Gpx2 Is an Overexpressed Gene in Rat Breast Cancers Induced by Three Different Chemical Carcinogens

Aya Naiki-Ito,1 Makoto Asamoto,1 Naomi Hokaiwado,1 Satoru Takahashi,1 Hiroko Yamashita,2 Hiroyuki Tsuda,2 Kumiko Ogawa,2 and Tomoyuki Shirai1

1Departments of Experimental Pathology and Tumor Biology, 2Oncology and Endocrinology, and 3Molecular Toxicology, Nagoya City University Graduate School of Medical Sciences, Mizuho-cho, Mizuho-ku, Nagoya, Japan

Abstract
Gene expression alterations are essential for the process of carcinogenesis. A carcinogen may have specific mechanisms for inducing tumors, which may involve inducing characteristic gene expression alterations. In this study, we attempted to identify genes crucial for mammmary carcinogenesis. For this purpose, we used human c-Ha-ras proto-oncogene transgenic rats (Hras128), which are highly sensitive to mammary carcinogens including N-methyl-N-nitrosourea, 7,12-dimethyl benz[a]anthracene, and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine. DNA microarray analysis revealed that glutathione peroxidase 2 (Gpx2) was commonly up-regulated in the mammary carcinomas induced by the three different carcinogens, and its up-regulation was confirmed by quantitative reverse transcriptase-PCR and Western blotting analysis. In addition, expression of Gpx2 was recognized in all 41 immunohistochemically examined cases of human breast cancer. Forced suppression of Gpx2 expression by siRNA resulted in significant growth inhibition in both rat and human mammary carcinoma cell lines with wild-type p53 cells. Thus, these data suggested that Gpx2 may be involved in mammmary carcinogenesis and cell proliferation in both rats and humans, indicating that Gpx2 may be a novel target for the prevention and therapy of breast cancer. [Cancer Res 2007;67(23):11353–8]

Introduction
Breast cancer is the most frequent type of cancer in women and, after lung cancer, represents the second leading cause of cancer death (1). Therefore, to reduce the incidence of death from this cancer among women, it is important to establish new approaches to its prevention and treatment.

Many genes have been reported to be involved in the mammary carcinogenic process. For instance, the receptor tyrosine kinase HER2 (Neu/ErbB2) is overexpressed in ~20% to 30% of primary breast cancers, and these patients have a poor prognosis (2, 3). The identification of overexpressed genes in the majority of breast cancer cases might lead to good candidates as target molecules for the prevention and therapy of the disease.

We have hypothesized that common gene expression changes in mammmary cancers, induced by different carcinogens, are critical for rat mammary carcinogenesis. Moreover, such common genes might also be involved in the development of human breast cancer.

Materials and Methods
Production of transgenic rats. Female Hras128 rats were produced by mating transgenic male with nontransgenic female Sprague-Dawley animals (Clea Japan, Inc.; ref. 4). Rats were maintained in plastic cages on hardwood chips in an air-conditioned, specific pathogen-free animal room at 22 ± 2°C and 50% humidity with 12:12 h light/dark cycle. All animal experiments were done under protocols approved by the Institutional Animal Care and Use Committee of Nagoya City University School of Medical Sciences.

Experimental protocols. The 7-week-old Hras128 transgenic rats were treated with MNU (a single i.p. injection of 50 mg/kg, body weight), DMBA (a single intragastric administration of 50 mg/kg of body weight), and PhIP (intragastric administration of 100 mg/kg of body weight, twice a week for 4 weeks). These chemicals were obtained from WAKO. Each carcinogen was given to four transgenic rats. Four nontreated transgenic rats and four littermate nontransgenic rats served as the controls. Rats were sacrificed at 14 weeks for sampling of the mammary cancers of MNU-treated transgenic rats and the normal mammary tissues of nontreated animals, and at 23 weeks for the transgenic rats treated with DMBA and PhIP. Mammary cancers were induced in Hras128 as described in Table 1.

Human breast cancer samples. From 2002 to 2003, breast cancer samples were collected from 41 female patients (32–79 years old) undergoing surgery for their disease at Nagoya City University Hospital; all patients had given their prior informed consent. Histologic grade of the breast cancer was assessed according to Elston and Ellis method (7).

