Table 3. Clinicopathological features of differentially expressed miRNAs associated with endometrial serous adenocarcinoma

No. of samples	Median expression		
	16	5	
Feature	Vascular invasion absent	Vascular invasion present	*P*-values
miR-10b*	12.5	6.2	0.048
miR-29b	12.9	5	0.013
miR-455-5p	12.6	5.8	0.032

P-values of <0.05 were considered significant.

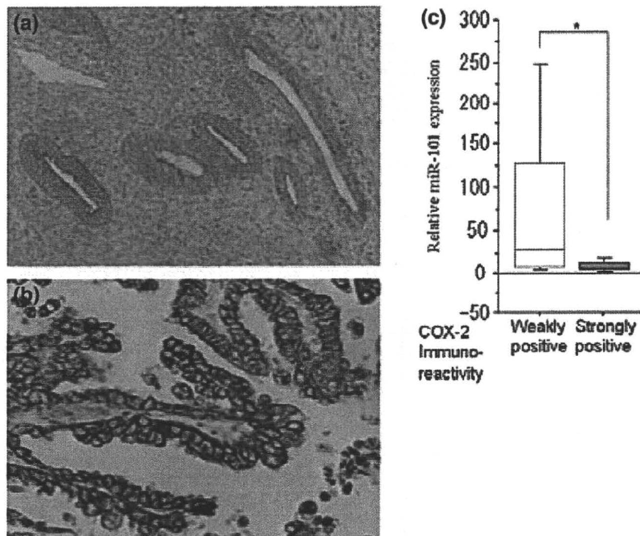

Fig. 3. Immunohistochemistry for cyclooxygenase (COX)-2 expression in endometrial tissues. (a) Immunoreactivity of COX-2 was weakly detected in the cytoplasm of normal endometrium glandular cells. (b) Strong immunoreactivity was detected in the cytoplasm of carcinoma cells. Original magnification, ×200 for (a) and (b). (c) miR-101 expression was significantly lower in tissues exhibiting COX-2 strong immunoreactivity. $^*P < 0.05$.

correlated with decreased overall survival (log-rank test, $P < 0.05$), whereas lower expression of miR-152, miR-29b, and miR-455-5p was significantly correlated with decreased progression-free survival. Multivariate analysis revealed that vascular invasion and miR-152 expression were statistically independent risk factors for overall survival ($P = 0.035$ and $P = 0.021$, respectively). Moreover, vascular invasion, miR-101 expression, and miR-152 expression were statistically independent factors for progression-free survival ($P = 0.018$, $P = 0.016$, and $P = 0.010$, respectively) (Table 4).

Restoration of miR-101 and miR-152 inhibits endometrial serous adenocarcinoma cell growth. SPAC-1-L cells were transfected with miR-101 and miR-152 precursor molecules, or a negative control to determine if either of the two miRNAs could suppress cancer cell growth. The proliferation assay revealed a significant reduction in cell growth following miR-101 ($P < 0.0001$) and miR-152 ($P = 0.01$) transfection (Fig. 5). The more striking decrease was observed after transfection of miR-101 precursor molecules. The functional analysis was performed in duplicate and each experiment was repeated independently in triplicate.

Discussion

We identified 54 miRNAs that are significantly down-regulated and 66 miRNAs that are significantly up-regulated in endometrial serous carcinoma compared to normal endometrial tissue. These miRNAs may therefore serve as potential markers for distinguishing endometrial serous carcinoma from normal endometrial tissue. Despite the cancer specimens being obtained from different patients, miRNA expression patterns were nearly homogenous across all cases. The endometrial serous adenocarcinoma miRNA expression profiles observed in the present study were consistent with the results of previous endometrial endometrioid cancer studies published by Boren *et al.* [25] and Wu *et al.*[26] Specifically, the observed down-regulation of miR-152 and miR-193 and the up-regulation of miR-106a, miR-205, miR-210, and miR-429 are in agreement with previous results, despite the histological difference between tissues. This concordance further supports our findings and underscores the relevance of these miRNAs in endometrial cancer. To the best of our knowledge, this is the first study to examine miRNA expression profiles and their association with clinical outcomes and prognosis in patients with endometrial serous adenocarcinoma.

The 21 endometrial serous adenocarcinoma tissues used for the miRNA microarray analysis constitute a limited sample size. However, they were all that fulfilled our designed criteria as histologically pure and typical from 2001 to 2006 at Tohoku University Hospital. The most consistently down- and up-regulated miRNAs in various cancers are miR-133a and miR-205, respectively. miR-133b has been shown to be significantly down-regulated in colorectal cancer,[27] although it is significantly up-regulated in gastric cancer.[28] Tongue squamous cell carcinoma cell lines transfected with miR-133a and miR-133b precursors display a reduction in proliferation rate. Computational target gene prediction has suggested that both miR-133a and miR-133b are target transcripts of pyruvate kinase type M2 (PKM2), a potential oncogene in solid cancers.[29] Wu *et al.*[26] reported that miR-205 is greatly enriched in endometrial endometrioid adenocarcinoma. These investigators reported that high levels of miR-205 expression are correlated with migration and invasion. Iorio *et al.*[30] demonstrated that DNA hypomethylation in ovarian tumors resulted in up-regulation of miR-205 compared to normal ovarian tissue. miR-205 has also been shown to be up-regulated in exosomes of ovarian serous carcinoma patients.[31] These results indicate that levels of these exosomal miRNAs are stable and do not significantly change with storage. The use of exosomal miRNA profiling could extend this approach to screening of asymptomatic individuals, as well as to monitoring disease recurrence.

Eleven miRNAs (miR-101, miR-10b*, miR-133a, miR-133b, miR-152, miR-29b, miR-34b, miR-411, miR-200a, miR-200b, and miR-205) were selected to validate the significance of their down- or up-regulation by microarray analysis or because of their reported interesting functions. Dysregulated miRNA expression may occur via a number of mechanisms, such as gene copy gain or loss.[32] germline mutation of precursor miRNA molecules,[17] promoter methylation,[33] aberrant miRNA processing due to altered expression of the miRNA biogenesis machinery,[34] or transcription factors.[35] In cancer specimens, the differential expression of nine out of 11 miRNAs compared to normal endometrial tissue expression reached statistical significance by qRT-PCR analysis. Thus, overall, the microarray data appear to be sufficient to warrant further analyses in the clinical setting.

The differential expression of some miRNAs was determined to be correlated with various clinicopathologic features unique to serous endometrial carcinoma. Among these miRNAs, miR-10b*, miR-29b, and miR-455-5p appear to be potentially involved in cancer progression. These were down-regulated in

