Expression of the Steroid and Xenobiotic Receptor and Its Possible Target Gene, Organic Anion Transporting Polypeptide-A, in Human Breast Carcinoma

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Abstract
Steroid and xenobiotic receptor (SXR) or human pregnane X receptor (hPXR) has been shown to play an important role in the regulation of genes related to xenobiotic detoxification, such as cytochrome P450 3A4 and multidrug resistance gene 1. Cytochrome P450 enzymes, conjugation enzymes, and transporters are all considered to be involved in the resistance of breast carcinoma to chemotherapeutic or endocrine agents. However, the expression of SXR/hPXR proteins and that of its target genes and their biological or clinical significance have not been examined in human breast carcinomas. Therefore, we first examined SXR/hPXR expression in 60 breast carcinomas using immunohistochemistry and quantitative reverse transcription-PCR. We then searched for possible SXR/hPXR target genes using microarray analysis of carcinoma cells captured by laser microscissors. Tohoku University Graduate School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi-ken 980-8575, Japan. Phone: 81-22-717-8050; Fax: 81-22-717-8051; E-mail: hsasano@patholo2.med.tohoku.ac.jp.

Introduction
The steroid and xenobiotic receptor (SXR; ref. 1) was originally isolated as a potential homologue of the Xenopus laevis benzoate X receptor (2). This receptor is also called human pregnane X receptor (hPXR; ref. 3) or human pregnane-activated receptor (4). SXR, encoded by NR1H2 (Nuclear Receptor Nomenclature Homepage), was shown to be activated by various medications, including potent inducers of cytochrome P450 3A, such as rifampicin and clotrimazole (1, 3, 4). SXR was also reported to be present in adult human liver, small intestine, and large intestine (1, 3, 4). We also showed recently the presence of SXR in both adult lung and kidney (5). SXR is well known to positively regulate transcription of cytochrome P450 3A4 (CYP3A4) and multidrug resistance gene (MDR1; refs. 6, 7) and is considered to be a key regulator of xenobiotic metabolism (8–11). SXR was also activated by a wide range of natural and synthetic steroids, such as estradiol and tamoxifen (1, 12, 13). Maglich et al. (14) reported recently that SXR may regulate not only cytochrome P450 and transporters but also many genes involved in all phases of detoxification process, including oxidative metabolism, conjugation, and transport, which suggested a central role for SXR in detoxification.

CYP1A1, CYP1B1, CYP2C9, and CYP3A4 mRNA transcripts have all been detected in human breast carcinoma (15–17). In addition, both P450 side chain cleavage (CYP11A1) and P450 aromatase (CYP19) play important roles in some human breast cancers (18). Phase II metabolic enzymes, glutathione S-transferase, and multidrug resistance protein 1 are expressed in human breast cancers and in MCF-7 breast cancer cells (19–21). Therefore, various genes related to detoxification known to be regulated by SXR in other tissues may also be regulated by SXR in breast cancers. SXR mRNA expression was reported in normal human breast and carcinoma tissues (22). SXR and its target genes CYP3A4 and CYP3A7 were also detected in estrogen-dependent endometrial carcinoma using immunohistochemistry and/or reverse transription-PCR (RT-PCR; ref. 13). However, a detailed examination of expression of SXR and its target genes has not been reported in human breast carcinoma tissues.

In this study, we first examined the expression of SXR mRNA and protein using real-time RT-PCR and immunohistochemistry in 60 human breast carcinomas and analyzed their correlation with clinicopathologic findings. SXR immunolocalization was also studied in early phase of breast cancer, such as ductal carcinoma in situ (DCIS), and intraduct proliferative lesions, such as atypical ductal hyperplasia (ADH), to further characterize its biological and/or clinical significance. We then did microarray analysis to identify gene expression patterns that could be related to SXR expression in human breast carcinoma cells isolated and captured by laser microscissors. The microarray expression data were examined using cluster analysis with the aim of identifying possible SXR-related responsive genes, such as P450 enzymes, transferases, conjugation enzymes, and transporters.

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and transporters. mRNAs encoding the SXR target genes CYP3A4 and MDR1 were also further characterized in 60 cases of breast cancer. Following these studies, organic anion transporting polypeptide-A (OATP-A), SLC01A2 or SLC21A3, was identified as one of the genes whose expression was most closely correlated with that of SXR using microarray clustering analysis above. Therefore, OATP-A was further evaluated using immunohistochemical methods and real-time RT-PCR in the same specimens examined for immunohistochemistry for SXR in human breast cancer.

