High Expression of Steroid Sulfatase mRNA Predicts Poor Prognosis in Patients with Estrogen Receptor-positive Breast Cancer¹

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ABSTRACT

Purpose: Prognostic significance of the intratumoral mRNA expression of three enzymes related to in situ estrogen biosynthesis, i.e., aromatase, sulfatase, and 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1), was evaluated in patients with invasive breast cancer.

Experimental Design: Aromatase, sulfatase, and 17β-HSD1 mRNA levels in tumor tissues (n = 181) and normal breast tissues (n = 34) were examined by a quantitative, real-time PCR assay and compared with various clinicopathological factors as well as prognosis.

Results: The sulfatase mRNA levels, but not the aromatase or 17β-HSD1 mRNA levels, were significantly associated with lymph node metastases (P < 0.005), histological grade III (P < 0.001), and poor prognosis (P < 0.005). The association between the sulfatase mRNA and poor prognosis was found to be significant (P < 0.001) only in patients with estrogen receptor (ER)-positive tumors but not in ER-negative tumors. In ER-positive tumors, the sulfatase mRNA levels was a significant prognostic factor independent of the lymph node status and histological grade by multivariate analysis.

Conclusions: The sulfatase mRNA levels can serve as a significant, independent prognostic factor only in ER-positive tumors. It is speculated that the up-regulation of sulfatase mRNA levels leads to a high intratumoral estrogen concentration and, thus, an enhanced stimulation of tumor growth through ERs.

INTRODUCTION

It is well established that estrogens play an essential role in the pathogenesis and growth of breast cancer (1, 2). Recently, several lines of evidences have demonstrated that intra-tumoral biosynthesis of E2³ plays an important role in the maintenance of intratumoral, high E2 levels in postmenopausal women (3–5). E2 is intratumorally synthesized mainly through the two pathways, i.e., aromatase pathway and sulfatase pathway. Tumor tissues uptake androstenedione, E1S, and E2S from the blood as precursors for E2 (6–7). In the aromatase pathway, androstenedione is aromatized into E1 by aromatase and catalyzed into E2 by 17β-HSD1 (8–11). In the sulfatase pathway, E1S is converted into E1 by sulfatase and catalyzed into E2 by 17β-HSD1, or E2S is directly converted into E2 (12).

A change in the expression levels of these three enzymes, i.e., aromatase, sulfatase, and 17β-HSD1, is speculated to affect the intratumoral E2 levels, resulting in the modulation of the growth and responsiveness to endocrine treatment of breast cancer. Thus, it seems to be of interest to study the prognostic significance of the intratumoral expression status of these estrogen biosynthesis-related enzymes, especially in ER-positive tumors, of which growth is dependent on estrogen. Indeed, Utsumi et al. (13) have recently reported that sulfatase mRNA expression can serve as a significant risk factor for recurrence, and more recently, Gunnarsson et al. (14) have reported a significant association of 17β-HSD1 mRNA expression with poor prognosis. The prognostic significance of aromatase mRNA expression has yet to be studied.

These preliminary results have prompted us to study the expression of all of the three enzymes related to the intratumoral biosynthesis of estrogen, firstly to confirm the previous reports, secondly to elucidate the prognostic significance of aromatase mRNA expression, and thirdly to determine which is the most useful prognostic factor among these three enzymes. Therefore, in this study, the mRNA expression levels of aromatase, sulfatase, and 17β-HSD1 in tumor tissues were assayed by a quantitative reverse transcription PCR assay, and their relationship with various clinicopathological parameters as well as prognosis was investigated.

MATERIALS AND METHODS

Surgical Specimens and Patients Characteristics. One hundred eighty-one female patients who underwent mastectomy or breast conserving surgery with invasive ductal breast cancer during the period from October 1995 to December 1999 were

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¹ The abbreviations used are: E2, estradiol; E1S, estrone sulfate; E2S, estradiol sulfate; E1, estrone; 17β-HSD1, 17β-hydroxysteroid dehydrogenase type 1; DFS, disease-free survival; ER, estrogen receptor.
recruited in this study. The details of patient characteristics are shown in Table 1. All tumors were histologically diagnosed as invasive ductal carcinoma, and histological grade was determined according to the modified Scarff-Bloom-Richardson criteria (15). Normal breast tissues were obtained from 34 breast cancer patients who underwent mastectomy or breast conserving surgery during the period from October 1999 to May 2001. All breast cancer and normal tissues were obtained at surgery and snap frozen until use. Informed consent was obtained from each patient before operation.