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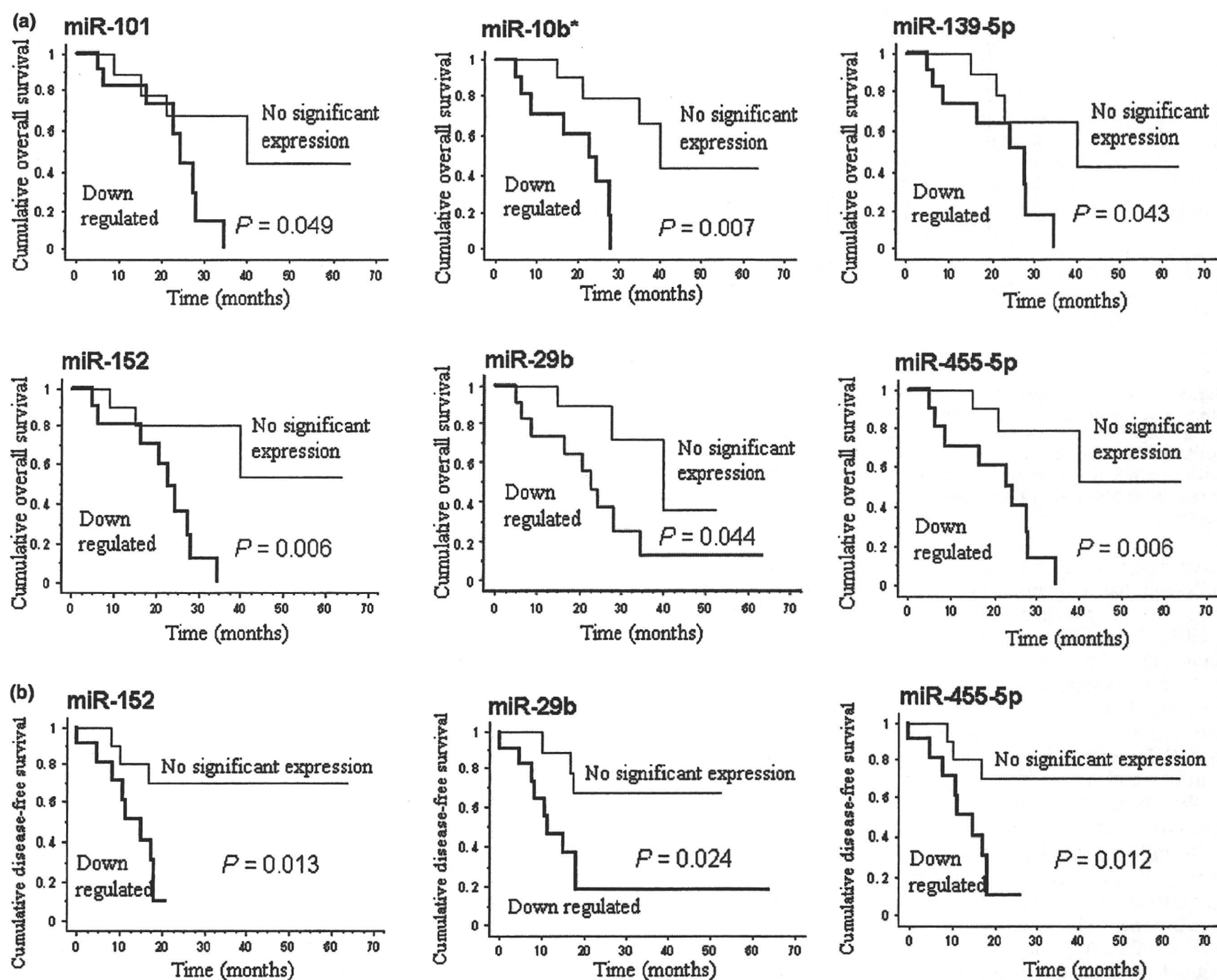

Fig. 4. Kaplan–Meier overall survival curve for patients with endometrial serous adenocarcinoma of based on miRNA expression. (a) Reduced expression of miRNA-101, miR-10b*, miR-139-5p, miR-152, miR-29b, and miR-455-5p was significantly correlated with shorter overall survival. (b) Decreased expression of miR-152, miR-29b, and miR-455-5p was significantly correlated with shorter disease-free survival. The log-rank test yielded significant *P*-values ($P < 0.05$).

Table 4. Multivariate analysis of predictors of overall survival and disease-free survival for endometrial serous adenocarcinoma patients

Variable	Overall survival		Disease-free survival	
	HR (95% CI)	*P*	HR (95% CI)	*P*
Stage (I/II *vs* III/IV)	0.318 (0.034 to 2.97)	0.315	0.170 (0.021 to 1.40)	0.099
Vascular invasion	33.0 (1.28 to 852.9)	0.035	53.2 (1.98 to 1425.4)	0.018
miR-101	189.5 (0.981 to 36586.7)	0.051	312.5 (2.969 to 32899.5)	0.016
miR-10b*	1.01 (0.037 to 27.4)	0.998	25.3 (0.832 to 767.7)	0.064
miR-139-5p	0.104 (0.005 to 2.10)	0.14	0.142 (0.010 to 2.00)	0.148
miR-152	0.005 (4.77E-5 to 0.440)	0.021	0.003 (4.37E-5 to 0.250)	0.01
miR-29b	3.65 (0.249 to 53.4)	0.345	5.83 (0.431 to 78.8)	0.185
miR-455-5p	0.349 (0.001 to 202.2)	0.746	0.033 (1.81E-4 to 6.04)	0.199

P-values of <0.05 were considered significant. HR, hazard ratio; 95% CI, 95% confidence interval.

endometrial serous adenocarcinoma patients with high vascular invasion, suggesting that their down-regulation occurs during the course of tumor progression and, in particular, during the acquisition of cancer metastatic potential.

Iorio *et al.*[18] found that miR-29 was down-regulated in aggressive breast cancer specimens, notably those that lacked estrogen and progesterone receptors (miR-29b) and those with vascular invasion (miR-29a). In contrast, other investigators

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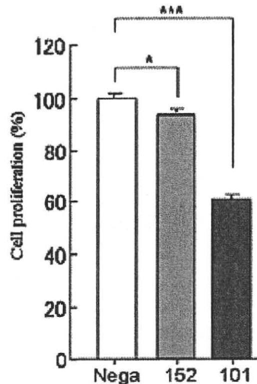

Fig. 5. Cell proliferation assay in SPAC-1-L cells. SPAC-1-L cells were transfected with pre-miR miR-101, pre-miR-152 precursor molecules, or a negative control for 72 h. Nega, transfected with negative control; 152, transfected with pre-miR-152 molecules; 101, transfected with pre-miR-101 molecules. Data are presented as the mean ± SD of three independent experiments. $*P < 0.05$ and $***P < 0.001$ *vs* controls.

have reported the up-regulation of miR-29b in breast cancer.[36] miR-29b has numerous predicted gene targets, including the majority of collagen mRNAs and insulin growth factor 1 (IGF1). In a cholangiocarcinoma cell line, miR-29b has been shown to down-regulate the expression of myeloid cell leukemia 1 (Mcl-1) protein, an anti-apoptotic member of the Bcl-2 family, although it does not affect Mcl-1 mRNA expression.[37] miR-29b acts directly at the Mcl-1 3′UTR, sensitizing cells to apoptosis.[37] Recently, Park *et al.*[38] reported that several miR-29 family members participate upstream of the p53 pathway.

