Sex steroid-producing enzymes in human breast cancer

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Abstract

It is well known that sex steroids are involved in the growth of breast cancers, and the great majority of breast carcinomas express estrogen (ER), progesterone (PR), and androgen (AR) receptors. In particular, recent studies have demonstrated that estrogens and androgens are locally produced in breast carcinoma tissues, and total blockade of in situ estrogen production potentially leads to an improvement in prognosis of breast cancer patients. Therefore, it is important to obtain a better understanding of sex steroid-producing enzymes in breast carcinoma tissues. In this review, we summarize recent studies on the expression and regulation of enzymes related to intratumoral production of estrogens (aromatase, 17β-hydroxysteroid dehydrogenase type 1 (17βHSD1), and steroid sulfatase (STS) etc) and androgens (17βHSD5 and 5α-reductase) in human breast carcinoma tissues, and discuss the biological and/or clinical significance of these enzymes. The cellular localization of aromatase in breast carcinoma tissues still remains controversial. Therefore, we examined localization of aromatase mRNA in breast carcinoma tissues by laser capture microdissection/real time-polymerase chain reaction. Aromatase mRNA expression was detected in both carcinoma and intratumoral stromal cells, and the expression level of aromatase mRNA was higher in intratumoral stromal cells than in carcinoma cells in the cases examined. We also examined an association among the immunoreactivity of enzymes related to intratumoral estrogen production and ERs in breast carcinoma tissues, but no significant association was detected. Therefore, the enzymes responsible for the intratumoral production of estrogen may not always be the same among breast cancer patients, and not only aromatase but also other enzymes such as STS and 17βHSD1 may have important therapeutic potential as targets for endocrine therapy in breast cancer patients.

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Introduction

Biologically active hormones are produced and secreted from the endocrine organs, transported through the circulation, and act on their target tissues where their specific receptors are expressed (Fig. 1A). This system is known as the endocrine system, and biological features of hormone-dependent target tissues are generally considered to be influenced by the plasma concentration of the biologically active hormones. In addition, hormones can also act in the same cell (autocrine) (Fig. 1B) or neighboring cells (paracrine) (Fig. 1C) without release into the circulation. A large proportion of androgens in men (approximately 50%) and estrogens in women (approximately 75% before the menopause, and close to 100% after the menopause) are synthesized in peripheral hormone-target tissues from abundantly present circulating precursor steroids (Labrie et al. 2003), where the enzymes involved in the formation of androgens and estrogens are expressed (Fig. 1D). These locally produced bioactive androgens and/or estrogens exert their action in the cells where synthesis occurs without release into the extracellular space. This phenomenon is different from the autocrine, paracrine and classical endocrine action, and is called ‘intracrine’. In classical endocrine systems, only a small amount of hormone is generally utilized in the target tissues, and thereafter the great majority is metabolized or converted to inactive forms. On the other hand,
an intracrine system requires minimal amounts of biologically active hormones to exert their maximum effects. Therefore, intracrine is an efficient mode of hormone action and plays important roles especially in the development of hormone-dependent neoplasms. It is also important to note that, in an intracrine system, serum concentrations of hormones do not necessarily reflect the local hormonal activity in the target tissues.

Sex steroids, such as estrogens and androgens, play important roles in various target tissues including reproductive organs. A majority of breast carcinoma tissues express estrogen (ER) and androgen (AR) receptors, and estrogens greatly contribute to the growth of breast cancers. Breast carcinoma tissues have been demonstrated to process intracrine activity. Locally produced biologically active estrogens act in breast carcinoma tissues. This mechanism has been considered to play a pivotal role in the proliferation of breast carcinoma cells. The blockade of this pathway potentially reduces cell proliferation of breast tumors, and it is very important to obtain a better understanding of sex steroid-related enzymes in breast carcinoma as potential therapeutic targets of endocrine therapy. Therefore, in this review we summarize the results of recent studies on the expression and regulation of the enzymes related to intratumoral production of sex steroids in human breast carcinoma tissues, and discuss the potential biological and/or clinical significance of intratumoral production of sex steroids in these carcinomas.

Intratumoral production of estrogens in breast carcinoma tissue

Circulating estrogens are mainly secreted from the ovary in premenopausal women. However, after the
menopause, estrogens are biosynthesized in peripheral tissues such as adipose tissue, skin, and muscle, through conversion of circulating inactive steroids (Sasano & Harada 1998). Intratumoral estradiol levels were not significantly different between premenopausal and postmenopausal breast cancer patients, but the intratumoral estradiol/estrone ratio was significantly higher in postmenopausal than in premenopausal breast cancers (Miyoshi et al. 2001). The ratio of estradiol concentration in tumor tissue/plasma was 23 in postmenopausal breast carcinomas, but was 5 in premenopausal breast carcinomas (Pasqualini et al. 1996, Pasqualini 2004). In addition, the concentration of estradiol was 2.3-times higher in breast cancer tissues than in the areas considered as morphologically normal (Chetrite et al. 2000). The great majority of breast cancers occur after the menopause and express ER, which suggests that the in situ production of estrogens plays an important role in the proliferation of breast cancer cells, especially in postmenopausal women.

Figure 2 summarizes the representative pathways of in situ production of sex steroids in human breast carcinoma tissues, which are currently postulated. High concentrations of circulating inactive steroids, such as androsterone/dione and estrone sulfate, are precursor substrates of local production of estrogens and/or androgens in breast carcinomas. Bioactive sex steroids, estradiol and 5α-dihydrotestosterone (DHT) are locally produced and act on the carcinoma cells through estrogen (ER) and androgen (AR) receptors respectively. 3β-HSD, 3β-hydroxysteroid dehydrogenase; EST, estrogen sulfotransferase (SULT1E1); STS, steroid sulfatase; 17β-HSD, 17β-hydroxysteroid dehydrogenase.