**Materials and Methods**

**Patients and tissue preparation.** Sixty specimens of invasive ductal carcinoma (IDC) of the breast were obtained from Japanese female patients who underwent mastectomy from 2000 to 2003 in the Department of Surgery, Tohoku University Hospital (Sendai, Japan). The mean age of the patients from whom specimens were obtained was 54.5 years (range, 32-78). Normal breast and adipose tissues adjacent to the carcinoma were also available for examination in 12 of 60 cases. None of the patients examined in this study received irradiation or chemotherapy before surgery. Relevant clinical data were retrieved from patient's charts. The histologic grade of each specimen was evaluated by three of the authors (T.S., T.M., and H.S.) based on the modified methods of Bloom and Richardson (23) and according to Elston and Ellis (24). Each six cases of DCIS and ADH were also obtained from patients from 1998 to 2002 in Tohoku University Hospital. Histopathologic diagnosis for DCIS and ADH were independently evaluated by three of the authors (B.S., T.M., and H.S.) based on the criteria of Dupont and Page (25) and Ottesen et al. (26), respectively. The number of cases corresponding to each grade of DCIS were as follows: grade I, n = 2; grade II, n = 1; and grade III, n = 3. The Committee on Ethics at the Tohoku University School of Medicine approved the research protocols, and informed consent was obtained from each patient before surgery.

**Immunohistochemistry.** Mouse monoclonal antibodies for SXR (PXRI) were kindly provided by Perseus Proteomics, Inc. (Tokyo, Japan). This antibody was produced by immunizing mice with a synthetic peptide corresponding to amino acids 1 to 40. Goat polyclonal antibody for OATP-A (C-15: sc-18428) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Other antibodies used in this study are as follows: monoclonal antibodies for estrogen receptor (ER) α (ER1D5), progesterone receptor (PR; MAB429), and Ki-67 (MIB1) were obtained from Immunotech S.A. (Marseille, France), Chemicon International, Inc. (Temecula, CA), and DakoCytomation Co. Ltd. (Kyoto, Japan), respectively. Rabbit polyclonal antibody for HER-2/neu (A0485) was purchased from DakoCytomation. The monoclonal antibody for steroid sulfatase (STS; KM1049; ref. 27, 28) was kindly provided by Kyowa Hakko Kogyo Co. Ltd. (Tokyo, Japan).

Tissue sections were immunostained by a biotin-streptavidin method with Histofine kit (Nichirei Co. Ltd., Tokyo, Japan). Experimental procedures employed in our present study have been described previously in detail (27, 28). Briefly, sections were deparaffinized with xylene and autoclaved at 120°C for 5 minutes in citric acid buffer or instant antigen retrieval H buffer (Mitsubishi Kagaku Iatron, Inc., Tokyo, Japan) for antigen retrieval. The dilutions of primary antibodies were as follows: SXR, 1:100; OATP-A, 1:50; ER, 1:2; PR, 1:150; Ki-67, 1:50; HER-2/neu, 1:200; and STS, 1:3200. The antigen-antibody complex was then visualized with 3,3'-diaminobenzidine, whereas OATP-A was detected with Vector Blue Alkaline Phosphatase Substrate Kit III (Vector Laboratories, Inc., Burlingame, CA) in Tris-HCl buffer (pH 8.2) in these double immunostainings.

To score SXR, ER, PR, and Ki-67, >500 carcinoma cells in each case were counted independently by the same three authors above (Y.M., T.S., and H.S.), and the percentage of immunoreactivity [i.e., labeling index (LI)] was determined. In the present study, interobserver differences were <5%, and the mean of the three values was obtained. The cases with 10% < ERs LI or PR LI were deemed ER- or PR-negative breast carcinomas according to the report by Allred et al. (30). In scoring OATP-A immunoreactivity, the carcinomas were tentatively classified into three groups (0, no immunoreactivity; 1, weak immunoreactivity; and 2, strong immunoreactivity). For scoring of HER-2/neu and STS, two groups were identified (0, no immunoreactivity; 1, positive carcinoma cells). Cases with discordant results among the observers were reevaluated by simultaneous examination using multiheaded light microscopy until concordant results were obtained.

**Real-time RT-PCR.** Total RNA was carefully extracted from 60 specimens of IDC and 12 specimens of adipose and normal tissues adjacent to the carcinoma using TRIzol (Invitrogen Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer’s instructions. The mRNA levels for all genes were measured using the SYBR Green Real-time PCR Detection System (Applied Biosystems, Foster City, CA). Primer sets of SXR, CYP3A4, MDR1, OATP-A, aromatase, and ribosomal protein L13A (RPL13A) used in this study were described previously (5, 28, 29, 31). Primer sets of ATP-binding cassette F subfamily 3 (ABCd3), hormone-sensitive lipase (LIPE), sulfotransferase A1A3 (SULT1A3), coactivator-associated arginine methyltransferase 1 (CARM1), and galactose-3-sulfotransferase 3 (GAL3ST3) were designed using OLGIO Primer Analysis Software (Takara Bio Inc., Shiga, Japan).