We observed a reduction of cell growth upon transfection of miR-101 and miR-152 precursor molecules into SPAC-1-L cells. miR-152 has been shown to be involved in aberrant hypermethylation in breast cancer patients.[39] One of its proposed target mRNAs is that of mutL homologue 1 (*MLH1*), a mismatch repair gene. Orbo *et al.*[40] demonstrated that MLH1 expression was significantly decreased in endometrial specimens from patients with a subsequent or coexisting endometrial carcinoma. Additional targets of miR-152 include latent transforming growth factor-β binding protein-4 (LTBP-4),[41] as well as autotoxin (ENPP2),[42,43] both of which have been implicated in cellular processes related to either oncogenesis, cell survival, migration, metastasis, and/or clinical outcome of human cancers.

miR-101 down-regulation is involved in COX-2 overexpression in human colon cancer cells,[44] and genomic loss of miR-101 has been shown to lead to overexpression of the histone methyltransferase EZH2 in prostate cancer.[45] Mcl-1 has also been characterized as a direct target of miR-101,[46] and it is potentially involved in miR-101–regulated apoptosis. Transfection of miR-101 precursor molecules caused a much larger decrease in proliferation than the miR-152 precursors. Therefore, miR-101 may be involved in apoptotic mechanisms, including Mcl-1. In our series, COX-2 immunohistochemical overexpression was significantly correlated with down-regulation of miR-101. In endometrial carcinoma, several studies have shown that COX-2 overexpression is associated with carcinogenesis and clinical outcomes.[47,48] Recently, Chakrabarty *et al.*[49] reported that miR-101a post-transcriptionally suppresses COX-2 expression in a human cancer cell line. In addition, hepatocyte growth factor (HGF) induces anoikis resistance in endometrial cancer cells, possibly through PI3K/Akt pathway-dependent up-regulation of COX-2 expression.[48] Although control of COX-2 protein expression is complicated, our result might be used as further confirmation of COX-2 as a candidate target of miR-101 in patients with endometrial serous carcinoma. The data suggest that miR-101 and miR-152 affect cell proliferation and play important roles in controlling carcinogenesis in endometrial serous carcinoma.

Overall, our report contributes to the understanding of miRNA expression patterns and their relationship to tumorigenesis in endometrial serous carcinoma. The identification of miRNAs as oncogenic or pro-metastatic factors holds the promise of revealing new diagnostic markers for human cancers and, quite possibly, novel targets for antitumor therapies. In conclusion, our data may serve as a foundation for the development of new pharmacologic and biologic therapy approaches for endometrial serous adenocarcinoma. A larger sample size is required to confirm the results of this study and to correlate them with clinical outcomes in patients with endometrial serous adenocarcinoma.

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CLINICAL TRIAL

Increased estrogen sulfatase (STS) and 17β-hydroxysteroid dehydrogenase type 1(17β-HSD1) following neoadjuvant aromatase inhibitor therapy in breast cancer patients

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Abstract Aromatase inhibitors (AIs) are considered the gold standard for endocrine therapy of estrogen receptor (ER) positive postmenopausal breast cancer patients. The therapy may enhance therapeutic response and stabilize disease but resistance and disease progression inevitably occur in the patients. These are considered at least partly due to an emergence of alternative intratumoral estrogen production pathways. Therefore, in this study we evaluated effects of exemestane (EXE) upon the enzymes involved in intratumoral estrogen production including estrogen sulfatase (STS), 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1), and estrogen sulfotransferase (EST) and correlated the findings with therapeutic responses including Ki67 labeling index (Ki67). 116 postmenopausal patients with invasive ductal carcinoma, stage II/IIIa, were enrolled in JFMC34-0601 clinical trials between March, 2006 and January, 2008. EXE of 25 mg/day was administered according to the protocol. Pre- and posttreatment specimens of 49 cases were available for this study. Status of STS, EST, 17β-HSD1, ER, progesterone receptor (PgR), human epidermal growth factor receptor type 2 (Her2), and Ki67 in pre- and post-specimens were evaluated. Specimens examined before the therapy demonstrated following features; ER+ (100%), PgR+ (85.7%), and Her2+ (77.6%). After treatment, the number of Ki67, PgR, and ER positive carcinoma cells demonstrated significant decrement in clinical response (CliR) and pathological response (PaR) groups. Significant increment of 17β-HSD1 and STS immunoreactivity was detected in all groups examined except for STS in PaR. EST showed significant increment in nonresponsive groups. Alterations of Ki67 of carcinoma cells before and after therapy were subclassified into three groups according to its degrees. Significant alterations of intratumoral enzymes, especially 17β-HSD1 and STS, were correlated with Ki67 reduction after neoadjuvant EXE therapy. This is the first study demonstrating significant increment of STS and 17β-HSD1 following AI neoadjuvant therapy of postmenopausal ER positive breast

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carcinoma patients. This increment may represent the compensatory response of breast carcinoma tissues to estrogen depletion.

Keywords Aromatase inhibitors · Breast cancer · Exemestane · Estrogen sulfatase · 17β-hydroxysteroid dehydrogenase · Ki67

Introduction

Breast cancer is the most common malignancy among women worldwide and the leading cause of cancer-related death in many countries [1, 2]. Approximately 60% of premenopausal and 75% of postmenopausal patients have sex steroid hormone-dependent breast carcinoma [3, 4]. Among these sex steroids, estrogens, especially estradiol or E2, a biologically potent estrogen, play pivotal roles in cell proliferation, development, and invasion of these hormone-dependent breast carcinoma cells [3–5].

Intratumoral estrogen production in breast carcinoma tissues has been advocated by Miller et al. in 1974 [6]. Its clinical significance was in dispute but the presence of local production of estrogens in breast carcinoma tissues has been subsequently reported by others [7, 8]. Aromatase, one of the enzymes involved in estrogen production, was subsequently demonstrated in adipocytes, stromal cells, and carcinoma cells of breast cancer tissues [9–13]. In addition, the other enzymes involved in intratumoral estrogen production (steroid or estrogen sulfatase; STS, estrogen sulfotransferase; EST and 17β-hydroxysteroid dehydrogenase type 1; 17β-HSD1, etc.) have been also reported to be overexpressed in human breast carcinoma tissues by a number of laboratories [10, 12, 14, 15].

Among these enzymes above, aromatase is the one catalyzing the rate limiting step in the biosynthetic pathway for estrogen [16] and has been considered an important critical target for pharmacological inhibitors which may cause estrogen deprivation for the postmenopausal patients with estrogen receptor (ER) positive breast carcinoma [3, 13, 15]. An introduction of aromatase inhibitors (AIs) in the treatment algorithms of these breast cancer patients has actually been considered one of the major achievements in breast cancer therapy through the last decades [17, 18]. Especially, third-generation AIs suppressed the aromatase activity in the magnitudes of more than 98%, which subsequently resulted in clinical benefits and relatively lower incidences of adverse effects [16, 19]. This therapy has been established as gold standard of endocrine therapy for all stages of ER positive postmenopausal breast cancer patients in numerous countries including Japan.

It is, however, also true that resistance to these endocrine therapies still occurs, which has resulted in serious clinical problems in the management of these patients above. The mechanisms of this endocrine resistance have been examined by many investigators from the standpoints of either de novo or intrinsic and acquired resistances. Mechanisms of intrinsic or de novo resistance were evident at an initial exposure to endocrine therapy even in some ER abundant tumor cases [4]. The exact mechanisms for this type of resistance have still remained unknown at this juncture. Acquired resistance usually develops during the course of endocrine therapy of the patients who initially respond to the AIs treatment. This mode of resistance has been, in general, explained by a possible adaptation of carcinoma cells to acquire the potential to proliferate despite the inhibition or suppression of aromatization or in situ depletion of estrogens, i.e., de novo acquirement of novel signaling mechanisms to develop a state of estrogen hypersensitivity in breast carcinoma cells, which subsequently circumvent the clinical effects of AIs [4, 16].

Multiple clinical trials have been recently designed in order to examine these resistance mechanisms of AIs [4, 20–24]. A number of putative theories have been proposed to explain the development of this resistance to AIs during the treatment but an adaptation of hormone-dependent breast carcinoma cells to estrogen withdrawal or depletion and develop estrogen hypersensitivity is considered to represent the common biological features [4, 23, 24]. It is also important to note that enzymes other than aromatase are indeed involved in intratumoral estrogen synthesis in human breast carcinoma tissue as described above. However, alterations of these enzymes before and after AIs therapy have not been known at all to the best of our knowledge. Therefore, in this study, we evaluated the changes of the enzymes involved in intratumoral estrogen production including STS, 17β-HSD1 and EST in breast carcinoma tissues before and after the neoadjuvant exemestane (EXE) treatment using immunohistochemistry (IHC). We then correlated the findings with the therapeutic responses of individual patients including clinical and pathological responses and alterations of Ki67 before and after the therapy of individual patients. We also correlated the findings with changes of ER, Progesterone receptor (PgR) and human epidermal growth factor receptor type 2 (Her2) in breast carcinoma.