Patients had a physical examination every 3 months for 2 years postoperatively, then every 6 months thereafter, and chest X-ray as well as abdominal ultrasonogram were obtained every 6 months postoperatively. Six patients received no adjuvant therapy. Tamoxifen (20 mg/day p.o. 2 years) was given to 92 patients, goserelin to 6 patients, and both to 3 patients. Six cycles of cyclophosphamide (100 mg/day p.o. days 1–14) plus methotrexate (40 mg/m² i.v. days 1 and 8) plus 5-fluorouracil (600 mg/m² i.v. days 1 and 8) were given to 14 patients, four cycles of cyclophosphamide (600 mg/m² i.v. day 1) plus epirubicin (60 mg/m² i.v. day 1) to 14 patients, and other chemotherapies to 3 patients. Forty-three patients were treated with tamoxifen plus cyclophosphamide + methotrexate + 5-fluorouracil or cyclophosphamide + epirubicin. Indication for adjuvant treatment was decided essentially according to St. Gallen recommendations (16, 17). None of the patients had been treated with neoadjuvant therapy.

The median follow-up period of 181 patients was 33 months (range, 27–62 months), and DFS of these patients was 81.8%. Twenty-five (13.8%) of 181 patients developed recurrences, i.e., 13 developed soft tissue metastases, 6 developed liver metastases, 4 developed bone metastases, 2 developed lung metastases, and 1 developed brain metastasis. Ipsilateral breast recurrences after breast conserving surgery were not counted as recurrences.

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<thead>
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<th>Table 1</th>
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</tr>
<tr>
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<td>74</td>
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<td>Postmenopausal</td>
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<td>≤2 cm</td>
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<tr>
<td>&gt;2 cm</td>
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<td>Lymph node metastasis</td>
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<td>I + II</td>
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<td>III</td>
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**Results**

**RNA Extraction, Reverse Transcription, and Real-Time PCR.** Total cellular RNA was extracted from the frozen tumor specimens using Trizol reagent (Molecular Research Center, Cincinnati, OH), and the 3 μg each of total RNA were used for synthesis of cDNA by Superscript II (Life Technologies, Inc., Rockville, MD) priming with oligo-(dT)15 primer. Primers and probes used for each real-time PCR amplification of aromatase, sulfatase, and 17β-HSD1 were described previously (18). PCR reactions were carried out using ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA) according to the method described previously (18, 19). To quantify gene transcripts precisely, the β-glucuronidase transcripts were monitored as the quantitative control, and each sample was normalized on the basis of its β-glucuronidase transcript content. The primer probe mixture for β-glucuronidase was purchased from Perkin-Elmer Applied Biosystems, and the method of PCR was followed by the manufacturer’s protocol. Finally, mRNA levels of aromatase, sulfatase, and 17β-HSD1 were shown as ratios to β-glucuronidase mRNA levels when 10−10 μg plasmid control for aromatase and 17β-HSD1, 10−6 μg for sulfatase, and 10−6 μg for β-glucuronidase defined as 1. Real-time PCR assays were conducted in duplicate for each sample, and the mean value was used for the calculation of mRNA expression levels.

**ER Assay.** ER levels in breast cancers were measured by enzyme immunoassay using the kit provided by Abbott Research Laboratories (Chicago, IL). The cutoff value for ER was defined as 5 fmol/mg protein.

**Statistical Methods.** The relationship between aromatase, sulfatase, and 17β-HSD1 mRNA expression levels and clinicopathological factors was assessed by Student t test or the Mann-Whitney test. DFS curves were calculated by the Kaplan-Meier method, and the log-rank test was used to evaluate the difference in DFS among the various patient subgroups. The proportional hazards model was used for the multivariate analysis. Statistical significance was assumed for P < 0.05.

