Inverse Correlation of Thioredoxin Expression with Estrogen Receptor- and p53-dependent Tumor Growth in Breast Cancer Tissues

Yasuo Matsutani, Akira Yamauchi, Rei Takahashi, Masaya Ueno, Kiyotugu Yoshikawa, Kazuo Honda, Hajime Nakamura, Hironori Kato, Hiroshi Kodama, Takashi Inamoto, Junji Yodoi, and Yoshio Yamaoka

Departments of Gastroenterological Surgery [Y. M., M. U., H. Ka., Y. Y.] and Pathology and Tumor Biology [R. T., K. Y.] and Division of the Science of Nursing [T. I.], Department of Biological Responses, Institute for Virus Research [H. N., J. Y.], Graduate School of Medicine, Kyoto University, Kyoto 606-8501, Japan; Second Department of Surgery, Kagawa Medical University, Kagawa 760-0701, Japan [A. Y.; First Department of Surgery, Ehime University School of Medicine, Ehime 791-0204, Japan [K. H.]; and Kodama Breast Clinic, Kyoto 606-8325, Japan [H. Ko.]

ABSTRACT

Estrogen receptor (ER) and p53 are important transcription factors in the growth regulation of tumor cells in breast cancer. We reported previously that thioredoxin (TRX) regulates the DNA binding activities of ER and p53 in vitro. We investigated the DNA binding activity of transcription factors, including activator protein 1, nuclear factor κB, and glucocorticoid receptor (10). Hayashi et al. (8) reported that TRX enhanced the DNA binding activity of ER in a human breast cancer cell line.

TRX was identified as a dithiol hydrogen donor for ribonucleotide reductase, an essential enzyme for DNA synthesis in Escherichia coli. Human TRX was cloned as a gene encoding an adult T-cell leukemia-derived factor from human T-cell leukemia virus I-transformed T cells (1). TRX was suggested to act as a cytoprotector and to scavenge reactive oxygen intermediates induced by various kinds of oxidative stresses such as UV and X-ray irradiation and viral infection (2–5). Recently, TRX was investigated not only as a cytoprotector or a cofactor of transcription factor but also as a growth promoter. In fact, TRX modulates the DNA binding activity of transcription factors, including activator protein 1, nuclear factor κB, and glucocorticoid receptor (6, 7). These findings suggest that TRX expression is linked to the ER- and p53-dependent regulation of tumor growth in breast cancer. In addition, TRX expression in ER+ and p53 intact (wild-type p53+) groups may mean better prognosis than in other conditions.

INTRODUCTION

Estrogen receptor (ER) and p53 are important transcription factors in the growth regulation of tumor cells in breast cancer. We investigated previously that thioredoxin (TRX) regulates the DNA binding activities of ER and p53 in vitro. The expression of pS-2, a trefoil factor, is also correlated with that of ER. To clarify the regulation mechanism of tumor growth in breast cancer, here we investigated the expression of TRX, ER, pS-2, and p53 and the mitotic index (MI) in 147 breast cancer tissues using immunohistochemical analysis. Of 123 TRX+ cases, ER+ cases (n = 62) showed a higher pS-2 score and lower MI than did ER− cases (n = 61). Furthermore, p53− cases (no mutation in p53; n = 76) also showed a lower MI than did p53+ cases (n = 47). There was no significant correlation between pS-2 and ER, MI and ER, or p53 and MI in the TRX− group. Among the ER+ and p53− cases (ER+/p53− group; n = 61), MI was lower in the TRX+ group (n = 46) than in the TRX− group (n = 15). However, in all other groups (n = 86) with abnormalities in the immunohistochemical expression of either p53 or ER, there was no significant correlation between MI and TRX expression. In the TRX+ and ER+/p53− group (n = 46), histological grading was lower than that in all other groups (n = 101). These findings suggest that TRX expression is linked to the ER- and p53-dependent regulation of tumor growth in breast cancer. In addition, TRX expression in ER+ and p53 intact (wild-type p53+) groups may mean better prognosis than in other conditions.