Aromatase
Aromatase (CYP19) is an enzyme located in the endoplasmic reticulum of estrogen producing cells, and is a key enzyme in the synthesis of estrogens, mainly aromatization of androstenedione to estrone (Fig. 2). In 63–72% of breast carcinoma specimens aromatase activity was comparable with or greater than that found in other tissues (Silva et al. 1989,
Miller et al. 1990, Miller 1991, Lipton et al. 1992, Bolufer et al. 1992). Aromatase activity was higher in the stromal than in the epithelial component in breast tumors (Purohit et al. 1995), but aromatase activity was also detected in several breast carcinoma cell lines (Kinoshita & Chen 2003, Sonne-Hansen & Lykkefeldt 2005). Positive correlations were reported between aromatase activity and ER (Miller et al. 1990, Miller 1991) or tumor grade (Silva et al. 1989). However, results of these studies have not necessarily been confirmed by other groups, and no consistent correlations between these two parameters have been established (de Jong et al. 2001). In addition, no significant association between aromatase activity and disease-free interval or overall survival has been reported. The level of aromatase mRNA expression was highest in a quadrant bearing carcinoma among four breast quadrants of mastectomy (Bulun et al. 1993), and aromatase mRNA levels in breast carcinomas were significantly increased compared with those in non-malignant tissues (Utsumi et al. 1996), which was consistent with the reported findings of aromatase activity in breast cancers described above. In order to examine the localization of aromatase mRNA in breast carcinoma tissues, we examined laser capture microdissection/real time-polymerase chain reaction (LCM/real-time PCR) for aromatase in breast carcinoma tissues (Fig. 3A). As shown in Fig. 3B, mRNA expression of aromatase was detected in both carcinoma and intratumoral stromal cells adjacent to the carcinoma cells. The level of aromatase mRNA expression was significantly ($P = 0.0040$) higher in intratumoral stromal cells than in carcinoma cells (Fig. 3C), and no significant association was detected in aromatase mRNA expression between intratumoral stromal cells and carcinoma cells (data not shown). Immunolocalization of aromatase was examined by several groups, but reported results of aromatase immunolocalization in breast cancers appear to be inconsistent (Table 1). Previously, Sasano et al. (1994) demonstrated aromatase immunoreactivity in stromal cells such as intratumoral fibroblasts (Fig. 4A) and adipocytes in breast carcinoma tissues. Santen et al. (1994) also demonstrated aromatase immunoreactivity predominantly in the stromal cells, On the other hand, Esteban et al. (1992), Lu et al. (1996) and Brodie et al. (2001) reported aromatase immunoreactivity in breast carcinoma cells. Shenton et al. (1998) examined two different antibodies for aromatase in breast carcinomas, and reported the different cellular immunolocalization. Recently, Sasano et al. (2003) validated several new aromatase antibodies for immunohistochemistry, and demonstrated that aromatase immunoreactivity was detected in various types of cells such as stromal cells, carcinoma cells (Fig. 4B) and normal duct epithelial cells. These discrepant results of aromatase immunolocalization in previous studies may be due to the different nature of the aromatase antibodies employed. Esteban et al. (1992) reported an inverse association between aromatase immunoreactivity and ER status in breast cancers, but no consistent correlations between aromatase immunoreactivity and known clinicopathological factors have been reported. Further investigations are required for clarification.

In addition, it remains unclear by which mechanism aromatase expression is increased in breast carcinoma tissues. Breast carcinoma cells secrete various factors that induce aromatase expression in adipose fibroblasts (Zhou et al. 2001), and the regulation of aromatase expression is partly considered as tumor-stromal interactions. For instance, prostaglandin E2 (PGE2) produced from breast carcinoma cells markedly stimulates aromatase expression in adipostromal cells (Zhao et al. 1996, Singh et al. 1999), and PGE2 production was partly regulated by estrogenic actions (Frasor et al. 2003). Various cytokines, such as interleukin (IL)-1, IL-6, IL-11 and tumor necrosis factor (TNF)-$\alpha$ (Reed & Purohit 2001, Simpson & Davis 2001), which are released from carcinoma cells and/or inflammatory cells were also demonstrated to be capable of significant induction of aromatase expression in breast cancers.

Previous studies also demonstrated the regulation of aromatase expression by various transcriptional factors. Transcription of aromatase is activated by steroidogenic factor 1 (SF1; designated NR5A1) in the ovary, which binds to a nuclear receptor half site (NRE) within their promoter regions to mediate basal transcription and, in part, cAMP-induced transcription (Parker & Schimmer 1997). However, SF1 is not expressed in breast carcinoma tissues (Clyne et al. 2002). Clyne and colleagues also examined various orphan nuclear receptors known to bind to such an NRE in 3T3-L1 preadipocytes, and reported the induction of aromatase expression by liver receptor homologue-1 (LRH-1; NR5A2) in the adipose stromal cells in breast cancers (Clyne et al. 2002, Zhou et al. 2005). Significant association was detected between LRH-1 and aromatase mRNA levels in the adipose tissues adjacent to the carcinoma, but not in the breast carcinoma tissues (Zhou et al. 2005), suggesting that LRH-1 may mainly regulate aromatase expression in adipose tissue adjacent to the breast carcinoma. Induction of aromatase expression by CCAAT/enhancer binding protein
(C/EBP) was also reported in adipose fibroblasts in breast cancer (Zhou et al. 2001). On the other hand, estrogen-related receptor-α (ERRα; NR3B1), which was mainly immunolocalized in breast cancer cells (Suzuki et al. 2004), had a positive regulatory function on aromatase in SK-BR-3 breast cancer cells (Yang et al. 1998), but not in 3T3-L1 preadipocytes (Clyne et al. 2002). In addition, Sebastian et al. (2002) reported up-regulation of aromatase by GATA-2 in vascular endothelial cells of breast cancer. Therefore, aromatase expression is possibly regulated by various transcriptional factors in breast cancer tissues, and the key regulator may be different according to the types of cells in breast carcinoma tissues.
androgens. To date, 12 isozymes of 17\textsuperscript{b}HSD have been cloned, and 17\textsuperscript{b}HSD-activation (17\textsuperscript{b}HSD\textsubscript{1}, 2, 4, 6 etc) of estrogens and/ or androgens is catalyzed by different 17\textsuperscript{b}HSD iso-zymes. Among these isozymes, 17\textsuperscript{b}HSD\textsubscript{1} enzyme uses NADPH as a cofactor, and catalyzes the oxidation of both estradiol and testosterone to androstenedione (Wu et al. 1993) (Fig. 2). Oxidative 17\textsuperscript{b}HSD activity is the preferential direction in normal breast tissues, but the reductive 17\textsuperscript{b}HSD pathway is dominant in breast cancers (Speirs et al. 1998, Luu-The et al. 1989). On the other hand, the 17\textsuperscript{b}HSD\textsubscript{2} enzyme uses NAD\textsuperscript{+} as a cofactor, and catalyzes the oxidation of both estradiol to estrone and testosterone to androstenedione (Peltoketo et al. 1988, Luu-The et al. 1989). Miyoshi et al. (2001) reported that the intratumoral estradiol/estrone ratio was significantly higher in postmenopausal than in premenopausal breast cancer tissue specimens. They suggested an association between up-regulation of 17\textsuperscript{b}HSD\textsubscript{1} and intratumoral estradiol levels in postmenopausal patients. Gunnarsson et al. (2001) also demonstrated that breast cancer patients with high levels of 17\textsuperscript{b}HSD\textsubscript{1} mRNA or loss of 17\textsuperscript{b}HSD\textsubscript{2} mRNA expression were associated with increased risk of developing a late relapse of breast cancer.