Materials and methods

Breast carcinoma cases

116 Japanese postmenopausal patients (55–75 years old), in whom the operable primary breast tumors had been histological diagnosed as primary invasive ductal carcinoma, TNM stage II-IIIA, had been enrolled into the study

of JFMC34-0601[25] of Japanese Foundation for Multidisciplinary Treatment of Cancer between March, 2006 and January, 2008. The menopausal status was defined by natural menopause: at least 1 year since the last menstrual period with the serum level of Follicle-stimulating hormone (FSH) and plasma E2 within the postmenopausal range (FSH $\geq$30 IU/L, E2 <10 pmol/l). None had received prior treatment with hormonal agents, chemotherapy or endocrine therapy for breast cancer nor were taking any medications including hormonal preparations at the time of study. None had the past history of breast cancer. All patients provided written informed consents to this study, which had been approved by the local ethics committee or institutional review board.

JFMC34-0601 trial was a multicenter phase II study by Japanese Foundation for Multidisciplinary Treatment of Cancer performed from March 2006 to January 2008. The study was conducted to evaluate the possible efficacy and safety of EXE treatment for 24 weeks administration in Japanese patients with breast carcinoma. 116 Japanese postmenopausal patients had been enrolled into this study and all the patients had been diagnosed as primary operable invasive ductal carcinoma of the breast. Primary clinical endpoints were objective response rates and safety after 24 weeks of the treatment. This trial study demonstrated that 24 weeks EXE treatment was more effective than 16 weeks administration.

According to the protocol of JFMC34-0601 multicenter phase II trial study, all 116 patients initially received EXE as an oral dose of 25 mg daily for 16 weeks and then additional 8 weeks treatment was subsequently given after clinical evaluation done at week 16. Tumor size was serially monitored by calipers and breast ultrasound before treatment, at 16 weeks and at 24 weeks after receiving the neoadjuvant therapy. At week 16, clinical response was assessed and if the patients were evaluated as clinical responders (complete response or partial response or stable disease), 8 weeks of the same treatment was subsequently added until reaching the total treatment period of 24 weeks. However, if the patients were classified as clinical nonresponders (progressive disease), these patients either underwent surgery or received another modes of treatment. All the patients but ten patients (6 patients discontinued the treatment because of adverse effects and 4 patients had been classified as nonresponders) continuously received the therapy until reaching the total period of 24 weeks treatment. At week 24, clinical response was reevaluated and all the patients underwent definitive surgery. The specimens available for examinations in this study were pretreatment core needle biopsies and post treatment surgical specimens which were obtained after the surgery at week 16 or 24. The pre- and posttreatment specimens of 49 patients among these patients were available for this study of pathological response and the Immunohistochemical evaluation. ER, PgR, and Her2 status was performed by individual institutions by means of standard procedures and retrieved the data for central review and analysis.

Clinical response

Clinical response was based on changes in tumor volume taken at 16 weeks and/or 24 weeks after the neoadjuvant therapy. Clinical response was defined as complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD) according to the Response Evaluation Criteria in Solid Tumors [26].

Pathological response

Tissue sections of the same tumors from pretreatment core needle biopsies and final surgical specimens were obtained and assessed for changes in cellularity and degree of fibrosis in hematoxylin-eosin stained slides. Pathological response was categorized, using the modified criteria described by Miller et al. [27], and assessed as follows: complete when there was no evidence of malignant cell at the original tumor site; partial response when histological decrement in cellularity and/or increment in fibrosis was detected; or no change/nonresponse, by two of the authors above (NC and MC).

Immunohistochemistry

One 4-μm section of each submitted paraffin blocks of pre- and posttreatment specimens were stained with hematoxylin-eosin to verify an adequate number of invasive breast carcinoma cells and the quality of fixation in order to determine the suitability of further immunohistochemical analyses. Serial tissue sections (4-μm) were then prepared from selected blocks and immunohistochemistry was performed to immunolocalize STS, 17β-HSD1, EST and Ki67 as described previously [10, 14, 28]. In brief, IHC staining was performed by streptavidin-biotin amplification method using a Histofine Kit (Nichirei, Tokyo, Japan). The lists of primary antibodies used in this study and concentrations with the antigen retrieval method were summarized in Table1. Tissue sections of full-term placenta were used as positive controls for STS, 17β-HSD1 and EST.

The immunostained slides were independently evaluated by three of the authors (NC, TS and HS), blinded to clinical outcome of individual patients. STS, 17β-HSD1, and EST immunoreactivity was evaluated using a semi-quantitative method as follows: score 2, >50% positive cells; 1, 1–50% positive cells; and 0, no immunoreactivity,

Table 1 The list of antibodies employed for immunostaining in this study

Biomarkers	Dilution	Providers
STS (KM1049)	0.37 μg/ml	Kindly provided by, Kyowa medix Co Ltd., Japan
17β-HSD1	1:400	Abnova, Taipei, Taiwan
EST	1:200	MBL, Nagoya, Japan
Ki67	1:100	DAKO, Denmark
ER	Undiluted	Roche diagnostic, Germany
PgR	Undiluted	Roche diagnostic, Germany
Her2	Undiluted	Roche diagnostic, Germany

STS Estrogen sulfatase, *17β-HSD1* 17β-hydroxysteroid dehydrogenase type 1, *EST* estrogen sulfotransferase, *Ki 67*, Ki 67 protein, *ER* estrogen receptor, *PgR* progesterone receptor, *Her2* human epidermal growth factor receptor type 2

The pretreatment methods for antigen retrieval were as follows:

17β-HSD1 and EST; microwave in citric buffer, Ki67; autoclave in citric buffer, ER, PgR and Her2; pretreatment by heat in automated machine, STS; no pretreatment was required

as previously described by Suzuki et al. [14]. Evaluation of Ki67 was performed by counting of 1,000 carcinoma cells or more from each cases and the percentage of immunoreactivity was subsequently determined as a labeling index (LI) [28].

In addition, the Ki67 LI was then subclassified, using the criteria described by Miller et al. [27], into three different groups according to the percentage of Ki67 alterations after treatment as follows: Group1; increased group, the Ki67 LI in this group was associated with increment after therapy. Group2; no change group, the Ki67 LI demonstrated unchanged or reduction for less than 40% of the pretreatment level. Group3; decreased group, the Ki67 LI demonstrated the reduction for more than 40% of the pretreatment level. ER and PgR immunoreactivity was scored by assigning proportion and intensity scores, according to Allred's procedure [29]. The membrane staining pattern was estimated in Her2 immunohistochemistry and scored on a scale of 0–3 [30]. ER, PgR, and HER2 were all independently evaluated by two of the authors (TS and HS).

Statistical analysis

The Mann–Whitney *U* test was used to compare the pretreatment IHC scores of all biological markers according to the clinical and pathological responses to EXE treatment in individual patients. The Wilcoxon matched-pairs signed-ranks test was employed in order to determine the mean differences between pre- and posttreatment IHC scores of individual biological markers in relation to the clinical and pathological responses status and alterations of Ki67 LI. The correlation among intratumoral enzymes (STS, 17β-HSD1 and EST) were analyzed using Spearman's rank nonparametric correlation test. The statistically significance was considered for the P value < 0.05.