**Real-time PCR.** Total RNA was extracted using the LightCycler System with software version 3.3.5 and FastStart DNA Master SYBR Green 1 (Roche Diagnostics GmbH, Mannheim, Germany). The PCR primer sequences of SXR, CYP3A4, MDR1, OATP-A, aromatase, and ribosomal protein L13A (RPL13A) used in this study were obtained from previously published studies (5, 28, 29, 31). Primer sets of ABCF3, aromatase (CYP19), RPL13A, and Ki-67 were designed using the primer set design software Primer Express (Applied Biosystems, Foster City, CA). The PCR primer sequences of SXR, CYP3A4, MDR1, OATP-A, aromatase, and ribosomal protein L13A (RPL13A) used in this study were evaluated as a ratio (%) compared with that of each positive control (27, 28, 31). Negative control experiments were done without cDNA substrate to examine the presence of exogenous contaminant DNA.

**Microarray analysis.** Twenty-three of 60 breast carcinomas were rapidly embedded in Tissue-Tek optimal cutting temperature compound (Sakura Finetechnical Co. Ltd., Tokyo, Japan) and frozen sectioned at a thickness of 8 μm. Approximately 5000 cells were laser transferred from the carcinoma cells and the intratumoral stromal cells. Each population of the cells was estimated to be >98% homogeneous as determined by microscopic visualization of the captured cells. Total RNA was extracted using an RNA microisolation protocol described by Niiro et al. (32). Protocols for quantitative RT-PCR were described above. Each primer set for PCR analysis is described in Table 1A. Internal controls were both RPL13A and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; ref. 27). For LCM/microarray analysis, after initial recovery and resuspension of the RNA pellet, a DNase digestion was done for 2 hours at 37°C using 10 units DNase (GenHunter, Nashville, TN) in the presence of 10 units RNase inhibitor (Invitrogen Life Technologies) followed by extraction and precipitation. The pellet was resuspended in 27 μl of RNAase-free H2O and used for high-density cDNA array analysis.

**Microarray analysis.** Total RNA was extracted from ~500 carcinoma cells prepared by LCM described above. Sample preparation and processing were done essentially as described in the Affymetrix GeneChip Expression Analysis Manual (Affymetrix, Inc., Santa Clara, CA), with the exception that the labeled cRNA samples were hybridized to the complete human U133 GeneChip set (Affymetrix), including 22,215 and 22,577 genes. Relative levels of gene expression were calculated by global normalization. All gene expression
data were clustered and results were visualized using GeneSpring version 7.2 (Silicon Genetics, Redwood City, CA).

In this study, we also focused on three categories, including P450 enzymes, transferases, and transporters. Genes (1,011 of 44,792) were selected from gene expression profiling for further analysis by reference to three Web databases: the Cytochrome P450 Homepage, Enzyme Nomenclature (33), and Human Membrane Transporter Database (34). Each case of breast carcinoma was ordered according to the level of SXR gene expression inferred by microarray and clustering analysis between each gene was done. Data from the three categories and SXR were subjected to hierarchical clustering analysis and visualization using the Cluster and TreeView programs (Stanford University) to generate tree structures.

### Table 1.

**A. Correlation between SXR and clinicopathologic variables in 60 breast carcinomas**

<table>
<thead>
<tr>
<th>Variable</th>
<th>n or range (mean ± SD)</th>
<th>SXR LI (mean ± SD)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>32-78 (54.5 ± 11.4)</td>
<td>0.20 (r = 0.15)</td>
<td></td>
</tr>
<tr>
<td>Menopausal status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Premenopausal</td>
<td>20</td>
<td>51.6 ± 28.9</td>
<td>0.32</td>
</tr>
<tr>
<td>Postmenopausal</td>
<td>40</td>
<td>42.8 ± 32.7</td>
<td></td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td>1.0-6.0 (3.3 ± 1.4)</td>
<td>0.57 (r = 0.10)</td>
<td></td>
</tr>
<tr>
<td>Lymph node status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>35</td>
<td>57.8 ± 29.0</td>
<td>0.01*</td>
</tr>
<tr>
<td>Negative</td>
<td>25</td>
<td>36.1 ± 30.1</td>
<td></td>
</tr>
<tr>
<td>Histologic grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>16</td>
<td>15.3 ± 18.7</td>
<td>0.008*  (II vs I)</td>
</tr>
<tr>
<td>II</td>
<td>26</td>
<td>49.0 ± 30.9</td>
<td>0.001*  (III vs I)</td>
</tr>
<tr>
<td>III</td>
<td>18</td>
<td>58.3 ± 30.5</td>
<td>0.52    (II vs III)</td>
</tr>
<tr>
<td>HER-2/neu immunoreactivity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>23</td>
<td>44.8 ± 30.7</td>
<td>0.87</td>
</tr>
<tr>
<td>Negative</td>
<td>37</td>
<td>46.2 ± 32.4</td>
<td></td>
</tr>
<tr>
<td>ER status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>43</td>
<td>41.9 ± 31.5</td>
<td>0.1</td>
</tr>
<tr>
<td>–</td>
<td>17</td>
<td>50.1 ± 28.0</td>
<td></td>
</tr>
<tr>
<td>PR status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>41</td>
<td>44.9 ± 31.6</td>
<td>0.68</td>
</tr>
<tr>
<td>–</td>
<td>19</td>
<td>41.0 ± 28.5</td>
<td></td>
</tr>
<tr>
<td>Ki-67 LI (24.6 ± 21.5)</td>
<td>3-93</td>
<td>0.10 (r = 0.24)</td>
<td></td>
</tr>
</tbody>
</table>