Both 17\textsuperscript{b}HSD\textsubscript{1} and 17\textsuperscript{b}HSD\textsubscript{2} immunoreactivity was focally detected in the epithelium of normal mammary glands (Ariga et al. 2000). In breast cancers, 17\textsuperscript{b}HSD\textsubscript{1} immunoreactivity was detected in carcinoma cells in 47–61% of cases (Poutanen et al. 1992a, Sasano et al. 1996, Suzuki et al. 2000a) (Fig. 4C) (Table 2). Suzuki et al. (2000a) reported that 17\textsuperscript{b}HSD\textsubscript{1} immunoreactivity was significantly correlated with ER\textalpha and progesterone receptor (PR), and inversely associated with histological grade and Ki67. No significant association was reported between 17\textsuperscript{b}HSD\textsubscript{1} immunoreactivity and menopausal status in breast cancers in these previous reports, although Miyoshi et al. (2001) reported this correlation employing quantitative real-time PCR. This discrepancy may partly be due to the fact that immunohistochemical results do not necessarily reflect the protein amount. Suzuki et al. (2000a) reported no 17\textsuperscript{b}HSD\textsubscript{2} immunoreactivity in 111 breast carcinoma tissues examined.

<table>
<thead>
<tr>
<th>Study</th>
<th>Characteristics of antibody</th>
<th>Pretreatment</th>
<th>Predominant localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Esteban et al. 1992</td>
<td>Rabbit polyclonal (provided by Dr N Harada)</td>
<td>Trypsin</td>
<td>Carcinoma cells</td>
</tr>
<tr>
<td>Sasano et al. 1994</td>
<td>Rabbit polyclonal</td>
<td>None</td>
<td>Stromal cells</td>
</tr>
<tr>
<td>Santen et al. 1994</td>
<td>Rabbit polyclonal</td>
<td>None</td>
<td>Stromal &gt; carcinoma cells and normal breast elements</td>
</tr>
<tr>
<td>Lu et al. 1996</td>
<td>Mouse monoclonal (provided by Dr E Simpson)</td>
<td>Microwave</td>
<td>Carcinoma &gt; stromal cells</td>
</tr>
<tr>
<td>Shenton et al. 1998</td>
<td>Mouse monoclonal (provided by Dr E Simpson)</td>
<td>Microwave</td>
<td>Carcinoma and stromal cells</td>
</tr>
<tr>
<td>Brodie et al. 2001</td>
<td>Mouse monoclonal (provided by Dr E Simpson)</td>
<td>Microwave</td>
<td>Carcinoma cells</td>
</tr>
<tr>
<td>Sasano et al. 2003</td>
<td>Mouse monoclonal (677)</td>
<td>None</td>
<td>Stromal, carcinoma, and normal epithelial cells</td>
</tr>
<tr>
<td></td>
<td>Mouse monoclonal (F2)</td>
<td>Autoclave</td>
<td>Stromal, carcinoma, and normal epithelial cells</td>
</tr>
</tbody>
</table>

**17\textsuperscript{b}HSD\textsubscript{1} and 17\textsuperscript{b}HSD\textsubscript{2}**

17\textsuperscript{b}HSD catalyzes an interconversion of estrogens or androgens. To date, 12 isozymes of 17\textsuperscript{b}HSD have been cloned, and 17\textsuperscript{b}-reduction (17\textsuperscript{b}HSD\textsubscript{1}, 3, 5, 7 etc) or oxidation (17\textsuperscript{b}HSD\textsubscript{2}, 4, 6 etc) of estrogens and/or androgens is catalyzed by different 17\textsuperscript{b}HSD iso-zymes. Among these isozymes, 17\textsuperscript{b}HSD\textsubscript{1} enzyme uses NADPH as a cofactor, and is considered mainly to catalyze the reduction of estrone to estradiol (Peltoketo et al. 1988, Luu-The et al. 1989). On the other hand, the 17\textsuperscript{b}HSD\textsubscript{2} enzyme uses NAD\textsuperscript{+} as a cofactor, and catalyzes the oxidation of both estradiol to estrone and testosterone to androstenedione (Wu et al. 1993) (Fig. 2). Oxidative 17\textsuperscript{b}HSD activity is the preferential direction in normal breast tissues, but the reductive 17\textsuperscript{b}HSD pathway is dominant in breast cancers (Speirs et al. 1998, Miettinen et al. 1999).
The gene coding for 17βHSD1 (HSD17B1) is located at 17q12-21, and frequent genetic rearrangement is known in this region (Kauraniemi et al. 2001). Recently, Gunnarsson et al. (2003) detected amplification of HSD17B1 in 32 (15%) out of 221 post-menopausal breast cancers. This gene amplification was related to decreased breast cancer survival for ER-positive patients who received adjuvant tamoxifen. Gunnarsson et al. (2003) also demonstrated that some carcinomas without HSD17B1 amplification expressed 17βHSD1 mRNA at high levels, suggesting other regulatory mechanisms for 17βHSD1 mRNA.