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2 To whom requests for reprints should be addressed, at Department of Gastroenterological Surgery, Graduate School of Medicine, Kyoto University, Shogoin, Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan. Phone: 81-75-751-3561; Fax: 81-75-751-3106.

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3 The abbreviations used are: TRX, thioredoxin; MI, mitotic index; ER, estrogen receptor; DGGE, denaturing gradient gel electrophoresis.

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human cancer tissues, including carcinomas of the liver (52%), uterine cervix (70.4%), pancreas (75%), and stomach (80%), where TRX was frequently expressed (15–18). However, there have been no in vivo studies on breast cancer tissues analyzing the relationship of TRX expression with prognostic factors such as ER, pS-2, p53, or MI.

ER and p53 are important target genes in the development of breast cancers. The transcriptional activity of both ER and p53 can be enhanced in the presence of TRX. Thus, precise examination of TRX expression and its relationship with ER, pS-2, p53, and MI should provide important clues to clarify the mechanism for growth regulation of breast cancer cells. The purpose of the present study was to clarify the role of TRX expression and its relationship with ER, pS-2, p53, and the growth rate of breast cancer cells in vivo using immunohistochemical analysis.

We found an ER- and p53-dependent, inverse relationship between expression of TRX and the cellular growth rate in breast cancer cells in vivo.

MATERIALS AND METHODS

Samples. Paraffin sections of 147 breast cancer tissues were collected from 147 patients who were surgically treated at the Kodama Breast Clinic (Kyoto Japan) and Kyoto University Hospital between 1995 and 1997. All cases were female. The ages of patients ranged from 26–88 years, and the average patient age was 49.7 years.

These tumors were histologically subtyped and staged according to the General Rule of the International Union Against Cancer (UICC). Histological grading was based on the criteria by Scarff-Bloom-Richardson, reading tubule formation, nuclear pleomorphism, and MI (19). MI was scored as follows: the average number of mitoses/field at objective 40 in 10 fields was used for scoring (1 ≤1 mitosis/field = 1, 2 mitoses/field = intermediate = 2, ≥3 mitoses/field = 3). Each diagnosis was based on an examination of H&E-stained sections. The clinical and pathological findings on 147 breast cancers are summarized in Table 1.

| Table 1 Clinical and pathological characteristics of breast cancer cases |
|-----------------------------------|------------------|
| TNM staging                       | No. of cases     |
| 1                                 | 92               |
| 2                                 | 32               |
| 3                                 | 22               |
| 4                                 | 1                |
| Histological grading              |                  |
| 1                                 | 58               |
| 2                                 | 62               |
| 3                                 | 27               |
| Mitotic index                     |                  |
| 1                                 | 77               |
| 2                                 | 35               |
| 3                                 | 35               |

Immunohistochemistry. Immunohistochemical staining of TRX, p53, and pS-2 proteins in breast cancer tissues was performed. Paraffin sections mounted on silanized slides were dewaxed and subjected to epitope retrieval by microwave treatment for 20 min for TRX and p53 (20) and by treatment with 5% pepsin (pH 1.50) for 30 min for pS-2.

Immunohistochemical staining was performed as described previously (17). Briefly, endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 10 min. After nonspecific reactions were blocked with 1% fetal bovine serum for 20 min, the slides were incubated with primary antibodies overnight at 4°C or with normal mouse immunoglobulin for negative controls. The primary antibodies were as follows: (a) for TRX, 1 μg/ml ADF-11 Ab antihuman recombinant TRX monoclonal antibody (provided by Fuji Rebio, Tokyo, Japan; Ref. 21); (b) for p53, 1 μg/ml DO-7 (A/S M 7001; DAKO, Glostrup, Denmark); and (c) for pS-2, 0.1 μg/ml Ab-1 (IM21L; Calbiochem-Novabiochem International, Cambridge, MA). The slides were then treated with biotin-labeled sheep antimouse immunoglobulin, followed by treatment with avidin-biotin horseradish peroxidase complex. The slides were washed in PBS after each step. Finally, the slides were dyed with 0.05% diaminobenzidine as a chromogen and counterstained with hematoxylin.