**B. Correlation between SXR and clinicopathologic variables in 43 ER-positive breast carcinomas cases**

<table>
<thead>
<tr>
<th>Variable</th>
<th>n or range (mean ± SD)</th>
<th>SXR LI (mean ± SD)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymph node status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>26</td>
<td>59.8 ± 27.8</td>
<td>0.005*</td>
</tr>
<tr>
<td>Negative</td>
<td>17</td>
<td>31.4 ± 29.2</td>
<td></td>
</tr>
<tr>
<td>Histologic grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>16</td>
<td>12.8 ± 17.7</td>
<td>0.003*  (II vs I)</td>
</tr>
<tr>
<td>II</td>
<td>19</td>
<td>48.7 ± 29.8</td>
<td>0.001*  (III vs I)</td>
</tr>
<tr>
<td>III</td>
<td>8</td>
<td>62.6 ± 32.6</td>
<td>0.24    (II vs III)</td>
</tr>
<tr>
<td>Ki-67 LI (18.4 ± 15.9)</td>
<td>3-62</td>
<td>0.017* (r = 0.49)</td>
<td></td>
</tr>
<tr>
<td>STS immunoreactivity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>13</td>
<td>32.3 ± 31.3</td>
<td>0.32    (1 vs 0)</td>
</tr>
<tr>
<td>1</td>
<td>28</td>
<td>42.6 ± 27.0</td>
<td>0.01*   (2 vs 0)</td>
</tr>
<tr>
<td>2</td>
<td>19</td>
<td>60.3 ± 33.9</td>
<td>0.05    (1 vs 2)</td>
</tr>
<tr>
<td>Aromatase mRNA expression †</td>
<td>0.0-14.9 (2.55 ± 1.60)</td>
<td>0.001* (r = 0.51)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variable</th>
<th>n or range (mean ± SD)</th>
<th>SXR LI (mean ± SD)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>OATP-A immunoreactivity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>13</td>
<td>25.4 ± 27.6</td>
<td>0.01*   (1 vs 0)</td>
</tr>
<tr>
<td>1</td>
<td>20</td>
<td>55.3 ± 30.8</td>
<td>0.009*  (2 vs 0)</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>74.0 ± 6.9</td>
<td>0.32    (1 vs 2)</td>
</tr>
</tbody>
</table>

*Significant difference.
†Correlation with SXR mRNA evaluated by quantitative RT-PCR.
University (Sendai, Japan). Human breast carcinoma cell lines MDA-MB-231, and MDA-MB-468 were purchased from American Type Culture Collection (Manassas, VA). The cell lines were maintained in RPMI 1640 (Sigma-Aldrich Co., St. Louis, MO) for MCF-7, T-47D, and ZR-75-1 or Leibovitz’s L-15 medium (Invitrogen Life Technologies) for MDA-MB-231 and MDA-MB-468 supplemented with 10% fetal bovine serum (FBS; JRH Biosciences Co., St. Lenexa, KS). The cells were harvested in phenol red– and FBS-free medium and plated on 100-mm cell culture dishes at an initial concentration of 3 × 10⁴ cells/mL. The test materials, rifampicin (Sigma-Aldrich), were dissolved in DMSO (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and serially diluted. To examine the effects of test materials on gene transcription and/or protein translation, cells were treated with test materials, 10⁻⁶ mol/L actinomycin D and 10⁻⁶ mol/L cycloheximide (Wako Pure Chemical Industries), respectively. Final DMSO concentrations never exceeded 0.05%. Different concentrations of test compounds were added, and the assay was terminated after 2, 4, 8, 12, 24, and 48 hours by removing the medium from wells and extracting total RNA using the TRIzol method.