Figure 4 Immunolocalization of sex-steroid producing enzymes in breast carcinoma tissue. (A) Aromatase immunoreactivity was detected in the cytoplasm of stromal cells adjacent to the carcinoma cells when the rabbit polyclonal antibody kindly provided by Dr N Harada (same antibody as used by Sasano et al. 1994) was used. (B) On the other hand, aromatase immunoreactivity was also detected in the cytoplasm of carcinoma cells when the mouse monoclonal (677) was used (same antibody as used by Sasano et al. 2003). (C–F) Immunohistochemistry for 17βHSD1 (C), STS (D), 17βHSD5 (E) and 5α-reductase type 1 (F) in breast carcinoma. Immunoreactivity for these enzymes was observed in the cytoplasm of carcinoma cells. Bars = 50 μm.
expression in breast carcinomas. Retinoic acid induces the expression of 17βHSD1 mRNA in T47D breast cancer cells (Reed et al. 1994), and a significant correlation was detected between retinoic acid receptor (RAR)-α and 17βHSD1 immunoreactivity (Suzuki et al. 2001). In addition, Simard and Gingras (2001) reported that IL-4 and IL-6 increased oxidative 17βHSD activity in ZR-75-1 cells, but IL-4 stimulated the reductive 17βHSD activity in T-47D cells. The effects of cytokines on 17βHSD activity appear to vary in different cell lines, and cytokines are considered to play important roles in the modulation of 17βHSD activity in breast carcinoma tissues (Purohit et al. 2002). An induction of 17βHSD1 expression by progestins has also been reported in several breast cancer cells (Poutanen et al. 1992a, Pasqualini 2003), which may partly explain the proliferative effects of progestins on breast cancer cells through the accumulation of estrogenic actions; however, further examinations are required to establish this hypothesis (Pasqualini 2003).

STS

STS is a single enzyme that hydrolyzes several sulfated steroids such as estrone sulfate, dehydroepiandrosterone (DHEA) sulfate, and cholesterol sulfate (Reed et al. 2005). A major circulating form of plasma estrogens in postmenopausal women is estrone sulfate, a biologically inactive form of estrogen. Estrone sulfate has a relatively long half-life in the peripheral blood, and the levels of estrone sulfate are 5 to 10 times higher than those of unconjugated estrogens such as estrone, estradiol and estriol during the menstrual cycle and in postmenopausal women (Pasqualini 2004). STS catalyzes estrone sulfate to estrone in breast carcinoma (Fig. 2), which contributes to local estrogen production. The enzymatic activity of STS is detected in the great majority of breast tumors, and is considerably higher than aromatase activity in breast tumors (Santner et al. 1984, Evans et al. 1994). Evans et al. (1994) reported no significant association between STS activity and time to recurrence or overall survival time in breast cancer patients examined in their study. STS activity was correlated with the level of STS mRNA expression in breast cancer cells (Pasqualini et al. 1994). The STS mRNA expression was higher in breast carcinoma tissues than that in normal tissues (Utsumi et al. 1999), which is also consistent with the findings of STS activity in breast cancers described above. Furthermore, STS mRNA expression has been reported to be significantly associated with poor clinical outcome of patients (Utsumi et al. 1999, Miyoshi et al. 2003). Reed et al. (2005) proposed that the sulfatase pathway might be more important than the aromatase route for intratumoral estrogen synthesis in breast cancers, because aromatase mRNA expression was reported to have no significant prognostic value.

STS mRNA expression was detected in breast carcinoma cells, but not in intratumoral stromal cells, by LCM/real-time PCR (Suzuki et al. 2003), which was consistent with immunohistochemical findings. STS immunoreactivity was detected in carcinoma cells in 59–88% of breast carcinoma cases (Saeki et al. 1999, Suzuki et al. 2003, Yamamoto et al. 2003) (Fig. 4D) (Table 3), and STS immunoreactivity was significantly associated with its mRNA level (Suzuki et al. 2003).

Table 2 Summary of immunohistochemical analysis of 17αHSD1 in breast carcinoma tissues

<table>
<thead>
<tr>
<th>Study</th>
<th>No. of cases examined</th>
<th>% of 17αHSD1 positive cases</th>
<th>Association between 17αHSD1 immunoreactivity and clinicopathological parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poutanen et al. 1992a</td>
<td>34</td>
<td>47%</td>
<td>Positive association: PR</td>
</tr>
<tr>
<td>Sasano et al. 1996</td>
<td>41</td>
<td>56%</td>
<td>None</td>
</tr>
<tr>
<td>Suzuki et al. 2000</td>
<td>111</td>
<td>61%</td>
<td>Positive association: ERα, PR, PR</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Inverse association: Histological grade, Ki67</td>
</tr>
</tbody>
</table>

Table 3 Summary of immunohistochemical analysis of STS in breast carcinoma tissues

<table>
<thead>
<tr>
<th>Study</th>
<th>No. of cases examined</th>
<th>% of STS positive cases</th>
<th>Association between STS immunoreactivity and clinicopathological parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saeki et al. 1999</td>
<td>25</td>
<td>88%</td>
<td>None</td>
</tr>
<tr>
<td>Suzuki et al. 2003</td>
<td>113</td>
<td>74%</td>
<td>Positive association: Tumor size, increased risk of recurrence</td>
</tr>
<tr>
<td>Yamamoto et al. 2003</td>
<td>83</td>
<td>59%</td>
<td>None</td>
</tr>
</tbody>
</table>
STS immunoreactivity was correlated with tumor size, and was significantly associated with an increased risk of recurrence (Suzuki et al. 2003). No significant correlation has been reported between STS and ER status in human breast cancer tissues in these studies. STS also allows the conversion of DHEA sulfate to DHEA; therefore, this conversion may be partly associated with a local accumulation of DHEA, a precursor substrate for both active estrogens and androgens, from circulating DHEA sulfate in breast cancer tissue.

Little is known about the regulatory mechanism of STS in breast cancers. IL-6 and TNFα stimulated STS activity and acted synergistically to increase enzyme activity, possibly via a post-transcriptional modification of the enzyme (Newman et al. 2000). In addition, progestins and anti-estrogens have been reported to inhibit the expression and/or activity of STS in breast cancer cells (Pasqualini & Chetrite 2005).