The staining specificity was verified as follows. Our monoclonal antibody detected a M12,000 band corresponding to the TRX protein without any significant background in Western blot analysis when the lysates of breast cancer tissues and cultured MCF-7 cells were used. Negative control staining without using primary antibody was also tested (data not shown). Tissue-specific or cell type-specific staining patterns of TRX in the present findings were mostly consistent with those of previous studies. For example, in addition to the normal mammary duct epithelium, surrounding fibroblastic cells and lymphocytes also showed slightly positive TRX staining. Furthermore, weak expression of TRX was found in normal dermal tissues and hepatocytes (15, 22).

Intensity of nuclear staining for p53, cytoplasmic staining for pS-2, and cytoplasmic and/or nuclear staining for TRX was scored in comparison with that in normal mammary gland epithelium and in stromal fibroblasts adjacent to the tumor.

The criteria for scoring immunostaining patterns were as follows.

For TRX staining, four-grade semiquantitative scoring was used to describe the overall staining intensity of the cytoplasm and the number of tumor cells with positive nuclear staining. The cytoplasm was scored as: (a) –, none; (b) +, slightly stained (cytoplasm with reticular staining pattern and blured margin); (c) ++, moderately stained (cytoplasm with homogeneous staining and clear margin); and (d) ++++, strongly stained. The nucleus was scored as: (a) –, none; (b) +, <50% of the cells; (c) ++, 50–90% of the cells; and (d) ++++, >90% of the cells. Cases with scores of ++ and ++++ in the cytoplasm and/or the nucleus were assessed as TRX+. Representative TRX expression patterns for each TRX score are shown in Fig. 1.

For p53 staining, three-grade semiquantitative scoring was used to describe the number of tumor cells with nuclear staining: (a) –, none; (b) +, <10% of the cells; and (c) ++, >10% of the cells. Cases with a score of ++ were assessed as p53+ (23).

For pS-2 staining, four-grade semiquantitative scoring was used to describe the number of tumor cells with cytoplasmic
Expression of TRX in Breast Cancers

staining: (a) −, none; (b) +, <10% of the cells stained; (c) + +, >10% and <50% of the cells stained or slightly more than 50% of cells stained homogeneously; and (d) + + +, cells with >50% strong staining. Each grade was assigned a score to examine the expression as follows: (a) −, 0; (b) +, 1; (c) + +, 2; and (d) + + +, 3 (24).

Two individuals (Y. M. and R. T.) performed microscopic analyses and scoring under a multtheaded discussion microscope. If the scores were different from each other, these individuals discussed and corrected the scores.

**ER Assay.** Surgically resected tumor tissues were immediately frozen and used for the ER assay. The ER status in each sample was determined by an enzyme immunoassay using monoclonal antibodies against ER (Abbott Laboratories, Abbott Park, IL). Results of the assay were not influenced by the presence of progesterone receptor. The cutoff line of the assay was determined to be 13 fmol/mg protein.

**Identification of p53 Gene Mutations.** The guanine cytosine clamped DGGE study was described previously (25). Briefly, DNA was prepared from frozen tissue sections by standard phenol-chloroform methods (26). Primers flanking p53 exon sequences were prepared according to a previous study (27). Genomic DNA (1 μg) was mixed with 50 pmol of each appropriate oligonucleotide primer, 0.2 mmol/liter each deoxyribonucleotide triphosphate, and 1.5 units of Taq DNA polymerase (Bioline, Toreno, Italy) in 50 μl of its standard potassium chloride buffer. Samples were incubated in a DNA thermal cycler (GeneAmp PCR system 2400; Perkin-Elmer, Foster City, CA) for a total of 40 cycles at 94°C, 58°C, and 72°C (3 s at each temperature). Four μl of the PCR products were subjected to electrophoresis on a 2% agarose gel to examine successful amplification of each fragment. The optimum gradient for each PCR product was determined with perpendicular DGGE according to the manufacturer’s instructions (D-Gene Denaturing Gel Electrophoresis System; Bio-Rad, Hercules, CA). The ranges of the denaturant of parallel DGGE and the optimum conditions of electrophoresis were reported previously (27). Gels were stained with ethidium bromide and photographed using Polaroid film.