Immunohistochemistry. Deparaffinized slide sections of rat and human mammary tissues were fixed with acetone (rat) or 10% formalin (human) and then incubated with 1:100 diluted Gpx2 (IMGENEX) antibody. Antibody binding was visualized by a conventional immunostaining method using an autoimmunostaining apparatus (Ventana HX System, Ventana Japan). For intensity of Gpx2 cytoplasmic immunoreactivity, clinical breast cancers were classified as weak (+), moderate (2+), or strong (3+) in each histologic grade.
Quantitative RT-PCR. One microgram of RNA was converted to cDNA with avian myoblastosis virus reverse transcriptase (Takara) in a 20-μL reaction mixture. Aliquots of 2 μL of cDNA samples were subjected to quantitative PCR in 20 μL using SYBR Premix ExTaq (Takara) in a LightCycler apparatus (Roche Diagnostics). The primers used were 5′-GACACGAGGAAACCGAAGCA-3′ and 5′-GGCCCTTCACAACGTCT-3′ for Gpx2 (rat), 5′-CCAGGACCTTGAGATTGAAT-3′ and 5′-GTGTCACGACG-CAGTTA-3′ for cytokeratin 19, 5′-GCCTCCTTAAAGTTGCCATA-3′ and 5′-GCCAGAGCTTACCCA-3′ for GPX2 (human), and 5′-GCATCCTGCAC-CACCAACTG-3′ and 5′-GCCTGCTTCACCACCTTCTT-3′ for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Initial denaturation was at 95°C for 60 s, followed by 40 cycles with denaturation at 95°C for 5 s, annealing at 55°C for 15 s, and elongation at 72°C for 30 s. Cytokeratin-19 mRNA levels were used to normalize for the sample cDNA content of rat tissues and cell lines and GAPDH mRNA levels for the human cell line samples.

Western blotting. A total of 20 μg protein per lane were separated on 12% acrylamide gels and electroblotted onto nitrocellulose membranes (Hybond-ECL, GE Healthcare UK Ltd.). Gpx2 expression levels were assessed with the same antibody used for immunohistochemical staining. β-Actin expression was evaluated to confirm equal amount of protein loadings by monoclonal anti–β-actin, AC-74 (Sigma-Aldrich Corp.).

Cell culture. The rat mammary carcinoma cell lines (C1, C2, C3, C6, C11, and C17), established from a DMBA-induced mammary carcinoma in Hras128 rats (8), and the human mammary carcinoma cell lines MCF-7, T47D, and MDA-MB-231 were maintained in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% FCS. The human mammary epithelial cell line (HMEC) was obtained from Lonza Walkersville, Inc. All cell cultures were maintained under 5% CO2/95% air at 37°C in a humidified incubator.

RNA interference and cell counts. Stealth Select RNAi targeting rat Gpx2, human GPX2, and control sequences were obtained from Invitrogen. Cells (5 × 10^4) from each of the two rat breast cancer cell lines, C2 and C11, were seeded in six-well plates and cultured for 24 h. They were then transfected with 100 pmol/well of siRNA using LipofectAMINE RNAiMAX (Invitrogen) at 10% cell confluence. Cell numbers were counted after 2 and 4 days. For the two human mammary carcinoma cell lines (MCF-7 and T47D), after preparing 100 pmol/well of siRNA samples, 3 × 10^5 MCF-7 cells and 2.5 × 10^5 T47D cells were seeded in six-well plates, and the cell numbers counted after 4 days. These experiments were done thrice.

Results

Mammary carcinomas induced by carcinogens. Mammary tumors were palpable in most of the Hras128 rats at the end of week 5 following treatment with the carcinogen. Faster growth and greater numbers of tumors (25.5 per rat) were noted in rats treated...
with MNU than in those treated with DMBA (16.25 per rat) or PhIP (12 per rat; Table 1). All mammary tumors were adenocarcinomas as shown in Fig. 1; there were no clear differences in tumor types among the three groups.

**GPX2 overexpressed in rat and human mammary carcinoma cell lines.** According to gene expression analysis for the rat mammary cancers using DNA microarray analysis, we found Gpx2 as an up-regulated gene in the mammary cancer induced by the three different mammary carcinogens, compared with the nontreated normal mammary glands in the Hras128 rats. Gpx2 mRNA expression was elevated >5-fold over the control value by quantitative RT-PCR (Fig. 2A), and Western blot analysis showed that Gpx2 protein levels were obviously high in all carcinogen-induced mammary cancers compared with normal mammary tissues of nontreated Hras128 (Fig. 2B).