**Statistical analysis.** Statistical analysis was done using the StatView 5.0 j software (SAS Institute, Inc., Cary, NC). Values for patient’s age, tumor size, SXR LI, ER LI, PR LI, Ki-67 LI, and mRNA levels for CYP3A4, MDR1, aromatase, and OATP-A were summarized as mean ± SD. Simple regression analysis was employed to assess the correlations between SXR and CYP3A4, MDR1, or OATP-A mRNA expression levels. Association between the degree of mRNA expression of SXR and these variables of each individual case was evaluated using one-way ANOVA and the Bonferroni test. Statistical differences between SXR mRNA expression and ER status, PR status, menopausal status, stage, lymph node status, histologic grade, and HER-2/neu immunoreactivity were evaluated in a cross-table using the χ² test.

**Results**

**Immunohistochemistry and quantitative RT-PCR for SXR.** SXR immunoreactivity was detected in the nuclei of carcinoma cells (Fig. 1A). The range and mean value of SXR LI in 60 breast carcinomas were 0 to 98% and 46.2% (SD, 31.5; 0% = 13 cases), respectively. No SXR immunoreactivity was detected in the nuclei or cytoplasm of stromal cells or in nonneoplastic glandular epithelia and adipocyte adjacent to the carcinoma. No SXR immunoreactivity was detected in all six ADH cases examined. Two of six DCIS cases showed SXR immunoreactivity (SXR LI under 15%). Histologic grade of these SXR-positive DCIS cases corresponded to grade III. SXR immunoreactivity was detected in positive controls [i.e., the nuclei of epithelial cells from human small intestine (data not shown)]. Association between SXR LI and clinicopathologic variables in 60 breast carcinomas are summarized in Table 1A. SXR LI was positively correlated with lymph node status (P = 0.01), histologic grade (II versus I, P = 0.008; III versus I, P = 0.001), and STS immunoreactivity (grade 2 versus 0, P = 0.01). No significant association was detected between SXR LI and patient age, menopausal status, tumor size, ER status, PR status, HER-2/neu status, and Ki-67 LI in this study. SXR mRNA expression levels were positively correlated with aromatase mRNA expression (P = 0.001) in all 60 breast carcinomas. We did further statistical analyses summarized in Table 1B regarding the correlation between SXR LI and clinicopathologic variables in ER-positive cases (43 cases) of the total 60 breast carcinomas (ER⁺/−+) to further examine the possible correlation between SXR and ER status in human breast carcinoma. In ER-positive breast carcinoma cases, SXR LI was more significantly correlated with lymph node status (P = 0.005) and histologic grade (I versus II, P = 0.003; I versus III, P = 0.001) than in ER⁻/− breast carcinomas. In addition, in ER-positive breast carcinomas, SXR LI was positively correlated with the status of proliferation of carcinoma cells determined by Ki-67 LI

**Cell lines and culture conditions.** Human breast carcinoma cell lines MCF-7, T-47D, and ZR-75-1 were provided from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku
SXR mRNA levels ranged from 0 to 54.0% of the positive controls with an average of 9.6 ± 12.0%. No expression was detected in 9 of 60 cases. Expression of SXR mRNA was closely correlated with SXR LI in 60 breast carcinomas (data not shown).

In 11 of 60 (18.3%) breast carcinomas, SXR immunoreactivity was detected in both nucleus and cytoplasm of carcinoma cells (Fig. 1A). There were no correlations between SXR immunoreactivity in nuclei and cytoplasm. No significant associations were detected between cyttoplasmic SXR immunoreactivity in carcinoma cells and clinicopathologic variables, mRNA transcripts described above, or OATP-A mRNA/protein. Cyttoplasmic SXR immunoreactivity was also detected in two SXR-positive DCIS cases.

Microarray analysis evaluated by hierarchical clustering. The genes whose expression clustered together with SXR (NR1I2) in the hierarchical clustering analysis were as follows (Fig. 2A): ABCF3, LIPE, OATP-A (SLCO1A2), and three expressed sequence tags (EST; accession nos. AI701949, BF508925, and BE349017). We then analyzed the clustering patterns of gene expression profiles of three categories: P450 enzymes, transferases, and transporters. Focused clustering analysis subclassified 1,011 genes into eight well-defined expression profiles or groups (Fig. 2B). The genes that were closely associated with SXR in the group d were as follows (Fig. 2C): OATP-A, SULT1A3, CARM1, and GAL3ST3. The expression of SXR was closely correlated with OATP-A in 23 breast carcinomas (Fig. 2C). The known SXR target genes CYP3A4 and MDR1 (ABCB1) were detected in other clusters (groups c and g, respectively) and were weakly correlated with SXR expression (Fig. 2C).