**Statistical Analysis.** Statistical analysis was done using the Mann-Whitney U test for pS-2 and ER, MI and ER, MI and p53, MI and TRX, and histological grading and TRX. The χ² test was used to determine the TRX+ rate. P < 0.05 was considered significant.

**RESULTS**

**TRX Expression of Breast Cancer Tissues.** Of 147 cases, 123 (83.7%) were TRX+. Fifty-one of 123 TRX+ cases (34.7%) showed nuclear staining without cytoplasmic staining, whereas cytoplasmic staining without nuclear staining was found in 30 cases (20.4%). The remaining 42 cases (28.6%) exhibited both nuclear and cytoplasmic staining. Normal mammary duct epithelia were used as internal positive controls (Fig. 1). In 91 of 94 (96.8%) cases, the normal mammary glands were TRX+. Sixty of 91 TRX+ cases (65.9%) showed nuclear staining without cytoplasmic staining, whereas cytoplasmic staining without nuclear staining was found in only 1 case (1.1%). The remaining 30 cases (33.0%) exhibited both nuclear and cytoplasmic staining. The nuclei of fibroblasts and lymphocytes in adjacent normal tissues were weakly stained, which was consistent with the findings of a previous study.

**Analysis of p53 Mutation by DGGE.** Mutations of p53 are not always detected by immunostaining. Therefore, we also analyzed the mutation of p53 by DGGE in 75 available cases that were immunohistochemically negative for p53. Five tumors were found to show abnormality on DGGE analysis, suggesting the presence of p53 mutation. For further analyses, these five cases were examined as members of the p53+ group.

**Relationship between ER and pS-2 and p53 and MI in TRX+ and TRX− Groups.** There was no correlation between TRX and ER expression (data not shown). All cases were therefore divided into TRX+ and TRX− groups, and then we examined the relationship between ER and pS-2 or p53 and MI.
the same conditions, ER+ cases had a higher pS-2 score (the TRX/H11002 correlation between pS-2 and ER, MI and ER, or p53 and MI in the other group, there was no significant correlation between 0.0036) and a lower MI (P/H11005 directly correlated with p53 expression (data not shown). Under the TRX/H11001 was suggested to be regulated through p53, the MI was lower in the ER+ group than it was in other groups with abnormalities in either the MI was examined. The MI was lower in the ER+ group than in other groups with abnormalities in either

in these groups. In the TRX+ group, the histological grading of breast cancers was inversely correlated with ER expression and directly correlated with p53 expression (data not shown). Under the same conditions, ER+ cases had a higher pS-2 score (P = 0.0036) and a lower MI (P = 0.0214) than ER− cases (Table 2). Furthermore, p53− cases had a lower MI (P = 0.0040) than p53+ cases.

In contrast to the TRX+ group, there was no significant correlation between pS-2 and ER, MI and ER, or p53 and MI in the TRX− group.

Relationship among p53, ER, and MI. We then analyzed the roles of TRX in regulation of tumor cell growth under estrogen- and p53-dependent conditions. To assess cell growth, the MI was examined. The MI was lower in the ER+/p53− group than it was in other groups with abnormalities in either p53 or ER (designated as the other group; Fig. 2). In the ER+/p53− group, in which estrogen-dependent cell growth was suggested to be regulated through p53, the MI was lower in the TRX+ group than in TRX− group (P = 0.0150; Table 3). In the other group, there was no significant correlation between MI and TRX expression. In the TRX+ group, the MI was lower in the ER+/p53− group than it was in the other group (P = 0.0001; data not shown). The histology of representative cases of the ER+/p53− group is shown in Fig. 3.