Results

The breast carcinoma specimens examined before the therapy demonstrated the following features after central review of the specimens; ER+ (100%), PgR+ (85.7%) and Her2+ (77.6%).

Clinical and pathological responsiveness

The relevant clinical findings of the patients were summarized in Table 2. All the patients in this study received 16 weeks of EXE treatment but 2 patients were evaluated as PD and the subsequent surgery was advocated at 16 weeks of treatment. Only 47 patients were continuously administrated for EXE treatment until 24 weeks. Clinical response was reevaluated and clinical responders were classified as PR in 27 cases or 55.1% while clinical nonresponders included 19 cases of SD (38.8%) and 3 cases of PD (6.1%) including those previously evaluated as PD (2 patients). Pathological responders and nonresponders corresponded to 22 cases (44.9%) and 27 cases (55.1%),

Table 2 Clinicopathological features of the patients examined (n = 49)

Patients evaluable for IHC	49
Mean age; years (range)	65.6 (56–77)
Tumor stage, *n* (%)	
T2	49 (100)
Nodal status, *n* (%)	
N0	39 (79.6)
N1	10 (20.4)
No distant metastasis, M0, *n* (%)	49 (100)
Clinical stage, *n* (%)	
Stage IIA	39 (79.6)
Stage IIB	10 (20.4)

Table 3 Correlation between clinical and pathological responses ($n = 49$)

Clinical response	Pathological response		Total
	Response	Nonresponse	
Complete response	0	0	0
Partial response	12	15	27
Stable disease	8	11	19
Progressive disease	2	1	3
Total	22	27	49

respectively. The correlation between clinical and pathological responses was demonstrated in Table 3.

Pretreatment evaluation of biological markers according to the responses to EXE

The means of individual biological markers, which were subjected to various responses to EXE treatment, were demonstrated in Table 4. No statistical significance was detected in all the markers examined between clinical and pathological response and nonresponse groups, except for Her2 scoring which was higher in pathological nonresponsive group than that of responsive group.

Associations between alterations of biological markers during the therapy and responses to exemestane treatment in individual patients

Alterations of immunohistochemical biomarkers examined in breast tumor tissues before and after EXE neoadjuvant treatment according to clinical and pathological responses were summarized in Tables 5 and 6. In clinical response group, the significant decrement of Ki67 LI ($P < 0.0001$), ER ($P = 0.0098$) and PgR expression ($P < 0.0001$) was detected. In addition, the statistically significant increment was demonstrated in STS ($P = 0.0084$) and 17β-HSD1 ($P = 0.0015$). EST also demonstrated some degrees of increment but this increase did not reach statistical significance ($P = 0.375$). Among clinical nonresponders, STS, 17β-HSD1 and EST were all significantly increased ($P = 0.0078$, $P = 0.0010$ and $P = 0.0313$, respectively). In addition, PgR and Ki67 LI demonstrated significant decrement ($P = 0.0034$ and $P = 0.0003$, respectively) but ER and Her2 status did not reveal any significant differences between before and after the therapy (Table 5). In pathological response group, the significant decrement of IHC scores were demonstrated in ER ($P = 0.0186$), PgR ($P < 0.0001$) and Ki67 LI ($P < 0.0001$). Among the enzymes examined, only 17β-HSD1 demonstrated statistically significant increment ($P = 0.0068$) (Table 6). In contrast, the intratumoral enzymes in pathological nonresponders, STS, 17β-HSD1 and EST were associated with statistically significant increment ($P = 0.0002$, $P = 0.0001$ and $P = 0.0156$, respectively). In addition, the significant decrement was also detected in PgR ($P = 0.0004$) and Ki67 LI ($P = 0.0003$) following the therapy among these nonresponder group.

Alterations of intratumoral enzymes and biological markers according to the changes of Ki67 labeling index

Differences of the individual enzyme between pre- and posttreatment were evaluated according to these categories of Ki67 LI described above. Immunoreactivity of STS,

Table 4 Correlation between immunohistochemical scores of biological markers in breast tumor before treatment and pathological and clinical responses to exemestane

Biological markers	Pathological response			Clinical response		
	R	NR	*P* value†	*R*	NR	*P* value†
STS	1.545 ± 0.5	1.259 ± 0.6	0.1745	1.333 ± 0.6	1.455 ± 0.6	0.5409
17β-HSD1	1.409 ± 0.5	1.296 ± 0.5	0.5581	1.333 ± 0.6	1.364 ± 0.5	0.9266
EST	1.727 ± 0.5	1.704 ± 0.5	0.8929	1.815 ± 0.4	1.591 ± 0.5	0.1756
Ki 67	14.045 ± 12.5	11.444 ± 9.5	0.6509	11.815 ± 11.8	13.591 ± 9.7	0.4448
ER	7.273 ± 1.3	7.370 ± 1.6	0.3247	7.704 ± 0.5	6.864 ± 2.0	0.1214
PgR	4.955 ± 2.4	4.889 ± 2.9	0.8561	5.444 ± 2.3	4.273 ± 3.0	0.2082
Her2	0.8636 ± 0.9	1.444 ± 0.8	0.0197*	1.259 ± 0.9	1.091 ± 0.9	0.5294

Data showed by means ± SD

† Mann–Whitney *U* test for the difference between R and NR. *R* response, *NR* Nonresponse; see text for the details

* P value <0.05 is considered significant

STS estrogen sulfatase, *17β-HSD1* 17β-hydroxysteroid dehydrogenase type 1, *EST* estrogen sulfotransferase, *Ki 67* Ki 67 protein, *ER* estrogen receptor, *PgR* progesterone receptor, *Her2* human epidermal growth factor receptor type 2

Table 5 Comparisons of pre- and posttreatment immunohistological scores of biological markers in tumors among groups of different clinical responses to Exemestane

Biological markers	Clinical response		Clinical nonresponse	
	Mean difference [95% CI]	*P* value†	Mean difference [95% CI]	*P* value†
STS	−0.4444 [−0.7206, −0.1683]	0.0084*	−0.3636 [−0.5820, −0.1453]	0.0078*
17β-HSD1	−0.4815 [−0.7109, −0.2521]	0.0015*	−0.5 [−0.7269, −0.2731]	0.0010*
EST	−0.7407 [−0.2264, 0.07822]	0.375	−0.2727 [−0.4749, −0.07058]	0.0313*
Ki 67	7.074 [3.453, 10.965]	<0.0001*	6.909 [3.301, 10.517]	0.0003*
ER	0.5556 [0.1401, 0.9710]	0.0098*	0.3636 [−0.1014, 0.8286]	0.1289
PgR	3.333 [2.346, 4.321]	<0.0001*	2.318 [0.8481, 3.788]	0.0034*
Her2	0.1481 [−0.1139, 0.4102]	0.3394	0.04545 [−0.1699, 0.2609]	0.8125

Data showed mean difference for pre IHC value-post IHC value with 95%CI [lower,upper values]; see text for the details

† Wilcoxon match-pairs signed-ranks test for the difference between groups

* *P* value <0.05 is considered significant

STS estrogen sulfatase, *17β-HSD1* 17β-hydroxysteroid dehydrogenase type 1, *EST* estrogen sulfotransferase, *Ki 67* Ki 67 protein, *ER* estrogen receptor, *PgR* progesterone receptor, *Her2* human epidermal growth factor receptor type 2

Table 6 Comparisons of pre- and posttreatment immunohistological scoring of biological markers in tumors among groups of different pathological responses to Exemestane