SXR, OATP-A, and MDR1 but not CYP3A4 expression levels as determined by quantitative RT-PCR analysis were significantly correlated with results of microarray in 23 breast carcinomas (data not shown). There were no significant associations between SXR expression levels obtained from microarray analysis and clinicopathologic variables in the 23 cases examined.

Distributions of SXR, CYP3A4, MDR1, and OATP-A mRNA transcripts in breast tissues. SXR, MDR1, and OATP-A mRNAs were detected exclusively in carcinoma cells, whereas CYP3A4 mRNA was detected in both carcinoma and stromal cells as shown by combined LCM and RT-PCR analysis (Fig. 1B). CYP3A4 and MDR1 mRNA expression was detected in both nonneoplastic breast and adipose tissues adjacent to carcinoma (Fig. 1C). SXR and OATP-A mRNA expression was absent from all nonneoplastic breast tissues and adipose tissues (Fig. 1C).

Correlations between SXR mRNA and CYP3A4, MDR1, and OATP-A mRNA transcripts. Correlations between SXR mRNA and CYP3A4, MDR1, and OATP-A mRNA levels were summarized in Table 2. A statistically significant positive correlation was detected between SXR and CYP3A4 (P = 0.030) or OATP-A (P = 0.004) but not between SXR and MDR1 (P = 0.080) expression. The same correlation was obtained in 37 cases in which specimens for microarray were not available (SXR versus OATP-A; P = 0.009, r = 0.48). A significant positive correlation was detected between SXR and OATP-A mRNA expressions but not between SXR and CYP3A4 mRNA expression in 12 human breast carcinoma cells (Table 2) isolated by LCM.

Regulation assay using breast carcinoma cell lines. SXR mRNA was detected in breast carcinoma cell lines examined in this study, with an exception of MDA-MB-468 (Fig. 3A). High levels of OATP-A mRNA were detected in ZR-75-1 and T-47D breast...
carcinoma cell lines (Fig. 3B). Rifampicin treatment elicited a
dose-dependent increase in OATP-A mRNA levels in both T-47D
(data not shown) and ZR-75-1 (Fig. 3C). OATP-A mRNA levels
induced by 10^{-5} mol/L rifampicin increased significantly and
peaked following 8 (T-47D) or 12 (ZR-75-1) hours of treatment
(Fig. 3E). Treatment with 10^{-5} mol/L actinomycin D significantly
decreased OATP-A mRNA induction by rifampicin, whereas
treatment with 10^{-5} mol/L cycloheximide did not (Fig. 3D).
ABCF3, LIPE, SULT1A3, GAL3ST3, CYP3A4, and MDR1 mRNAs
were detected in T-47D. ABCF3, SULT1A3, and GAL3ST3 mRNAs
were detected in ZR-75-1. CARM1, LIPE, CYP3A4, and MDR1
mRNAs were detected in MCF-7. LIPE (48 hours in T-47D),
SULT1A3 (24 hours in MCF-7 and T47D and 48 hours in T-47D),
and GAL3ST3 (24 hours in T-47D) mRNA levels were significantly
induced by treatment of 10^{-5} mol/L rifampicin (Fig. 3F).

Treatment with 10^{-5} mol/L actinomycin D could not inhibit
all these findings above (data not shown). ABCF3 (data not
shown), CARM1 (data not shown), CYP3A4 (Fig. 3G), and MDR1
(Fig. 3G) mRNA levels were not significantly induced by the
treatment of 10^{-5} mol/L rifampicin.

Immunohistochemistry of OATP-A. OATP-A immunoreactivity
was detected in the cell membrane and/or cytoplasm of breast
carcinoma cells (Fig. 1A) but not in nonneoplastic epithelial
cells, stroma, or adipocytes adjacent to the carcinoma. Double
immunohistochemistry revealed coexpression of SXR and OATP-A
in carcinoma cells (Fig. 1A). The numbers of cases with positive
OATP-A immunoreactivity in each groups of 60 breast carcinomas
were summarized in Table 3. Expression of OATP-A mRNA was
closely correlated with OATP-A immunoreactivity (data not shown).
There was a significantly positive association between OATP-A
immunoreactivity and SXR immunoreactivity evaluated as SXR LI
(Table 3). A significantly positive association between OATP-A
immunoreactivity and SXR LI was also detected in 37 breast
carcinomas in which the specimens for microarray analyses were
not available (Table 3).