We then compared the expression levels of TRX between the ER+/p53− group and the other group. Positive TRX expression was found more often in the other group than in the ER+/p53− group (P = 0.0256; Table 3). Relationship among ER, p53, TRX, and Histological Grading. Histological grading was compared between four groups, which were divided by TRX expression and ER/p53 status. Histological grading was lowest in the TRX+ group and among the ER+/p53− group (Fig. 4).

DISCUSSION

We reported previously that TRX regulates the DNA binding of ER and p53 in vitro (8, 12). In the present study, we demonstrate TRX expression was associated with a lower MI in ER+ and p53 intact cases than in cases that had abnormalities in ER or p53. These findings strongly suggest that TRX is involved in the ER- and p53-dependent regulation of tumor growth in breast cancer in vivo.

The present findings showed that normal mammary glands exhibited TRX+ staining. There are several studies showing that malignant tissues such as uterine cancer, hepatocellular carcinoma, pancreatic cancer, and gastric cancer express higher levels of TRX than surrounding noncancerous tissues. However, endocrine glands such as the pituitary gland (28) and ovary (29) are also strongly positive for TRX staining. Therefore, it is possible that TRX expression is stronger in normal mammary glands than in cancer tissues.

Table 2 Relationship between ER and pS-2, ER and MI, and p53 and MI

<table>
<thead>
<tr>
<th>TRX+ (n = 123)</th>
<th>TRX− (n = 24)</th>
<th>Total (n = 147)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pS-2 expression</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ER−</td>
<td>1.213 ± 1.127 (n = 61)</td>
<td>1.714 ± 1.380 (n = 7)</td>
</tr>
<tr>
<td>P = 0.0036b</td>
<td>1.706 ± 1.160 (n = 17)</td>
<td>1.785 ± 1.046 (n = 79)</td>
</tr>
<tr>
<td>ER+</td>
<td>1.806 ± 1.022 (n = 62)</td>
<td>2.143 ± 1.069 (n = 7)</td>
</tr>
<tr>
<td>P = 0.0213b</td>
<td>1.882 ± 0.928 (n = 17)</td>
<td>1.582 ± 0.778 (n = 79)</td>
</tr>
<tr>
<td>MI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ER−</td>
<td>1.836 ± 0.840 (n = 61)</td>
<td>2.000 ± 0.970 (n = 18)</td>
</tr>
<tr>
<td>P = 0.5537</td>
<td>1.915 ± 0.803 (n = 47)</td>
<td>2.133 ± 0.983 (n = 6)</td>
</tr>
<tr>
<td>ER+</td>
<td>1.500 ± 0.719 (n = 62)</td>
<td>1.160 ± 0.635 (n = 46)</td>
</tr>
<tr>
<td>P = 0.7146</td>
<td>1.069 ± 0.721 (n = 92)</td>
<td>1.046 ± 0.778 (n = 79)</td>
</tr>
<tr>
<td>MI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p53−</td>
<td>1.513 ± 0.757 (n = 76)</td>
<td>1.046 ± 0.778 (n = 79)</td>
</tr>
<tr>
<td>P = 0.0040b</td>
<td>1.833 ± 0.983 (n = 6)</td>
<td>1.906 ± 0.815 (n = 53)</td>
</tr>
<tr>
<td>p53+</td>
<td>1.915 ± 0.803 (n = 47)</td>
<td>1.069 ± 0.721 (n = 92)</td>
</tr>
</tbody>
</table>

*a* Values are given as the mean ± SD. *b* Statistical significance.

