briefly, pTRE3G containing NR0B1 cDNA was transfected into A549 cells with linear puromycin marker (Clontech). Puromycin-resistant clones were selected as Tet-Express inducible cells. NR0B1 expression induced by the transfection of Tet-Express with Xfect transfection reagent (Clontech) was confirmed by immunoblotting as described below. Cells were harvested and the nuclear extractions were prepared; they were isolated on 10% SDS-polyacrylamide gels, transferred into immobilin (Millipore, Bedford, MA), and incubated with anti-NR0B1 (Abcam Ltd, Cambridge, UK) or anti-Lamin A/C (Cell Signaling, Beverly, MA) antibodies. After washing, the blots were incubated with an appropriate peroxidase-labeled secondary antibody (MBI, Nagoya, Japan), and then reacted with Renaissance reagents (NEN, Boston, MA) before exposure.

Quantification of mRNA levels by real-time reverse-transcription PCR (RT-PCR). Total RNA was extracted from cells transfected with Tet-Express or incubated with GW9662 (20 μM overnight) using an RNeasy kit (Qiagen, Valencia, CA). RNA was reverse-transcribed into cDNA by Superscript III (Invitrogen). The mRNA levels for aldehyde dehydrogenase 3A1 (ALDH3A1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were measured using TaqMan gene expression assays (Applied Biosystems, Foster City, CA). The amount of ALDH3A1 mRNA was normalized to that of GAPDH mRNA. The mRNA amount of cells transfected with empty vector alone or supplemented with DMSO were used as a control. The data were represented as folds of control.

Aldefluor assay. ALDH activity was detected using the Aldefluor assay kit (Stemcell Technologies, Vancouver, Canada) as described by the manufacturer. Briefly, cells were suspended in Aldefluor assay buffer containing ALDH substrate and BODIPY-aminoacetalddehyde (BAAA). The BAAA was taken up by living cells and converted by intracellular ALDH into BODIPY-aminoacetate, which yields bright fluoresce. The brightly fluorescent ALDH-expressing cells were detected with FACS Calibur or FACS Aria II (BD Biosciences, Franklin Lakes, NJ). As a negative control, cells were stained under identical conditions with the specific ALDH inhibitor, diethylaminobenzaldehyde (DEAB; Sigma). Data were analyzed by Cell Quest software (BD Biosciences).

Patients. Fifty-two patients with p-Stage IA lung adenocarcinoma who had undergone surgery at the Department of General Thoracic Surgery, Osaka University, between 1995 and 2003, were included in this study. No patients had chemotherapy or radiation therapy prior to surgery, and complete resection of tumors was performed. The clinical characteristics of the patients are shown in Table I. Survival data were available for all patients. The mean follow-up duration after surgery was 6.2±0.3 years. The study was approved by the ethics review board of the Graduate School of Medicine, Osaka University.

Immunohistochemical analysis. Histologic specimens were fixed in 10% formalin and routinely processed for paraffin-embedding. Paraffin-embedded specimens were stored in the dark room in the Department of Pathology of Osaka University Hospital at room temperature, and sectioned at 4 μm thickness at the time of staining. After antigen retrieval with Pascal pressurized heating chamber (Dako A/S, Glostrup, Denmark), the sections were incubated with anti-NR0B1 or anti-PPARγ antibody (Abcam), subjected to the treatment with ChemMate EnVision kit (Dako), DAB (Dako) was used as a chromogen. As a negative control, staining was carried out in the absence of primary antibody. Immunohistochemically stained sections were evaluated independently by two pathologists (Y.S. and E.M.) in a blinded manner, without any knowledge
Table I. Clinical characteristics of 52 cases of Stage IA lung adenocarcinoma.

<table>
<thead>
<tr>
<th></th>
<th>Number of patients</th>
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<tbody>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>20</td>
</tr>
<tr>
<td>Female</td>
<td>32</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma in situ</td>
<td>27</td>
</tr>
<tr>
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<td>6</td>
</tr>
<tr>
<td>Invasive adenocarcinoma</td>
<td>19</td>
</tr>
<tr>
<td>Recurrence</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>11</td>
</tr>
<tr>
<td>No</td>
<td>41</td>
</tr>
<tr>
<td>Prognosis</td>
<td></td>
</tr>
<tr>
<td>Deceased</td>
<td>6</td>
</tr>
<tr>
<td>Alive</td>
<td>46</td>
</tr>
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of the clinicopathological parameters or patient outcomes. Cases were categorized into three groups (score 0, score 1, and score 2) according to the extent of staining in each section. Cases without any staining were categorized as score 0, those with <10% positive cells among tumor were categorized as score 1 and those ≥10% were grouped as score 2.