Discussion

Dotzlaw et al. (22) showed the presence of SXR (hPXR) mRNA
and its variant mRNA, termed hPXR2, in both human breast tumor
biopsy specimens and the matched adjacent nonneoplastic breast

Table 2. Correlations between SXR mRNA and CYP3A4,
MDR1, and OATP-A mRNA levels in breast carcinoma

<table>
<thead>
<tr>
<th></th>
<th>Mean ± SD (%)</th>
<th>60 cases (whole breast carcinoma tissues)</th>
<th>12 cases (breast carcinoma cells isolated by LCM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P  r</td>
<td>60 cases</td>
<td>12 cases</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>5.7 ± 6.5</td>
<td>0.030* 0.30</td>
<td>0.34 0.30</td>
</tr>
<tr>
<td>MDR1</td>
<td>7.6 ± 9.8</td>
<td>0.080 0.25</td>
<td>NE  NE</td>
</tr>
<tr>
<td>OATP-A</td>
<td>3.6 ± 4.1</td>
<td>0.004* 0.61</td>
<td>0.007* 0.73</td>
</tr>
</tbody>
</table>

Abbreviation: NE, not examined.
*Significantly different.

Figure 3. A, SXR mRNA levels in breast carcinoma cell lines. B, OATP-A mRNA levels in breast carcinoma cell lines. M, MCF-7; T, T-47D; Z, ZR-75-1; 231, MDA-MB-231; 468, MDA-MB-468. C, rifampicin (RIF) increased OATP-A mRNA in a dose-dependent (10^{-7}-10^{-5} mol/L) manner in ZR-75-1. *, P < 0.05 versus vehicle control (C); †, P < 0.05 versus rifampicin. D, rifampicin (10^{-5} mol/L) induction of SXR mRNA in ZR-75-1. *, P < 0.05 versus vehicle control (C); †, P < 0.05 versus rifampicin. E, OATP-A mRNA levels at various times after rifampicin treatment in T-47D and ZR-75-1. The levels of OATP-A mRNA transcript induced by rifampicin (10^{-5} mol/L) peaked in after 8 hours (T-47D; †) to 12 hours (ZR-75-1; †). *, P < 0.05 versus vehicle control (0 hour). F, summary of mRNA levels clustered with SXR after rifampicin (10^{-5} mol/L) treatment in breast cancer cell lines (T-47D is shown), LIPE (48 hours; C, solid line), SULT1A3 (24 and 48 hours, C, dashed line), and GAL3ST3 (24 hours; C, mRNA levels (T-47D is shown)), †, P < 0.05 versus vehicle control (0 hour). G, there were no significant changes in both CYP3A4 (C) and MDR1 (C) mRNA levels (T-47D is shown). †, P < 0.05 versus vehicle control (0 hour). Representative of three separate experiments. Columns, mean; bars, SD (A-D). Points, mean; bars, SD (E-G).
tissues. However, we did not detect either SXR mRNA or protein in normal human breast tissues. We therefore previously reported that the obligate SXR heterodimeric partner, retinoid X receptor, was only very weakly expressed in normal breast cells (36, 37). SXR was also detected in hormone-dependent endometrial carcinoma cell but not in normal endometrium (13). Results from this study and previously reported studies above all suggest that SXR is predominantly expressed in carcinoma cells but not in nonneoplastic cells. It is unknown whether variant forms of SXR expressed in different human tissues were functional (11). Therefore, we focused on the major functional form of SXR in this study of human breast cancer. Kawana et al. (38) showed that SXR is localized in the cytoplasm of mouse liver and is translocated into the nuclei after administration of its ligands. Therefore, SXR present in the cytoplasm is considered to play a less functional role than nuclear SXR, but cytoplasmic SXR was not correlated with any of the factors examined, including nuclear SXR in our present study. Therefore, further investigation will be required to clarify its biological clinical significance in human breast cancer.