Gpx2 expression in rat and human mammary carcinoma cell lines was examined by quantitative RT-PCR. Gpx2 was highly expressed in all rat mammary carcinoma cell lines established from a DMBA-induced mammary carcinoma in Hras128 rats, C1, C2, C3, C6, C11, and C17 (8). In contrast, normal rat mammary tissues from nontreated transgenic rats exhibited very low levels of Gpx2 (Table 2). Among these cancer cell lines, we selected C2 and C11 for further experiments in vitro because of their higher mRNA expression levels of Gpx2. Gpx2 protein expressions were confirmed by Western blot analysis. These rat breast cancer cell lines expressed Gpx2 protein. However, there is no clear difference among protein levels of the cell lines (data not shown). Interestingly, the human mammary carcinoma cell lines MCF-7, T47D, and MDA-MB-231 also showed overexpression of Gpx2 compared with the HMEC. Furthermore, Gpx2 expression in the estrogen-dependent cell lines MCF-7 and T47D tended to be higher than the estrogen-independent MDA-MB-231 (Table 2).

**Immunohistochemical analysis of GPX2 in rat and human mammary carcinomas.** Gpx2 protein was clearly detected in the cytoplasm of tumor cells. Stromal tissue also exhibited partially positive staining. All 18 tumors were positive for Gpx2.
In contrast, Gpx2 was not immunohistochemically detected in normal mammary tissues of the nontreated transgenic and nontransgenic rats (Fig. 2C). These results suggest that Gpx2 may play a critical role in the proliferation of mammary cancer cells. To clarify the relevance of this finding in human cases, expression levels of GPX2 were assessed in 41 human breast cancers (all cases were invasive ductal carcinoma; 18 papillotubular carcinomas, 10 solid-tubular carcinomas, and 13 scirrhous carcinomas) by immunohistochemistry. Cytoplasmic positive immunostaining was clearly detected in all the human tumor tissues (Fig. 3B, D, F). Fourteen (32%) cases of low histologic grade (grade 1) invasive ductal carcinomas were strongly positive for GPX2 (average

<table>
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<tr>
<th>Cell lines (rat)</th>
<th>p53</th>
<th>Gpx2</th>
<th>Cell lines (human)</th>
<th>p53</th>
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<tr>
<td>C1</td>
<td>Wild</td>
<td>134.8 ± 6.8</td>
<td>MCF-7</td>
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<td>C2</td>
<td>Wild</td>
<td>472.9 ± 143.2</td>
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<tr>
<td>C3</td>
<td>Wild</td>
<td>9.1 ± 0.9</td>
<td>MDA-MB-231</td>
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<tr>
<td>C6</td>
<td>Wild</td>
<td>367.3 ± 68.4</td>
<td>HMEC</td>
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<tr>
<td>C11</td>
<td>Mutant</td>
<td>519.0 ± 118.6</td>
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<tr>
<td>C17</td>
<td>Wild</td>
<td>86.4 ± 5.2</td>
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<td>Control mammary tissue of transgenic</td>
<td>0.2 ± 0.1</td>
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NOTE: GPX2 expression of three human mammary carcinoma cell lines was relative to its expression of HMEC.

Figure 3. Histologic appearance of human breast cancers and GPX2 immunohistochemical staining. A, C, E, G, and I, H&E; B, D, F, H, and J, immunohistochemistry. GPX2 staining was positive in the cytoplasm of human breast cancer cells regardless of tumor type. A to F, invasive ductal carcinoma: A and B, papillotubular carcinoma; C and D, solid-tubular carcinoma; E and F, scirrhous carcinoma. G and H, a representative normal mammary gland with GPX2-positive surrounding breast cancer lesion. I and J, negative for GPX2 in noncancerous mammary lesions. K, inverse correlation between the low and high histologic grade samples for GPX2 intensity in clinical breast cancer.
intensity, 2.6 ± 0.5) and the other high-grade (grade 2 and 3) tumors (27 cases; 68%) had weaker GPX2 expression (average score, 1.8 ± 0.7; Fig. 3K). These data showed inverse correlation between the low-grade and the high-grade samples for GPX2 intensity (Student’s t test, \( P = 0.00014 \)). Interestingly, normal-appearing breast epithelial cells surrounding the cancer often gave positive GPX2 staining (Fig. 3G and H); however, samples of fibrocystic change derived from noncancer patients did not show positive staining of GPX2 (Fig. 3I and J).