Biological markers	Pathological response		Pathological nonresponse	
	Mean difference [95% CI]	*P* value†	Mean difference [95% CI]	*P* value†
STS	−0.2727 [−0.5526, 0.007132]	0.1055	−0.5185 [−0.7479, −0.2891]	0.0002*
17β-HSD1	−0.4545 [−0.7188, −0.903]	0.0068*	−0.5185 [−0.7200, −0.3170]	0.0001*
EST	−0.04545 [−0.2118, 0.1209]	0.75	−0.2593 [−0.4360, −0.08256]	0.0156*
Ki 67	7.636 [4.216, 11.056]	<0.0001*	6.481 [2.758, 10.205]	0.0003*
ER	0.6818 [0.1450, 1.219]	0.0186*	0.2963 [−0.04751, 0.6401]	0.1094
PgR	2.909 [1.859, 3.959]	<0.0001*	2.852 [1.536, 4.167]	0.0004*
Her2	0.04545 [−0.1699, 0.2609]	0.8125	0.1481 [−0.1139, 0.4102]	0.3394

Data showed mean difference for pre-IHC value-post-IHC value with 95% CI [lower,upper values]; see text for the details

† Wilcoxon match-pairs signed-ranks test for the difference between groups

* *P* value <0.05 is considered significant

STS Estrogen sulfatase, *17β-HSD1* 17β-hydroxysteroid dehydrogenase type 1, *EST* estrogen sulfotransferase, *Ki 67* Ki 67 protein, *ER* estrogen receptor, *PgR* progesterone receptor, *Her2* human epidermal growth factor receptor type 2

17β-HSD1, EST, ER, PgR, and Her2 in pretreatment specimens was not significantly different among these three different groups of Ki67 LI changes (Nonparametric ANOVAs; Data not shown). In group 1 or those whose Ki67 LI increased after the therapy, no statistically significant differences of intratumoral enzymes and biomarkers were detected in the specimens between before and after the treatment. In group 3 or those whose Ki67 LI decreased with more than 40% of the pretreatment level, the significant increment of STS and 17β-HSD1 were demonstrated ($P = 0.0008$ and $P = 0.0003$, respectively). In addition, ER and PgR scorings following the therapy demonstrated significant decrement compared to pretreatment ($P = 0.0013$ and $P < 0.0001$, respectively) in group 3 patients. In group 2 or those whose Ki67 LI unchanged or decreased with less than 40% of the pretreatment level, only 17β-HSD1 was associated with statistically significant increment ($P = 0.0313$). Moreover, among the enzymes examined and among the other biomarkers examined, only PgR status was significantly decreased following the therapy ($P = 0.0117$) in group 2 patients. EST and Her2 scorings were not different among these three different groups of Ki67 LI alterations (Table 7).

Correlation among STS, 17β-HSD1 and EST immunoreactivity before and after EXE treatment

Results were summarized in Table 8. The status of three enzymes examined in this study was significantly correlated among each others in tissue specimens before the

Table 7 Comparisons of pre- and posttreatment immunohistological scoring of biological markers in tumors according to the degrees of Ki67 changes before and after Exemestane treatment

Biomarkers	Increase		No change		Decrease	
	Mean difference [95% CI]	P value†	Mean difference [95% CI]	P value†	Mean difference [95% CI]	P value†
STS	−0.3333 [−1.417, 0.7507]	0.5000	−0.4000 [−0.7694, −0.3062]	0.1250	−0.4242 [−0.6231, −0.2253]	0.0008*
17β-HSD1	−0.3333 [−0.8753, 0.2087]	0.5000	−0.6000 [−0.9694, −0.2306]	0.0313*	−0.4848 [−0.6855, −0.2842]	0.0003*
EST	−0.5000 [−1.075, 0.07489]	0.0756	−0.1000 [−03262, 0.1262]	>0.9999	−0.1212 [−0.2685, −0.02606]	0.1563
ER	0.1667 [−0.8653, 1.199]	>0.9999	−0.1000 [−0.3262, 0.1262]	>0.9999	0.6970 [0.2956, 1.098]	0.0013*
PgR	2.667[−1.298, 6.631]	0.2500	2.800 [0.9286, 4.671]	0.0117*	2.939 [1.905, 3.973]	<0.0001*
Her2	0.000 [−0.6638, 0.6638]	>0.9999	0.2000 [−0.2524, 0.6524]	0.3750	0.09091 [−0.1145, 0.2963]	0.4648

Degrees of Ki67 changes were determined by the changes of labeling index after EXE treatment, i.e., pre value -post value, and subclassified into three groups. Data showed mean difference for pre IHC value–post IHC value with 95%CI [lower,upper values]; see text for the details

† Wilcoxon match-pairs signed-ranks test for the difference between groups

* P value <0.05 is considered significant

STS Estrogen sulfatase, *17β-HSD1* 17β-hydroxysteroid dehydrogenase type 1, *EST* estrogen sulfotransferase, *Ki 67* Ki 67 protein, *ER* estrogen receptor; *PgR* progesterone receptor, *Her2* human epidermal growth factor receptor type 2

Table 8 Correlation between intratumoral enzymes involved in estrogen production before and after treatment with Exemestane

	STS vs. EST	STS vs. 17β-HSD1	EST vs. 17β-HSD1
Before treatment	$r = 0.5402$	$r = 0.5374$	$r = 0.4156$
	$P < 0.0001$*	$P < 0.0001$*	$P = 0.0030$*
After treatment	$r = 0.2743$	$r = 0.5983$	$r = 0.3403$
	$P = 0.0565$	$P < 0.0001$*	$P = 0.0167$

Data showed by r value and P value calculated by Spearman's rank nonparametric correlation test

* P value <0.05 is considered significant correlation

therapy. However, in tumor tissues following the therapy, only the status of STS and 17β-HSD1 was significantly correlated each other.

Discussion

This is the first study to demonstrate significant alterations of the enzymes other than aromatase involved in intratumoral estrogen production following aromatase inhibitor administration. Several clinical studies have been reported using exemestane as primary endocrine therapy in operable breast cancer patients but results of clinical and pathological responses to exemestane varied among these studies [31–34]. Alterations in tumor histopathological features following aromatase inhibitors administration include the changes in cellularity, degree of fibrosis, histological grading [17, 27], and treatment-related changes of cell proliferation, apoptosis, and hormone receptor expression were also described [31–37].

In tumor specimens following aromatase inhibitors therapy, one of the histological features most frequently or significantly affected was considered the number of mitotic figures or Ki67 positive carcinoma cells, which decreased in the great majority of cases [17, 36, 37], usually more pronounced than tamoxifen therapy [35]. In IMPACT study [36], Dowsett et al. evaluated the alterations of the number of Ki67 positive carcinoma cells using immunohistochemistry in 10% formalin-fixed and paraffin-embedded tissue sections of both pretreatment and after 2 weeks of neoadjuvant anastrozole treatment. Fifty-two out of 56 patients (93%) were associated with some degree of Ki67 labeling index reduction over the only 2-week period of therapy. Reported results of subsequent IMPACT studies further highlighted the clinical or therapeutic importance of evaluating the changes of Ki67 labeling index of carcinoma cells before and after the treatment [37]. We also demonstrated marked decrement in cell proliferation evaluated by the changes of Ki67 labeling index between before and after the therapy in all clinical and pathological response and nonresponse groups, which is also consistent with results of previously reported studies [17, 33, 36–39]. Therefore, an inhibition of aromatase activity and subsequent in situ decreased tissue estrogen availability were considered to affect the expression of molecules present in the downstream of ER signaling pathways related to cell proliferation regardless of response to treatment [3, 17, 33, 36–39] (Fig. 1).