### 3β-Hydroxysteroid dehydrogenase

3β-Hydroxysteroid dehydrogenase (3βHSD) is a membrane-bound enzyme responsible for the interconversion of 3β-hydroxy- and 3-keto-5α-androstane steroids. Two isoforms of 3βHSD, i.e. 3βHSD1 and 3βHSD2, have been characterized in humans. 3βHSD2 is mainly expressed in the adrenal glands and gonads (Rheaume et al. 1991), and is a crucial step in the biosynthesis of various steroid hormones such as progesterone, estrogens, androgens, glucocorticoids and mineralocorticoids. On the other hand, 3βHSD1 is predominantly expressed in the placenta and various non-classical steroidogenic tissues such as skin and breast (Rheaume et al. 1991). In the non-classical steroidogenic tissues, 3βHSD is considered mainly to catalyze DHEA into androstenedione, and to increase the local tissue levels of androstenedione, a precursor substrate for both bioactive estrogens and androgens (Labrie et al. 2003) (Fig. 2). Therefore, 3βHSD may also play a part in the initial step of the intracrine transformation in breast carcinoma. Limited information is available on the expression of 3βHSD in breast carcinoma tissues. However, 3βHSD activity has been detected in breast carcinomas (Gunasegaram et al. 1998). Sasano et al. (1994) reported that 3βHSD immunoreactivity, which recognized both 3βHSD isoforms, was localized in breast carcinoma cells in 12 (33%) of 33 breast cancer tissues, but was not significantly associated with the ER or PR status.

3βHSD activity has been detected widely among peripheral tissues, although 3βHSD protein itself was not necessarily detected (Milewich et al. 1991). 17βHSD2 also possesses 3βHSD activity, and can catalyze DHEA to androstenedione (Suzuki et al. 2000b). 17βHSD2 expression appears to be negligible in breast cancer tissues, but dual activity of certain steroid-specific oxidoreductases may be associated with 3βHSD activity in breast cancers.

Previous studies demonstrated enhancement of 3βHSD2 transcription by SF1 (Leers-Sucheta et al. 1997) or LRH-1 (Sirianni et al. 2002). Expression of 3βHSD1 was also regulated by cAMP or protein kinase-C (Tremblay & Beaudoin 1993). However, in contrast to 3βHSD2, regulation of 3βHSD1 by SF1 has not been clarified. The regulatory mechanism of 3βHSD1 in human breast cancers is unclear, but Gingras et al. (1999, 2000) reported induction of 3βHSD1 gene transcription by IL-4 and IL-13 in breast cancer cells.

### Estrogen-metabolizing enzymes: estrogen sulfotransferase

The potency of steroid hormones is generally reduced in the metabolic process towards inactive products in the same tissue sites as the synthesis and/or action, which contributes to the modulation of overall biological actions of steroid hormones. Therefore, it is very important to examine the expression of estrogen-metabolizing enzymes as well as that of estrogen-producing enzymes to assess the local estrogen levels in breast cancers.

Estrogen sulfotransferase (EST; SULT1E1) is a member of the superfamily of steroid-sulfotransferases; it sulfonates estrogens to biologically inactive estrogen sulfates (Aksoy et al. 1994, Falany et al. 1995) (Fig. 2). EST has the lowest Kₘ values for estrogens of the 10 known human sulfotransferase (SULT) isoforms (Adjei et al. 2003), and is also considered to be involved in the regulation of in situ estrogen levels in human breast carcinoma. The concentration of estrone sulfate was significantly (7–11 times) higher in breast cancer tissues than in plasma (Pasqualini et al. 1996), and the enzymatic activity of EST was detected in some breast cancer cell lines (Falany & Falany 1996, Chetrite et al. 1998), breast carcinoma tissues, and normal breast tissues (Adams et al. 1979, Tseng et al. 1983). EST enzymatic activity was associated with ER status in breast cancer tissues (Adams et al. 1979, Tseng et al. 1983). MCF7 breast cancer cells transfected with EST possess EST activity at levels similar to normal human mammary epithelial cells, and are associated with much lower estrogen-stimulated DNA synthesis or cell proliferation than control MCF7 cells that do not possess EST, suggesting that the
loss of EST expression in the transformation of normal breast tissues to breast cancer may be an important factor in increasing the growth responsiveness of pre-neoplastic or tumor cells to estrogen stimulation (Qian et al. 1998, Falany et al. 2002).

EST mRNA expression was detected in breast cancer tissues (Suzuki et al. 2003, Yoshimura et al. 2004), and was significantly associated with EST immunoreactivity (Suzuki et al. 2003). EST immunoreactivity was detected in carcinoma cells in 44% of human breast carcinomas, and was also present in the epithelial cells of normal glands (Suzuki et al. 2003). EST immunoreactivity was inversely correlated with tumor size or lymph node status, and was significantly associated with a decreased risk of recurrence or improved prognosis (Suzuki et al. 2003). However, EST immunoreactivity was not significantly correlated with ER status in breast cancer tissues (Suzuki et al. 2003), which was inconsistent with the results of the EST enzymatic activity described above (Adams et al. 1979, Tseng et al. 1983). It may be partly due to the fact that other members of the steroid-sulfotransferase superfamily, such as thermostable phenol sulfotransferase (P-PST; SULT1A1) and the monoamine sulfating form of phenol sulfotransferase (M-PST; SULT1A3) can also sulfonate estrogens to estrogen sulfates. Spink et al. (2000) reported expression of P-PST and M-PST in several breast cancer cell lines, but the biological significance of P-PST and M-PST in breast cancer tissues remains largely unclear at this juncture.

Other estrogen-metabolizing enzymes: CYP1A1, CYP1B1, and CYP3A4

The CYP superfamily is classified into families and subfamilies based on amino acid similarity, and 14 families have been reported in mammals. CYPs are involved in synthesis of steroid and bile acids and hydroxylation of fatty acids, or elimination of xenobiotics and steroids from the body (Bistolas et al. 2005). Among the CYP superfamily, CYP1A1, CYP1B1, and CYP3A4 oxidatively metabolize estradiol, and these findings suggest the possible association with regulation of local estrogen levels in breast cancer tissues.

CYP1A1 catalyzes C-2, C-6α and C-15α hydroxylation of estradiol. CYP1A1 mRNA expression was detected in 25–46% of normal breast tissues (Huang et al. 1996, Iscan et al. 2001) and 5–53% of breast carcinoma tissues (Huang et al. 1996, Hellmold et al. 1998, Iscan et al. 2001). Using quantitative RT-PCR analysis, Modugno et al. (2003) reported that CYP1A1 mRNA expression was elevated in non-tumor tissue among pairs in which the tumor expressed ER. In immunoblotting analysis, CYP1A1 protein was detected in 36% of breast cancer tissues (Hellmold et al. 1998), and the CYP1A1 protein level was significantly lower in breast cancer tissue as compared with morphologically normal adjacent tissues (El-Rayes et al. 2003).