**RESULTS**

**Relationship between Aromatase, Sulfatase, and 17β-HSD1 mRNA Levels and various Clinicopathological Parameters.** Aromatase, sulfatase, and 17β-HSD1 mRNA expression levels in tumor tissues (n = 181) and normal breast tissues (n = 34) were quantified by a real-time PCR assay. The mRNA levels (mean ± SE) of aromatase, sulfatase, and 17β-HSD1 in breast tumors (0.67 ± 0.09, 10.34 ± 1.16, and 3.65 ± 0.66) were significantly (P < 0.005, P < 0.001, and P < 0.001) higher than those in the normal breast tissues (0.26 ± 0.07, 4.78 ± 1.11, and 1.45 ± 0.34).

The relationship between mRNA levels in these three enzymes and the various clinicopathological parameters of breast tumors is shown in Table 2. Sulfatase mRNA expression levels in tumors with lymph node metastasis were significantly (P < 0.005) higher than those without lymph node metastasis. In addition, ER negativity and histological grade III were significantly (P < 0.05 and P < 0.001) associated with the high sulfatase mRNA expression. On the other hand, we found no significant association of aromatase mRNA or 17β-HSD1 mRNA levels with any clinicopathological parameters.
Univariate Analysis of Prognosis. Breast tumors were dichotomized into high and low mRNA expression groups for aromatase, sulfatase, and 17β-HSD1 using the cutoff value, which corresponded to the mean ± 2 SD in the normal breast tissues (1.02, 17.7, and 5.42, respectively). According to this cutoff value, 32, 29, and 24 tumors of 181 tumors were classified into the high aromatase, sulfatase, and 17β-HSD1 mRNA expression group, respectively. The high sulfatase mRNA group showed a significantly (P < 0.005) lower 5-year DFS rate (67.4%) than the low sulfatase mRNA group (84.4%; Fig. 1A, Table 3). On the other hand, no significant association was observed between prognosis and aromatase mRNA or 17β-HSD1 mRNA levels, whereas the high 17β-HSD1 mRNA group showed a nonsignificant trend toward a decrease in a 5-year DFS rate than the low 17β-HSD1 mRNA group (P = 0.079).

The association between the sulfatase mRNA levels and prognosis was additionally analyzed according to the ER status (Fig. 2). Such a subset analysis revealed that prognosis of the high sulfatase mRNA group was significantly poorer than that of the low sulfatase mRNA group in the ER-positive subset (P < 0.001) but not in the ER-negative subset (P = 0.40). The ER-positive subset was additionally broken down according to the menopausal status. A significant association between the high sulfatase mRNA expression and poor prognosis was observed both in premenopausal patients (P < 0.01, Fig. 3A) and in postmenopausal patients (P < 0.005, Fig. 3B).

Multivariate Analysis of Prognosis. Because lymph node metastasis, high histological grade, ER negativity, and high sulfatase mRNA levels were significantly associated with a poor prognosis by univariate analysis, they were additionally analyzed by multivariate analysis to see their independence of other risk factors. Multivariate analysis has revealed that the expression of sulfatase mRNA is a significant (P = 0.039), prognostic factor independent of the other factors (Table 4). Multivariate analysis, which has been done in the ER-positive subset, has also shown that the sulfatase mRNA expression is a significant (P = 0.019) prognostic factor (Table 5).

DISCUSSION
We have been able to show that the sulfatase mRNA expression is significantly associated with poor prognosis, which is consistent with the report of Utsumi et al. (13). In addition, interestingly, we have found that such an association is seen only in ER-positive tumors but not in ER-negative tumors. It is speculated that high sulfatase activity increases the intratumoral E2 levels, resulting in growth stimulation of ER-positive but not ER-negative breast tumors.

James et al. (20) reported that transfection of sulfatase into MCF-7 human breast cancer cells led to an increase in cell proliferation and anchorage-independent growth in vitro. They also reported that this growth-stimulative effect seen in sulfatase-transfected MCF-7 cells was blocked by antiestrogens, indicating that the constitutive expression of sulfatase enhanced the E2 production and, then, stimulated the cell growth through ERs. These reports are consistent with our present findings that patients with high sulfatase mRNA levels are associated with a poor prognosis in ER-positive but not ER-negative tumors.