**GPX2 silencing effects in rat and human mammary carcinoma cell lines.** Next, we investigated the role of GPX2 by using siRNA to knockdown its expression in the rat mammary carcinoma cell lines C2 and C11. Down-regulation of GPX2 expression resulted in the dramatic inhibition of cell proliferation in C2 with wild-type p53 but not in C11 with mutated p53 (Fig. 4A). We also examined the effects of silencing GPX2 in the human mammary carcinoma cell lines MCF-7 and T47D. Interestingly, MCF-7 with wild-type p53 showed a large number of dead cells following GPX2 knockdown, which resulted in a decrease of cell numbers, whereas T47D with mutant p53 did not have any significant effect on cell growth (Fig. 4B).

**Discussion**

In the present study, we have attempted to identify genes that are important for mammary carcinogenesis. Chemical carcinogens induce mammary cancer by means of their own characteristic mechanisms, including changes in gene expression. However, we believe that common important mechanisms for mammary carcinogenesis probably exist, and that such mechanisms are also likely to be involved in human cases. Therefore, we used DNA microarray analysis to identify gene expression changes in mammary cancers, which had been induced in Hras128 transgenic rats by three different carcinogens; this strain is very sensitive to the chemical induction of mammary carcinomas (Table 1).

Among the genes that showed statistically significant expression changes, we focused particularly on Gpx2, the expression of which was elevated in all of the induced mammary carcinomas but was
very reduced in normal mammary glands (Fig. 2). GPX2 is a selenium-dependent glutathione peroxidase belonging to the glutathione peroxidase (GPX) family; it reduces H₂O₂ and alkyl hydroperoxides and possesses anti-inflammatory activity (9, 10). Because GPX2 has been reported to be expressed in selected tissues including mammary glands and the mucosal epithelium of the gastrointestinal tract (GI), it is also known as GPX-GI (11, 12).

There have already been a few reports showing an involvement of Gpx2 in cancer; mice with target disruption of Gpx2 together with Gpx1 have been associated with inflammation-induced cancer formation; the expression of Gpx2 was shown to be reduced in the epithelium of prostatic intraepithelial neoplasia in Nkx3.1 mutant mice; and overexpression of GPX2 has been found in human colorectal adenomas, Barrett’s mucosa of the esophagus, and rat hepatocarcinogenesis (13–18). In addition, inactivation of Gpx2 has been associated with UV-induced squamous cell carcinoma of skin (19), and in the public data base Gene Expressing Omnibus (GEO), a similar result to our present data, which shows that Gpx2 is overexpressed in MNU-induced breast cancers of Wistar-Furth female rats, is deposited (GDS1363).

Immunohistochemically, Gpx2 was clearly detected in the mammary cancers of Hras128 rats (Fig. 2C), and expression of this protein was located in the cytoplasm of cancer cells in all the human breast cancer tissues examined (Fig. 3A–J). These positive findings indicate that GPX2 could play an important role in not only rat but also human breast cancer development. In human cases, normal-appearing mammary tissues surrounding the breast cancer often show GPX2 positivity (Fig. 3G and H). On the other hand, negative staining for GPX2 was shown in nonmammary glands of a patient without cancer (Fig. 3I and J). These results may suggest that similar alteration of GPX2 expression has already occurred in normal-appearing tissues adjacent the cancer lesion.

To clarify how GPX2 might function in the proliferation of mammary cancer cells, we carried out GPX2 silencing by using siRNA in both rat and human mammary carcinoma cell lines. Yan and Chen (20) have recently reported that suppression by GPX2 of oxidative stress–induced apoptosis in MCF-7, a human breast cancer cell line, was wild-type p53 dependent. Therefore, in the present study, mammary cancer cell lines with both wild-type p53 [C2 (rat) and MCF-7 (human)] and mutant p53 [C11 (rat) and T47D (human)] were analyzed to investigate any association between GPX2 and p53 (8, 21). Interestingly, knockdown of GPX2 expression caused the inhibition of cell proliferation in both rat and human mammary carcinoma cell lines with wild-type p53 but not with mutation of p53 (Fig. 4). These results were compatible with the report by Yan and Chen.

According to a review article by Lacroix M, et al., (21), 20% to 35% of breast cancers express a mutant p53; breast cancer patients with p53 mutations have been associated with poor prognosis (22). Therefore, GPX2 could be a target gene for therapy of breast cancer with wild-type p53, especially in the early stages of mammary carcinogenesis including precancerous condition.

In conclusion, the present data show that GPX2 may be involved in breast cancers with wild-type p53 and indicate that GPX2 may be a novel target for the prevention and therapy of breast cancer.

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References


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