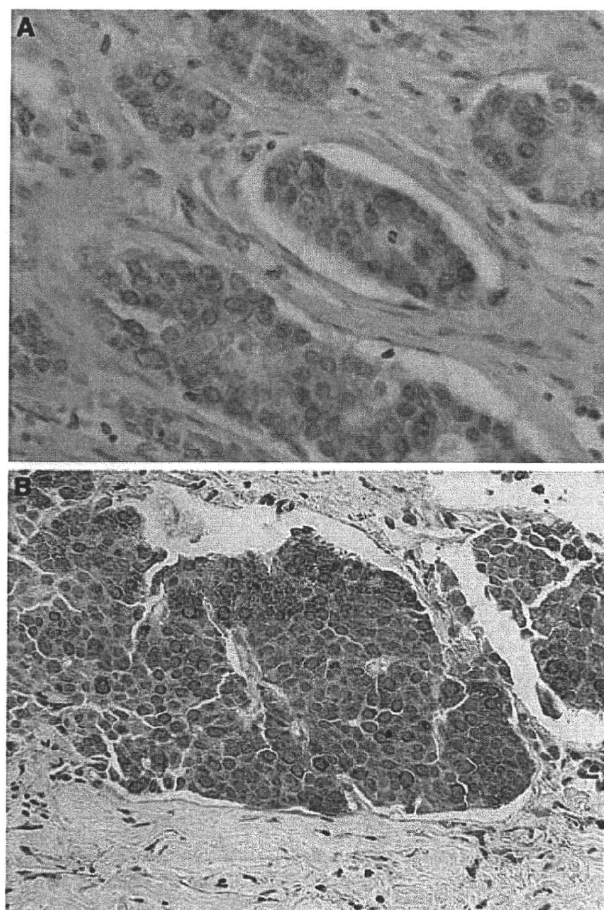

Fig. 1 Representative illustrations of immunohistochemistry: (**a**) 17β-HSD1 and (**b**) STS in the case of invasive ductal carcinoma. Immunoreactivity was detected in the cytoplasm of invasive ductal carcinoma cells in both enzymes. Original magnification ×200

Changes of the enzymes other than aromatase involved in intratumoral estrogen biosynthesis following aromatase inhibitor therapy have been considered or postulated important in relation to the development of treatment resistance [3] but have remained virtually unknown. To the best of our knowledge, this is the first study demonstrating a significant increment of STS and 17β-HSD1 following aromatase inhibitor neoadjuvant therapy of ER positive postmenopausal breast carcinoma patients. We hypothesized that this increment of STS and 17β-HSD1 detected in this study may be due to the compensatory response of breast carcinoma tissues to estrogen depletion and may represent an attempt of breast carcinoma to increase intratumoral estrogen concentrations using the estrogen producing or metabolizing pathways other than aromatase. In particular, a significant increment of STS and 17β-HSD1 following exemestane treatment was detected in the group associated with decreased Ki67 labeling index in our present study (Table 7). This increment of the enzymes above was not detected in the group associated with increased Ki67 labeling index, i.e., those associated with an absence of suppression of tumor cell proliferation. However, it awaits further investigations such as the intratumoral regulation of STS and 17β-HSD1 under estrogen depletion in order to substantiate this interesting hypothesis.

The simultaneous increase in STS and EST expression detected in our present study may be considered due to intratumoral metabolism and synthesis of estrogens. Both of these enzymes play pivotal roles in intratumoral estrogen production in the hormone-dependent breast carcinoma. STS hydrolyzes estrone sulfate (E1-S) to estrone, while EST sulfonates estrogens to inactive estrogen sulfates [15]. Therefore, an increment of STS levels in breast carcinoma cells may result in increased intratumoral estrogen production, but EST expression may also increase as one of the counterbalance effects or responses to an increment of intracellular estrogen, especially in nonresponder groups. However, it awaits further investigations to study the mechanisms of this simultaneous increment of both enzymes.

Among these enzymes examined, in particular, 17β-HSD1 was an only intratumoral enzyme whose expression increased regardless of clinical response, pathological response or Ki67 changes of the patients. Suzuki et al. [40] reported that the status of 17β-HSD1 immunoreactivity in carcinoma cells was significantly correlated with that of ER and PgR, suggesting estradiol, synthesized by 17β-HSD1 in carcinoma cells, act on these cells locally in breast carcinomas. In addition, reductive 17β-hydroxysteroid dehydrogenases are the last step in estrogen activation and thus play pivotal roles in biological behavior of ER positive breast carcinoma cells [41]. Sasano et al. [15] also reported that the status of intratumoral aromatase, 17β-HSD1, EST and STS in human breast cancer tissues varied markedly among different cases and, especially, no significant correlation was detected between intratumoral aromatase and 17β-HSD1. Therefore, an inhibition of 17β-HSD1 may be considered to confer clinical or therapeutic benefits upon the patients in whom over-expression of intratumoral 17β-HSD1 but not of aromatase was present in breast cancer tissues. The analysis of STS and 17β-HSD1 using immunohistochemistry is therefore considered important because these inhibitors may not work unless these enzymes or targets are not present or overexpressed in breast carcinoma cells. Therefore, an analysis of these enzymes as potential surrogate markers of treatment may be required for the successful clinical outcome of treatment when specific inhibitors against these enzymes will be clinically available.

In conclusion, results of this study indicated that an increment of STS and 17β-HSD1 may represent at least

one of the mechanisms why hormone-dependent breast carcinoma cells developed resistance to AIs, which also suggest a possible adaptation of carcinoma cells in response to intratumoral estrogen depletion as a result of effective aromatase inhibitor therapy.

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Total-Circumference Intraoperative Frozen Section Analysis Reduces Margin-Positive Rate in Breast-Conservation Surgery

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Objective: One problem existing in breast-conservation surgery is ipsilateral breast tumour recurrence, and one of its major risk factors is surgical margin positivity. We therefore investigated whether total-circumference surgical margin examination can reduce surgical margin-positive rates.
Methods: A total of 122 cases were examined after BCS was performed between March 2004 and March 2006. After partial mastectomy, specimens were taken from the remnant breast side along the total-circumference of the mammary gland (width, approximately 5 mm). Intraoperative frozen section analysis was performed for those specimens. Margin-positive cases were defined as those showing malignancy within $\leq$5 mm of the final margin as revealed by final diagnosis.
Results: If intraoperative frozen section analysis had not been performed, 33 cases (27%) would have been diagnosed as margin-positive. However, it reduced the number of margin-positive cases to 12 (9.8%), and final margin-positivity rates were thus significantly reduced ($P < 0.001$). As for the accuracy of intraoperative frozen section analysis, sensitivity was 78.6%, specificity was 100%, correct diagnosis rate was 95.1%, positive predictive value was 100% and negative predictive value was 94.0%. False-negatives were caused by the detection of malignancy as revealed in permanent specimens. Margin-positive sites were not limited to the nipple and distal (peripheral) sites, with equivalent margin-positive cases found laterally.
Conclusions: Total-circumference surgical margin examination by IFSA for BCS significantly reduced margin-positive rates from 27% to 9.8%.