It is generally thought that the intratumoral high estrogen levels are maintained mostly by the intratumoral estrogen biosynthesis in postmenopausal patients, but they are maintained...
mostly by the uptake of estrogen secreted from the ovaries in premenopausal patients. Thus, a change in the intratumoral estrogen biosynthesis induced by sulfatase expression is speculated to affect the tumor growth in postmenopausal patients but is unlikely to do so in premenopausal patients. However, interestingly, we have found that the high sulfatase mRNA expression is associated with poor prognosis both in premenopausal and postmenopausal patients with ER-positive tumors in the present study. These results suggest a possibility that, even in premenopausal patients, the intratumoral estrogen biosynthesis plays an important role in the growth stimulation of breast tumors. Indeed, the expression levels of sulfatase mRNA and aromatase mRNA in tumor tissues of premenopausal patients are as high as those of postmenopausal patients (18). In our study, the fact that response rate to luteinizing hormone-releasing hormone agonist (goserelin) plus tamoxifen is superior to that to goserelin alone seems to indicate a possibility that intratumoral estrogen biosynthesis plays a significant role in the growth stimulation of breast tumors even in premenopausal patients (21). The relative contribution of the intratumorally synthesized E2 and the ovary-originating E2 to the intratumoral total E2 levels still remains to be established.

We have been able to show a nonsignificant trend that tumors with the high 17β-HSD1 mRNA levels have a poorer prognosis than those with the low 17β-HSD1 mRNA levels. Our results seem to be essentially consistent with those reported by Gunnarsson et al. (14) who showed a significant association between 17β-HSD1 mRNA expression and prognosis. Although sulfatase mRNA levels were not assayed in their report (14), we have studied both 17β-HSD1 mRNA and sulfatase mRNA levels in the present study and have shown that sulfatase mRNA levels are a clinically more useful prognostic indicator than 17β-HSD1 mRNA levels.

Only a few reports have been available that investigated the prognostic significance of aromatase expression in breast cancer. Silva et al. (22) and Lipton et al. (23) reported no significant relationship between aromatase activity and patient outcome. To the best of our knowledge, this is the first report that studied the relationship between aromatase mRNA levels and prognosis.

Because aromatase had been considered as a pivotal enzyme for the intratumoral estrogen biosynthesis, a significant relationship between its mRNA expression and prognosis was expected, but we failed to demonstrate such a relationship, which is consistent with the reports of Silva et al. (22) and Lipton et al. (23), suggesting a possibility that the sulfatase pathway is more important than the aromatase pathway in the intratumoral estrogen biosynthesis. Miller et al. (24) showed that the relative contribution of the aromatase pathway to the intratumoral estrogen biosynthesis varied widely from 0 to 75% with a median value of 37%, suggesting that the aromatase pathway is a major pathway in a smaller proportion of tumors than expected and that the sulfatase pathway plays a more important role than expected.

Ideally, a new prognostic factor is to be tested in the patients without adjuvant therapy. However, almost all breast cancer patients are recently treated with adjuvant therapy according to the recommendation of the consensus meeting of St. Gallen (16, 17, 25) and NIH (26). Thus nowadays, it is practically almost infeasible to evaluate a new prognostic factor.
intratumoral E2 levels. Thus, it is possible that tumors with the estrogen action by tamoxifen possibly because of the high to aromatase inhibitor (27) suggests an incomplete blockade of ER-positive tumors that fail in tamoxifen treatment still respond rather than a prognostic factor. The well-known fact that some with poor prognosis only in patients with ER-positive tumors were treated with tamoxifen. Our observation respond to tamoxifen because virtually most patients with ER-

levels are a real prognostic factor or a predictive factor to sulfatase mRNA levels in premenopausal (A) and postmenopausal (B) groups.

Table 4 Univariate and multivariate analysis of various prognostic factors

<table>
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<th>Univariate analysis</th>
<th>Multivariate analysis</th>
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<tr>
<td></td>
<td>Relative risk P</td>
<td>Relative risk P</td>
</tr>
<tr>
<td>Lymph node status</td>
<td>2.89 0.011</td>
<td>1.78 0.259</td>
</tr>
<tr>
<td>Histological grade</td>
<td>5.46 &lt;0.001</td>
<td>3.72 0.004</td>
</tr>
<tr>
<td>ER status</td>
<td>3.23 0.005</td>
<td>2.43 0.035</td>
</tr>
<tr>
<td>Aromatase</td>
<td>0.88 0.821</td>
<td>2.43 0.039</td>
</tr>
<tr>
<td>Sulfatase</td>
<td>3.37 0.004</td>
<td>2.43 0.039</td>
</tr>
<tr>
<td>17β-HSD1</td>
<td>2.23 0.088</td>
<td></td>
</tr>
</tbody>
</table>

a Relative risk of lymph node positive against lymph node negative, histological grade III against grade I + II, and mRNA high against mRNA low for aromatase, sulfatase, and 17β-HSD1.