The results of our present study all suggest that at least the direct correlations between SXR and its well-known target genes, such as CYP3A4 or MDRI, were relatively low in human breast carcinoma or parenchymal cells. Our results also suggest that OATP-A might be a primary SXR response gene in human breast carcinoma cells. Expression of mouse OATP-2 mRNA (SLC21A6) was up-regulated by lithocholic acid through a PXR-dependent mechanism in mouse liver (39). In our LCM/microarray analysis, other forms of OATP, such as OATP-B, OATP-C, and OATP-8, were clustered in groups other than OATP-A (data not shown). The patterns of genes induced by SXR activation are considered to depend on multiple factors, such as ligands, tissues, and cell types (40–42). Further investigation, such as the analysis of SXR target genes using other procedures and cell types, are required for clarification, but we showed possible target gene of SXR in human breast carcinoma in this study. OATP-A was reported to be expressed in various human tissues, including liver, brain, kidney, lung, and testis (43), and has been implicated in the transport of bile acids (44), bromosulphthalein (43), and estrone (E1) sulfate (E1S; ref 45) in these tissues. Expression of OATP-A was also reported in the human breast carcinoma cell line ZR-75-1 (29). OATP-A is also considered to be involved in uptake of E1S in breast carcinoma cells. E1S is a major circulating plasma estrogen that is converted into the biological active estrogen, E1, by STS. SXR LI was positively correlated with STS immunoreactivity. This finding suggests that E1 may be transported into carcinoma cells by OATP-A and transformed into E1 by STS. These findings also suggest that SXR may therefore play an important and unexpected role in the up-regulation of in situ estrogen actions in breast carcinoma cells rather than its generally accepted roles, such as promoting the elimination of steroids and xenobiotic compounds. However, there were various OATPs involved in uptake of metabolic estrogens, such as sulfate and/or glucuronide forms in human tissues (46). In addition, the potential contributions of OATP-A to overall amounts of intratumoral estrogen remain unclear. Therefore, further examination will be necessary to clarify the biological significance of OATP-A in human breast cancer.

We also detected a significant positive correlation between SXR and steroid synthesis enzyme, P450 aromatase. In addition, LIPE may also be considered to be an up-regulator for in situ steroid hormone levels (47, 48) in breast carcinoma cells. These findings above all suggest that SXR may serve as a hormone sensitivity factor for human breast carcinoma cells. In ER-positive breast carcinomas cases examined in our present study, SXR LI was significantly correlated with lymph node status, histologic grade, and proliferation marker of carcinoma cells, Ki-67 LI. Therefore, high SXR LI in ER-positive breast carcinoma cases may represent aggressive biological behavior through estrogen production induced by SXR system. In addition, SXR LI in the possible cascade of breast carcinoma development determined by our present study was summarized as follows: IDC (grade III > II > I) > DCIS > ADH = normal gland. Therefore, these results above all indicate that SXR expression may be correlated with progression or differentiation of breast carcinoma tissues.

The SXR pathway is involved in the resistance of chemotherapy or endocrine therapy using paclitaxel (8) or tamoxifen (12). Therefore, some investigators have proposed SXR status as predictor for chemotherapy or endocrine therapy. However, it is also true that SXR activity could be affected by many other drugs (11), vitamins (42, 49), hormones (1, 13), and endocrine disruptors (50). All the patients examined in our present study received neither chemotherapy nor endocrine therapy before surgery. However, due to the nature of this study, it is nearly impossible to determine how much substrates, such as drugs, hormones, and diet, had been consumed by patients in their past. Therefore, further investigations using pathologic specimens form post-therapeutic or chemotherapy/endocrine therapy resistance patients may contribute to elucidation of possible correlation between ligand-SXR and SXR-target genes in breast carcinoma.

### Table 3. Correlations between SXR LI and OATP-A immunoreactivity in breast carcinoma tissues

<table>
<thead>
<tr>
<th>OATP-A immunoreactivity</th>
<th>n</th>
<th>SXR LI* (mean ± SD)</th>
<th>n</th>
<th>SXR LI† (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade 0</td>
<td>19</td>
<td>27.1 ± 29.7</td>
<td>12</td>
<td>24.5 ± 22.0</td>
</tr>
<tr>
<td>Grade 1</td>
<td>23</td>
<td>52.0 ± 31.7*</td>
<td>15</td>
<td>60.2 ± 30.5†</td>
</tr>
<tr>
<td>Grade 2</td>
<td>18</td>
<td>75.9 ± 11.7†</td>
<td>10</td>
<td>61.6 ± 23.7†</td>
</tr>
</tbody>
</table>

*60 cases of breast carcinoma.
†37 cases in which specimens for microarray were not available.
*Significantly difference versus grade 0.
†Significantly difference versus grade 1.

Acknowledgments

Received 3/30/2005; revised 9/13/2005; accepted 10/14/2005.

Grant support: Health and Labor Sciences Research Grants on Risk of Chemical Substances (H16-Kagaku-002), Ministry of Health, Labor, and Welfare, Japan grant-in-aid. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. Shin-ichi Hayashi (Division of Molecular Medical Technology), Dr. Tohru Furukawa (Division of Molecular Pathology), Dr. Tohru Onogawa (Division of Gastroenterological Surgery), and Dr. Takaaki Abe (Division of Nephrology, Endocrinology, and Vascular Medicine; all from the Tohoku University School of Medicine) for critical comments and Chika Tazawa, Megumi Matsui, and Katsuhiko Ono (Department of Pathology, Tohoku University School of Medicine) for skillful technical assistance.
References


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