Statistical analysis. Statistical analysis for experimental studies was performed with the Student's t-test. The values are shown as the mean ± standard error (SE) of at least three experiments. Statistical analysis for clinical samples was performed using JMP ver. 9.0.2 software (SAS Institute Inc., Cary, NC). Five-year overall survival (OS) and disease-free survival (DFS) were calculated by the Kaplan-Meier method, and the differences in survival curves were analyzed by the log-rank test.

Results

Inhibitory effect of NR0B1 on the transactivation ability of PPARγ in lung adenocarcinoma cell lines. To examine the effect of NR0B1 on the transactivation ability of PPARγ, the reporter plasmid containing luciferase gene under the control of PPARγ recognition elements (PPRE) was transfected to the A549 lung adenocarcinoma cell line. When the PPARγ expression plasmid was cotransfected, the luciferase activity increased approximately 3-fold (Fig. 1A). However, the coexpression of NR0B1 dose-dependently interfered with the increase of luciferase activity by PPARγ (Fig. 1A). The comparable results were obtained in PC9, another lung adenocarcinoma cell line (Fig. 1B).

Kim et al (36) demonstrated that PPARγ is physically bound to NR0B1 via its DNA binding and hinge domains. The domain of NR0B1 mediating the interaction with PPARγ has yet to be identified. NR0B1 possesses repeated LxxLL motifs in its N-terminal half, and interacts with various factors, such as estrogen receptor, via these LxxLL motifs. Then, the mutant NR0B1 lacking LxxLL motifs (del-NR0B1) was constructed (Fig. 2A). Flag-tagged wild-type NR0B1 or del-NR0B1 was coexpressed with HA-tagged PPARγ in HEK293T cells and the nuclear extract was analyzed. The immunoprecipitated product with anti-HA antibody contained Flag-tagged wild-type NR0B1, but it did not contain Flag-tagged del-NR0B1 (Fig. 2B). This indicated that the interaction of NR0B1 with PPARγ was mediated via its N-terminal domain containing LxxLL motifs.

Next, the wild-type NR0B1 or del-NR0B1 was cotransfected with PPARγ. In contrast to wild-type NR0B1, del-NR0B1 did not interfere with the PPARγ transactivation ability, indicating that the inhibitory effect of NR0B1 was mediated via its N-terminal domain containing LxxLL motifs.

Effect of NR0B1 and PPARγ on the expression of ALDH3A1. Tumorigenic potential is correlated with ALDH3A1 expression in lung adenocarcinoma (38). Therefore, the effect of NR0B1 and PPARγ on the expression of ALDH3A1 was examined. Tet-Express inducible system of NR0B1 was established in A549 cells (Fig. 3A). Without NR0B1, the overexpression of PPARγ decreased the ALDH3A1 expression level (Fig. 3B). The induced expression of NR0B1 increased the ALDH3A1 expression, but this increase interfered with the overexpression of PPARγ (Fig. 3B). These results indicated that the
Effect of NR0B1 on ALDH3A1 expression was inhibited by PPARγ. Moreover, the functional interaction between NR0B1 and PPARγ was examined with PPARγ inhibitor GW9662. When GW9662 was added, the expression level of ALDH3A1 increased. The induced expression of NR0B1 also increased ALDH3A1 expression. The induced NR0B1 expression and the treatment of GW9662 additively increased ALDH3A1 expression (Fig. 3C).

ALDH activity was measured with Aldefluor assay. ALDH activity was examined in the combination of NR0B1 induction and GW9662. Consistent with the result of ALDH3A1 expression, the proportion of ALDH-positive cells increased when NR0B1 was induced and when GW9662 was treated, respectively (Fig. 4A and B). The simultaneous induction of NR0B1 and treatment of GW9662 further increased the proportion of ALDH-positive cells (Fig. 4A and B).