On the other hand, CYP1B1 shows activity towards the C4-hydroxylation of estradiol. Expression of CYP1B1 mRNA was detected in 100% of breast carcinoma tissues (Hellmold et al. 1998, Iscan et al. 2001), and the level of expression was significantly higher in non-tumor tissues than in tumor tissues (Modugno et al. 2003). CYP1B1 immunoreactivity was detected in carcinoma cells in 77–82% of breast cancers by immunohistochemistry (McFadyen et al. 1999, Oyama et al. 2005). McFadyen et al. (1999) found no significant association between CYP1B1 immunoreactivity and clinicopathological factors, including tumor grade and ER status in the breast carcinomas, while Oyama et al. (2005) reported an inverse correlation between CYP1B1 immunoreactivity and clinical stage in breast cancers.

CYP1A1 and CYP1B1 metabolize not only estrogens but also some environmental carcinogens. It is well known that CYP1A1 and CYP1B1 are under the regulation of aryl hydrocarbon receptor (AhR), and these two enzymes are induced by AhR agonists such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Angus et al. 1999, Kristensen & Borresen-Dale 2000). However, CYP1B1 expression is constitutively detected in breast carcinoma tissues as described above, and CYP1B1 is considered as a major form of the CYP1 family in breast cancer. Regulation of CYP1A1 and CYP1B1 expression by estrogen has also been reported in breast cancer cells (Angus et al. 1999). Recently, Tsuchiya et al. (2004) demonstrated the induction of CYP1B1 mRNA expression by estradiol in ER-positive MCF7 cells, but not ER-negative MDA-MB-435 cells, and suggested that the induction of CYP1B1 mRNA was caused by the ER-mediated pathway rather than the AhR-mediated pathway.

CYP3A4 plays a pivotal role inactivating and detoxifying various xenobiotics and endobiotics. CYP3A4 also catalyzes the C2, C4, C6β, C12, C15α, and C16β-hydroxylation of estrogen. Several groups reported the expression of CYP3A in breast carcinoma tissues, but these results appear to be inconsistent. Hellmold et al. (1998) detected CYP3A4 mRNA expression in 73% of breast carcinoma tissues, and Modugno et al. (2003) reported that the expression level of CYP3A4 mRNA was significantly higher in
non-tumor tissues than in tumor tissues. However, Iscan et al. (2001) reported no mRNA expression of CYP3A4 in breast tumor tissues or normal breast tissues. In immunoblotting analysis, El-Rayes et al. (2003) reported that CYP3A4 protein levels were significantly lower in breast carcinoma tissues than in morphologically normal adjacent tissues, while Hellmold et al. (1998) did not detect CYP3A protein in 15 breast carcinoma tissues. In immunohistochemical analysis, CYP3A4 immunoreactivity was detected in 84–100% of breast carcinoma tissues, and normal mammary epithelium was focally positive (Galant et al. 2001, Kapucuoglu et al. 2003). Galant et al. (2001) reported an inverse association between CYP3A immunoreactivity and the proliferation index. On the other hand, Oyama et al. (2005) did not detect CYP3A immunoreactivity in 34 cases of Japanese patients with breast cancers, and suggested that, based on their findings, CYP3A expression in breast cancer was possibly dependent on the ethnicity of the patient.

CYP3A4 gene expression is induced by nuclear receptors such as steroid and xenobiotic receptor/pregnane X receptor (SXR/PXR; NR1I2) and constitutive androstane receptor (CAR; NR1I3), and is repressed by proinflammatory cytokines in hepatocytes (Raunio et al. 2005). Masuyama et al. (2003, 2005) reported induction of CYP3A4 by SXR/PXR in endometrial cancer, but the regulation mechanism of CYP3A4 in breast cancer still remains unclear.

4-Hydroxy-estradiol, which is metabolized by CYP1B1 or CYP3A4 from estradiol, is further converted to the 3,4 estradiol quinone. This compound is recognized as a genotoxic mutagenic carcinogen, and possibly induces breast cancer (Liehr 2000). Therefore, metabolism of estradiol by CYP1B1 and CYP3A4 may not necessarily be associated with reducing the progression of breast tumors. Further examinations are required to clarify the biological significance of CYP1B1 and CYP3A4 in human breast cancer tissues.

**Intratumoral production of androgens in breast cancer**

Various previous studies demonstrated that androgens predominantly exerted anti-proliferative effects on the mitogenic effects of estrogens in breast cancer cell lines, although some divergent findings have been reported according to the specific cell line used, the androgen used and its dose, and estrogen status (Ortmann et al. 2002, Somboonporn & Davis 2004). This inhibitory effect is mediated by AR, and is partly associated with increased levels of p21 and/or p27 (Lapointe & Labrie 2001, Greeve et al. 2004). Previously, Isola (1993) reported that approximately 80% of breast carcinomas expressed AR, suggesting the presence of androgenic actions in human breast carcinoma tissues. Plasma concentrations of potent androgens such as 5α-dihydrotestosterone (DHT) are very low in normal women and in breast cancer patients (Labrie et al. 2003). However, DHT concentrations were significantly (threefold) higher in breast cancer tissues than in plasma (Recchione et al. 1995), suggesting the possible local production of DHT and an important biological role of DHT in breast carcinoma tissues. Two steroidogenic enzymes, namely 17βHSD5 and 5α-reductase, are considered to be the main enzymes involved in local androgen production in human breast cancer tissues (Fig. 2).