Table 5 Univariate and multivariate analysis of various prognostic factors in the ER-positive subset

<table>
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<th>Multivariate analysis</th>
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<tr>
<td></td>
<td>Relative risk P</td>
<td>Relative risk P</td>
</tr>
<tr>
<td>Lymph node status</td>
<td>3.15 0.075</td>
<td>2.66 0.137</td>
</tr>
<tr>
<td>Histological grade</td>
<td>3.85 0.038</td>
<td>2.56 0.170</td>
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<tr>
<td>Sulfatase</td>
<td>7.52 0.002</td>
<td>5.03 0.019</td>
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</table>

a Relative risk of lymph node positive against lymph node negative, histological grade III against grade I + II, and mRNA high against mRNA low for sulfatase.

among the patients without adjuvant therapy. Thus, strictly speaking, it is currently unknown that the sulfatase mRNA levels are a real prognostic factor or a predictive factor to respond to tamoxifen because virtually most patients with ER-positive tumors were treated with tamoxifen. Our observation that the sulfatase mRNA levels were significantly associated with poor prognosis only in patients with ER-positive tumors but not in those with ER-negative tumors seems to indicate that the sulfatase mRNA levels are a predictive factor to tamoxifen rather than a prognostic factor. The well-known fact that some ER-positive tumors that fail in tamoxifen treatment still respond to aromatase inhibitor (27) suggests an incomplete blockade of the estrogen action by tamoxifen possibly because of the high intratumoral E2 levels. Thus, it is possible that tumors with the high sulfatase expression could be tamoxifen resistant. The relationship between the sulfatase mRNA levels and response to tamoxifen needs to be clarified in a future study. Elucidation of the relationship of aromatase, sulfatase, and 17β-HSD1 expression with the intratumoral E2 levels appears to be of pivotal importance for the better understanding of the biological significance of up-regulation of these enzymes. However, unfortunately, this important issue has rarely been studied. Only a few reports have been available that studied the relationship between the intratumoral E2 levels and aromatase expression or activity. These reports have shown no significant association between them (28, 29).

The relationship between the intratumoral E2 levels and sulfatase or 17β-HSD1 expression has never been reported yet. In postmenopausal women, it has been reported that E1 and E2 concentrations in tumors are higher than those in sera; on the contrary, E1S concentrations in sera are higher than those in tumors (30, 31). These facts seem to indicate that E1 and E2 are locally synthesized from a precursor (E1S) in the blood. Because serum E1S levels are relatively high, it is speculated that up-regulation of intratumoral sulfatase could sufficiently convert E1S into E1, leading to high E2 concentrations in tumors. However, the association between the intratumoral sulfatase activity and E2 concentrations has yet to be reported.

The limitation of this study lies in that mRNA expression of aromatase, sulfatase, and 17β-HSD1 in whole tumor samples was assayed, and, thus, mRNA expression from both tumor cells and stromal cells was included. However, we think that measurement of mRNA expression in both tumor cells and stromal cells might be relevant to estimate the intratumoral hormonal milieu because both tumor cells and stromal cells express these enzymes (32, 33).

In conclusion, we have demonstrated that the intratumoral sulfatase mRNA levels, but not the aromatase and the 17β-HSD1 mRNA levels, can serve as a significant prognostic factor only in ER-positive breast cancer patients. It is tempting to speculate that the up-regulation of sulfatase mRNA levels leads to a high intratumoral estrogen concentration and, thus, an enhanced stimulation of tumor growth through ERs. Although a significant correlation between mRNA levels and enzymatic activities have been demonstrated in cultured cells with respect to aromatase and sulfatase (34, 35), such a correlation has never been reported in tumor tissues. Thus, strictly speaking, it is currently unknown whether the association between sulfatase mRNA levels and prognosis reflects the association between sulfatase activities and prognosis. Our preliminary results need
to be confirmed by a future study, including a larger number of patients with a longer follow-up period.

REFERENCES


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