Table 3 Relationship between TRX expression and MI

<table>
<thead>
<tr>
<th>ER+/p53− group (n = 61)</th>
<th>The other group (n = 86)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRX− (n = 24)</td>
<td>1.933 ± 0.961 (n = 15)</td>
</tr>
<tr>
<td>MI</td>
<td>1.326 ± 0.635 (n = 46)</td>
</tr>
<tr>
<td>P</td>
<td>P = 0.0150b</td>
</tr>
</tbody>
</table>

*a* Values are given as the mean ± SD. *b* Statistical significance.
Maruyama et al. (30) showed high nuclear and cytoplasmic TRX expression in human endometrium of the secretory phase of the menstrual cycle, which might be under the strong influence of estradiol. Therefore, TRX expression in the nuclei of normal mammary duct epithelium in the current study might be induced by the stimulation of estradiol in this particular tissue, but its biological significance remains unknown.

TRX translocates from the cytoplasm into the nucleus when cells are exposed to oxidative stress such as cis-diaminedichloroplatinum(II) (12), HgCl2 (31), UVB irradiation (32), or ischemia (33), and it is localized in the nuclei to protect DNA from oxidative stress that is easily generated in ischemic tissues. In addition to DNA protection, TRX may play an important role for cell function in the nucleus including DNA synthesis or transcription, and cytoplasmic TRXs may act as a reservoir for nuclear TRX. Because we examined the steady-state expression levels of TRX in cancer tissues using immunohistochemical analysis, both nuclear and cytoplasmic TRX were included in the assay.

It is of particular interest that TRX is preferentially expressed in estrogen-responsive tissues such as the uterine endometrium (30) and the mammary gland, as shown in the current study, whereas normal hepatocytes and gastric epithelia express extremely low amounts of TRX. These findings imply the involvement of estrogen-dependent mechanisms; however, its significance should be further investigated.

Expression levels of TRX appear to be tightly correlated with the rate of cell growth. According to our preliminary findings on mouse embryonic liver by histopathological analysis, the expression level of TRX is lower during the proliferative phase of hepatocytes and gradually increases but markedly decreases when the cells become mature and cease dividing. Considering the finding that the function of pS-2 is also linked to the maturation of mucosal epithelium of the gastrointestinal tract, the significance of TRX and its downstream genes, pS-2 and p53, in the development of breast carcinoma could readily be implicated. Such a mechanism could partially explain the role of TRX expression in the ER- and p53-intact breast cancer tissues.

In an in vitro study of ER+ endometrial stromal cells of the uterus, expression of TRX mRNA can be induced by estrogen stimuli (30), which is consistent with our finding that ER+ normal mammary duct epithelium expresses high levels of TRX in vivo. Lack of estrogen-dependent TRX expression even in ER+ cancer cells in the present study may suggest the existence of an alternative ER-independent pathway down-regulating TRX expression, although further examination is necessary.

The present findings demonstrated that the lowest MI in the TRX+ condition was found in the ER+/p53 group. Completely opposite effects of TRX on cell growth and histological grading, depending on the ER/p53 status, may implicate the presence of some feedback mechanism that could compensate
for the loss of the p53 or ER function by accumulating TRX. This may be why ER-independent TRX induction was suggested in the present study. Although transfection of TRX accelerates colony formation of breast cancer cell line MCF-7 in soft agarose (13), the colony formation rate may not be associated with the proliferation rate. Indeed, same studies showed that transfected TRX did not accelerate the growth rate of MCF-7 cells on a flat plastic plate. Moreover, TRX partially inhibits the tumorigenicity of MCF-7 cells in SCID mice (13). Because MCF-7 cells are ER+ and wild-type p53+ (p53 intact), TRX may play an important role in growth suppression in ER+ and p53 intact conditions.

The present findings suggest that expression of TRX is linked to the ER- and p53-dependent growth of breast cancer cells in vivo and that TRX may act as a suppressor of cell proliferation. Namely, TRX expression may mean better prognosis in the ER+ and p53 intact (wild-type p53+) condition than in other conditions. TRX may control cell growth by accelerating the transcription of ER and p53 with subsequent induction of pS-2 and p21 expression.

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REFERENCES


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