**17βHSD5**

It is well known that testosterone is mainly secreted from the Leydig cells of the testis, and it is biosynthesized from androstenedione by 17βHSD3 (Geissler et al. 1994). However, testicular Leydig cells provide approximately 50% of the total amount in men, and the rest of the amount is converted from circulating androstendione in peripheral tissues (Labrie et al. 2003). 17βHSD3 is predominantly expressed in the testis, while the same enzymatic reaction in peripheral tissues is catalyzed by different enzymes, namely 17βHSD5 (Duflot et al. 1999). 17βHSD5 is identical to 3αHSD2. 17βHSD5 is a member of the aldo-keto reductase (AKR) superfamily, and is formally termed AKR1C3, while 17βHSD1 to 3 are members of the short-chain dehydrogenase/reductase (SDR) superfamily (Penning et al. 2001).

mRNA expression of 17βHSD5 was detected in 65–83% of breast carcinoma tissues (Ji et al. 2004, Vihko et al. 2005). In particular Vihko et al. (2005) reported that 17βHSD5 mRNA expression was significantly higher in breast tumor specimens than in normal tissues. They also demonstrated that a group of patients with overexpression of 17βHSD5 mRNA had a worse prognosis than other patients (Vihko et al. 2005). 17βHSD5 immunoreactivity was detected in normal mammary gland (Pelletier et al. 1999) and breast carcinoma cells in 53% of cases (Suzuki et al. 2001a) (Fig. 4E). Immunoreactivity of 17βHSD5 was significantly associated with that of 5α-reductase type 1 and type 2 (Suzuki et al. 2001a), but was not significantly associated with other clinicopathological factors such as patient age, menopausal status, clinical stage, tumor size, lymph node status, histological...
grade, ER, PR, AR, Ki67, and HER2, examined in 60 breast carcinoma tissues (T Suzuki, Y Miki, Y Nakamura, T Moriya, K Ito, N Ohuchi, H Sasano, unpublished data).

17βHSD5 also possesses 3αHSD and 20αHSD activities (Luu-The et al. 2001). The 3αHSD and 20αHSD activities are involved in the inactivation of progesterone (Wiebe et al. 2000, Luu-The et al. 2001, Suzuki et al. 2002). The biological significance of 17βHSD5 in these activities, however, still remains unclear in breast cancer tissues.

5α-Reductases

5α-Reductase catalyzes the conversion of testosterone to a more potent androgen DHT (Russell & Wilson 1994) (Fig. 2), and is considered as an important regulator of local actions of androgens. Two isoforms of 5α-reductase have been cloned and characterized in mammals. 5α-Reductase type 1 is located on the distal short arm of chromosome 5, and is mainly expressed in the liver and skin (Russell & Wilson 1994, Jin & Penning 2001). On the other hand, type 2 5α-reductase is located in band p23 of chromosome 2, and is expressed in the liver, prostate, seminal vesicle, and epididymis (Russell & Wilson 1994, Jin & Penning 2001).

Activity of 5α-reductase was previously detected in human breast carcinoma cell lines (MacIndoe & Woods 1981), and 5α-reductase activity was elevated 4–8 times in breast cancer tissues compared with non-tumorous breast tissues (Wiebe et al. 2000). mRNA expression of 5α-reductase type 1 was detected in all the breast carcinoma tissues examined (Suzuki et al. 2001a, Lewis et al. 2004, Ji et al. 2004), while that of 5α-reductase type 2 was detected in 38–100% of the tumors (Suzuki et al. 2001a, Lewis et al. 2004). Lewis et al. (2004) also demonstrated that mRNA expression levels of 5α-reductase type 1 and type 2 were significantly higher in the tumors than in corresponding normal tissues. Immunoactivity for 5α-reductase type 1 was detected in 58% of breast carcinomas (Fig. 4F), while that of 5α-reductase type 2 was detected in only 15% of breast carcinomas (Suzuki et al. 2001a), suggesting that 5α-reductase type 1 may mainly determine 5α-reductase activity in breast carcinoma tissues. 5α-Reductase type 1 immunoactivity was significantly correlated with AR, and inversely associated with histological grade or tumor size in breast carcinoma tissues (Suzuki et al. 2001a). Therefore, breast carcinomas positive for 5α-reductase type 1 may partly maintain some androgen regulatory mechanisms.

5α-Reductase metabolizes progesterone to 5α-dihydroprogesterone (5α-DHP) (Russell & Wilson 1994), suggesting that this enzyme is also involved in the local regulation of progesterone actions. Wiebe et al. (2000) reported that in breast cancer progesterone was metabolized to 5α-DHP and 3α-hydroxyprogesterone (3α-HP) by 5α-reductase and 3α-HSD respectively, and the ratio of 5α-DHP:3α-HP was nearly 30-fold higher in tumorous than in non-tumorous breast tissues. They also reported that 5α-DHP stimulated, whereas 3α-HP inhibited, proliferation and detachment of breast cell lines in vitro (Wiebe et al. 2000). These findings suggest that some progesterone metabolites exhibit different bioactive properties from progesterone, and 5α-reductase may be partly associated with the proliferation effect of progesterone in breast cancer cells (Wiebe et al. 2005).

Regulation of in situ estrogen production in breast carcinomas as an endocrine therapy

It is well known that estrogen deprivation therapy is an effective treatment for breast cancer, and various types of endocrine therapy are currently available in breast cancer patients (Fig. 5). Ovarian suppression is important for the treatment of premenopausal breast
cancers, and ovarian ablation or treatment with luteinizing hormone releasing hormone (LH-RH) agonists is frequently considered in premenopausal patients (Robertson & Blamey 2003). On the other hand, since biological effects of estrogens are mediated through ER, anti-estrogens such as tamoxifen have been used as endocrine therapy in hormone-receptor-positive breast carcinomas of both pre- and postmenopausal women (Eneman et al. 2004).