Immunohistochemical findings in clinical specimens. Expression of NR0B1 and PPARγ was examined immunohistochemically in 52 clinical cases of lung adenocarcinoma. Cases were categorized into three groups (score 0, score 1, and score 2) according to the proportion of positive cells (Fig. 5A). In the NR0B1 score, 24 cases were categorized as score 0, 14 as score 1, and 14 as score 2. In the PPARγ
score, 21 cases were categorized as score 0, 23 as score 1, and 8 as score 3, respectively. Consistent with our previous report (25), the high NR0B1 score was correlated with poor prognosis in both DFS and OS (Fig. 5B, p=0.003 and 0.005, respectively). To evaluate the functional interaction between NR0B1 and PPARγ, the PPARγ score was subtracted from the NR0B1 score (NR0B1 score - PPARγ score), and the resultant value was referred to as ‘N-P score’. The low ‘N-P score’ corresponds to high PPARγ and low NR0B1 scores, whereas the high ‘N-P score’ to low PPARγ and high NR0B1 scores. Cases were again divided into two categories; cases with ≤0 ‘N-P score’ were categorized as ‘low N-P’, and cases with ≥1 ‘N-P score’ were categorized as ‘high N-P’ (Fig. 5C). The former included 39 cases, whereas the latter 13 cases. The ‘high N-P’ was correlated with poor prognosis in both DFS and OS (Fig. 5C).

Eleven out of the 14 cases with NR0B1 score 1 and 4 out of the 14 cases with NR0B1 score 2 were grouped as ‘low N-P’. The former 11 cases were alive (1 case with recurrence), and the latter 4 cases were alive without recurrence. These results indicated that high NR0B1 cases with favorable prognosis could be categorized as ‘low N-P’ group.

**Discussion**

NR0B1, an orphan nuclear receptor, is expressed in a side population of lung adenocarcinoma. We previously reported that the knockdown expression of NR0B1 reduced tumorigenic and anti-apoptotic potential in lung adenocarcinoma cell lines, and that the high expression of NR0B1 was a poor indicator of prognosis in clinical cases of lung adenocarcinoma. NR0B1 is known to form heterodimers with various factors, such as estrogen and progesterone receptors. NR0B1 has previously been reported to interact with PPARγ in adipose tissue (36).

In lung adenocarcinoma, PPARγ inhibits the growth of tumor cells and induces apoptosis (32-35) suggesting that NR0B1 and PPARγ might present opposite effects on tumor cells. In the present study, we examined the interaction between NR0B1 and PPARγ in lung adenocarcinoma.

The luciferase activity enhanced by PPARγ was reduced by the coexpression of NR0B1, indicating the inhibitory effect of NR0B1 on the transactivation ability of PPARγ. Immunoprecipitated experiments revealed that the N-terminal region of NR0B1 containing three LxxLL motifs mediated the physical interaction with PPARγ. Deletion of this region
abolished the inhibitory effect to PPARγ in luciferase assay. The N-terminal region of NR0B1 appeared to be essential for physical and functional interaction with PPARγ in lung adenocarcinoma. The N-terminal region is essential for the interaction of NR0B1 with various factors, such as estrogen receptors, and this was applicable to the interaction with PPARγ.

ALDH activity is correlated with tumorigenic potential in various types of tumors (38). Among 19 distinct isoforms, ALDH3A1 is responsible for ALDH activity of lung adenocarcinoma (38,39). The level of NR0B1 induced by Tet-Express was correlated with ALDH activity and ALDH3A1 expression levels. This was consistent with our previous report that NR0B1 is involved in the malignant potential of lung adenocarcinoma. In contrast to NR0B1, PPARγ was inversely correlated with ALDH activity and ALDH3A1 expression levels, which were reduced with the overexpression of PPARγ and increased with the addition of PPARγ inhibitor GW9662. The additive effect of NR0B1 and GW9662 indicated that PPARγ inhibited the tumorigenic potential of NR0B1 in lung adenocarcinoma. NR0B1 and PPARγ appeared to be antagonistic in malignant attitude.

We previously reported that the high NR0B1 score was a negative prognostic indicator, when all stages of lung adenocarcinoma were included. This was applicable even when only stage IA cases were included in the present study. Furthermore,
we compared the evaluation using two factors (NR0B1 and PPARγ scores) with that using a single factor (only NR0B1 score). When evaluated with two factors, 11 of the 14 NR0B1 score 1 cases and 4 of the 14 NR0B1 score 2 cases were categorized to the group with favorable prognosis. All of the re-categorized cases were alive, and most of them were free from recurrence. These results suggested that the evaluation of the two factors was a more accurate indicator than that of a single factor. NR0B1 and PPARγ may interact with each other also in clinical samples of lung adenocarcinoma.

Taken together, NR0B1 and PPARγ were antagonistic with each other in malignant attitude. The interaction was mediated through the N-terminal region of NR0B1. The high NR0B1 and low PPARγ expression was strictly correlated with poor prognosis of Stage IA lung adenocarcinoma.

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