The importance of in situ estrogen production has been demonstrated in breast carcinomas, as described in the above sections of this review, and the inhibition of this pathway is considered clinically useful for reducing the progression of breast tumors especially in postmenopausal women. The third-generation aromatase inhibitors, such as anastrozole, letrozole and exemestane, are currently available (Brueggemeier et al. 2005). Results of large multicenter trials such as the ATAC trial, the NCIC MA-17 trial, and the Intergroup Exemestane Study, all demonstrated that aromatase inhibitors are significantly associated with the improved disease-free survival and good tolerability in breast cancer patients (Baum et al. 2002, 2003, Goss et al. 2003, Baum 2004, Coombes et al. 2004, Howell et al. 2005), and anastrozole demonstrated superior efficacy to tamoxifen in the ATAC trial. ER status in the breast carcinoma is the most influential parameter to determine the administration of aromatase inhibitors at this juncture. However, it is true that additional factors are required to improve the clinical effects of aromatase inhibitor in breast cancer patients. Previous studies reported an association between aromatase activity in breast carcinoma tissues and the response to treatment with aromatase inhibitors (Bezwoda et al. 1987, Miller & O’Neill 1987), but it may not necessarily be a useful surrogate marker to determine clinically the treatment of aromatase inhibitor, because of the lack of robust assays for tumor aromatase (Miller et al. 2003). In this regard, immunohistochemistry for aromatase is expected to be the attractive routine method, considering the great success in detecting ER, PR and HER2 in breast cancer tissues. However, further examinations are certainly required to establish a standardized approach, including the determination of aromatase antibody, the immunohistochemical procedure and the evaluation system. Cyclooxygenase-2 (COX2) is partly associated with the synthesis of PGE₂, a potent stimulator of aromatase, in breast cancers (Diaz-Cruz et al. 2005), and therefore, clinical trials are currently underway to examine synergistic effects between COX2 inhibitor and aromatase inhibitor in postmenopausal women (Arun & Goss 2004).

Table 4 Association among immunoreactivity of enzymes related to intratumoral estrogen production and ERs in breast carcinoma tissues

<table>
<thead>
<tr>
<th>Immunoreactivity</th>
<th>17αHSD1</th>
<th>STS</th>
<th>EST</th>
<th>ERα</th>
<th>ERα</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aromatase</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NA</td>
</tr>
<tr>
<td>17αHSD1</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.05</td>
<td>&lt;0.05*</td>
</tr>
<tr>
<td>STS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>EST</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>ERα</td>
<td>NA</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.01*</td>
</tr>
</tbody>
</table>

Data were taken from a series of our examinations (Sasano et al. 1994, Sasano et al. 1996, Suzuki et al. 2000a, Suzuki et al. 2003), and adapted.

Aromatase is a key enzyme of intratumoral production of estrogen in breast cancers. However, Yamaguchi et al. (2005) reported that estrogen signals in breast cancer cells were not always correlated with aromatase expression in stromal cells of carcinoma tissues, suggesting that even complete suppression of aromatase does not fully block the estrogenic actions in breast cancer tissues. When we examined an association among the immunoreactivity of enzymes related to intratumoral production and ERs in breast carcinoma tissues, no significant association was detected (Table 4). Therefore, enzymes responsible for the intratumoral production of estrogen may not always be the same among breast cancer patients, and other estrogen-producing enzymes, with the exception of aromatase, including STS and 17βHSD1 may also have important therapeutic potential as endocrine therapy for total blockade of local estrogen in breast cancer tissues. STS inhibitors were reported to be effective in suppressing the proliferation of estrogen-dependent MCF7 cells when estrone sulfate was the source of estrogen, and STS inhibitors are currently being developed by several groups (Nussbaumer & Billich 2004, Reed et al. 2005). The design of 17βHSD1 inhibitors has also been attempted (Qiu et al. 2002, Poirier 2003).

Sakamoto et al. (2002) showed that proliferation of ER-positive breast cancer cells increased in medium to which average levels of postmenopausal plasma steroids had been added, but the growth was not stimulated in the transformed cells overexpressing 17βHSD2 or EST in the same medium. These findings suggest that induction of 17βHSD2 or EST can also effectively contribute to the decrement of intratumoral estrogen production in breast cancer. In addition, Pasqualini (2003) reported that the progesterin,
medrogestone, stimulates EST in breast cancer cells through decreasing estrogen-dependent cell proliferation. Therefore, induction of estrogen-metabolizing enzymes is also considered to result in a decrement of estrogenic actions in breast carcinoma tissues, which may eventually contribute to an improvement in the prognosis of breast cancer patients.

Activation of ER by growth factors, such as epidermal growth factor (EGF), insulin-like growth factor-I (IGF-I), and transforming growth factor-α (TGFα) (Surmacz & Bartucci 2004, Osborne et al. 2005) has also been demonstrated. HER2-overexpressing breast cancers frequently become resistant to tamoxifen (Osborne et al. 2003), and aromatase inhibitors were more effective in these patients (Ellis et al. 2001). Cross-talk between ERα and growth factor receptor signals is postulated to be important especially in the mechanism of tamoxifen resistance in breast cancer, and double blockade using both ER-targeted therapies and therapies targeting the growth factor receptor cascade is currently being tested in clinical trials (Osborne et al. 2005).

In contrast to estrogens, androgens are generally known to inhibit the proliferation of breast carcinoma cells. A strong correlation between AR and ER was detected in breast cancer tissues (Isola 1993, Suzuki et al. 2001a), and frequently estrogen-dependent breast cancers are also dependent on androgenic actions. When androgens were combined with anti-estrogens in breast cancer patients, a higher response rate and a longer time to disease progression have been reported compared with the administration of an anti-estrogen alone, and the additive inhibitory effects of androgens and anti-estrogens on breast carcinoma are exerted, in part, by different mechanisms (Labrie et al. 2003). On the other hand, Sonne-Hansen and Lykkesfeldt (2005) recently reported that proliferation of MCF7 cells was significantly stimulated by testosterone, and the testosterone-mediated growth effect was completely inhibited by aromatase inhibitors. They also reported that androstenedione did not significantly stimulate MCF7 cell proliferation, and suggested that the preferred substrate for aromatase in MCF7 cells is not androstenedione but testosterone (Sonne-Hansen & Lykkesfeldt 2005). 17βHSD5 locally produces testosterone from androstenedione, and also converts progesterone to its inactive metabolite 20α-hydroprogesterone. Therefore, 17βHSD5 may partly be associated with a pro-estrogenic state (Penning et al. 2001) in addition to the in situ androgen production in breast carcinoma, and may also become a therapeutic target to decrease the intratumoral estrogen production in breast cancer.

The clinical significance of aromatase inhibitors demonstrated that inhibition of estrogen-producing enzymes is effective in reducing the progression of breast carcinomas, and the endocrine therapy for breast cancer could be improved if inhibitors for other related enzymes become clinically available. Therefore, it will become very important accurately to evaluate the expression of various sex-steroid producing enzymes in resected surgical pathology specimens, to determine the treatment by appropriate inhibitors for the enzymes in individual breast cancer patients.

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