Key words: breast cancer – breast-conservation surgery – frozen section – surgical margin

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INTRODUCTION

In recent years, breast-conservation surgery (BCS) has become a standard operative procedure for breast cancers. One problem that arises with BCS is ipsilateral breast tumour recurrence (IBTR), and one of the major risk factors for such recurrence is surgical margin positivity (1–5). Margin examination by intraoperative frozen section analysis (IFSA) is anticipated to significantly facilitate the correct identification of margin status. As a result, margin examination is currently performed in individual facilities. However, many issues require further investigation. For instance, methods of specimen submission and evaluation of accurate diagnosis rates are yet to be standardized. Moreover, few detailed reports have examined margin diagnosis within BCS, and no reports have yet clarified the use of IFSA examining the total-circumference (i.e. 'circumferential' examination) of the surgical margin.

Ever since we established an environment for performing intraoperative pathological examinations, we have been submitting total-circumference margins shaved from the

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mammary gland of the remnant breast for IFSA, and performing margin diagnoses based on these specimens. The present study examined the extent to which circumferential margin examination can reduce surgical margin-positive rates.

METHODS

The present research was performed in the Department of Breast and Endocrine Surgery at Tohoku University Hospital, in the 2-year period from March 2004 to March 2006, and involved a retrospective study of 122 cases in which BCS had been performed (including three cases of simultaneous dual-breast cancer). During the same periods, 57 patients had received total mastectomy and they were excluded from this study. And cases in which preoperative adjuvant chemotherapy or endocrine therapy had been performed were excluded from analysis, as were cases in which surgical excision biopsy of the main tumour had been performed. Background factors of subjects are shown in Table 1.

Table 1. Characteristics of the 122 patients

Age, years (median)	32–87 (56)
Tumour size, *n* (%)	
Tis	31 (25.4)
T1 (≤2 cm)	74 (60.7)
T2 (>2 cm, ≤5 cm)	17 (13.9)
Histology, *n* (%)	
DCIS	31 (25.4)
IDC	81 (66.5)
ILC	2 (1.6)
Mucinous carcinoma	6 (4.9)
Apocrine carcinoma	2 (1.6)
Oestrogen receptor status, *n* (%)	
Positive	103 (84.4)
Negative	19 (15.6)
Progesterone receptor status, *n* (%)	
Positive	90 (73.8)
Negative	32 (26.2)
HER2 status, *n* (%)	
Positive	24 (19.7)
Negative	98 (80.3)
Menopausal status, *n* (%)	
Premenopausal	45 (36.9)
Postmenopausal	77 (63.1)

DCIS, ductal carcinoma *in situ*; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma.

Preoperative Evaluation

For the preoperative diagnosis of lesion spread, mammography, ultrasonography, computed tomography (CT) and magnetic resonance imaging were used concomitantly. An overall determination was made for each finding, and the excision range was determined. Particularly with CT, imaging was performed using body surface markers, three-dimensional images were created, and detailed width diagnoses were made (6).

Surgical Method

For surgery, wide excision or quadrantectomy was performed after making a skin flap, in principle securing a 2-cm margin from the conjectured lesion. As for lymph node dissection, depending on the case, sampling of Level I to Level III dissection was performed (7).

Adjuvant Therapy

Indication for adjuvant chemotherapy was decided in consideration with histological evidence and according to the report of St Gallen Oncology Conference or Guideline of National Comprehensive Cancer Network. As for adjuvant radiotherapy, most patients underwent except those who refused or contraindicated patients. In this study, 35 patients did not undergo adjuvant radiotherapy.

Margin Diagnosis

After partial mastectomy, specimens were taken from the remnant breast side of the mammary gland with a width of approximately 5 mm, and these specimens were submitted for IFSA (Fig. 1). The outermost surface of the specimen was set as the evaluation surface [Fig. 1 (asterisks)].

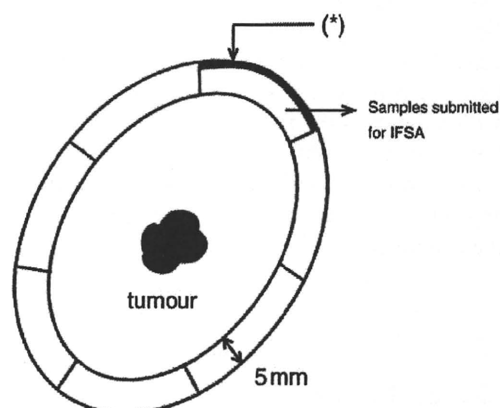

Figure 1. Schematic diagram of a total-circumference surgical margin examination. After partial mastectomy, specimens were taken from the remnant breast side of the mammary gland with a width of approximately 5 mm, and these specimens were submitted for IFSA. The outermost surface of the specimen (asterisks) was set as the evaluation surface.

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Table 2. Results of intraoperative frozen section analysis (IFSA) for the 122 cases and classification of diagnosis pattern

Excised sample margin	IFSA	Final margin diagnosis	No. of cases	Classification of diagnosis[a]	
−	−	−	79		
−	+	−	6	A	
+	−	−	15	B	33 cases (27%)[b] (B–F)
+	+	−	10	C	
+	+	+	4	D, E	
+	−	+	4	F	12 cases (9.8%)[c] (D–H)
−	−	+	3	G	
−	+	+	1	H	
		Total	122		

[a]Cases with lesions detected in the excised sample margin, IFSA or final margin diagnosis were divided into types A–H.
[b]Cases in which margin positivity could have been diagnosed without IFSA.
[c]Cases in which margin positivity was diagnosed in the final specimen.

According to the adipose content of the submitted specimen, penetration was made with the proper surfactant, and the sample was frozen in liquid nitrogen. For each specimen, a minimum of two slides were prepared and stained using haematoxylin and eosin. As for margin diagnosis, the basic policy set was that additional excisions would be made of margin-positive portions until the specimen was found to be margin-negative. These frozen specimens were defrosted after evaluation and embedded in paraffin to create permanent specimens. An optical microtome was then used to create sections, and slides were prepared. The diagnosis obtained using these final specimens was considered final.

Pathological Examination of Surgical Samples

For the pathological examination of surgical samples, whole serial slices (width, 5 mm) were created and examined under microscopy. Margin-positive status was defined as the existence of cancer cells (either invasive or non-invasive carcinoma) within 5 mm of the final margin.

Written consent was obtained from all patients for the use of specimens in the present investigation. The χ^2 test was used for statistical analyses, and values of $P < 0.05$ were determined significant.

RESULTS

The median number of slices submitted for IFSA was seven slices per case (range, 4–12 slices per case). An average of eight slides per case (range, 8–24 slices per case) were prepared and stained using haematoxylin and eosin. It took an average of 53 min per case to perform total-circumference IFSA.

As for margin status according to the need for IFSA, cases were divided into three groups:

Group 1: Could have been determined as margin-negative without IFSA (79 cases).

Group 2: Determined as margin-negative from IFSA (31 cases).

Group 3: Margin-positive despite performance of IFSA (12 cases).

A more detailed investigation of Groups 1–3 was therefore undertaken.

Group in which Margin-Negative Status could have been Determined without IFSA

For this group [79 cases (65%), Table 2], preoperative imaging diagnoses were valid, and excisions could thus be made while securing a sufficiently safe margin. Margin diagnoses from excised sample margin, IFSA and final diagnosis were all negative.

Group in which Margin-Negative Status was Determined from IFSA

Upon investigation of cases that were finally determined as margin-negative based on IFSA, patterns A, B and C were identified:

(i) Although margins in the excised sample were negative, skip lesions were identified on slices submitted for IFSA. Since these were considered IFSA-positive, additional excision was performed, and a final diagnosis of margin-negative was obtained. Six cases showed this pattern (Table 2 and Fig. 2A). All these skip lesions were confirmed as cancer occupying in only one or several ducts. There were not multifocal lesions.

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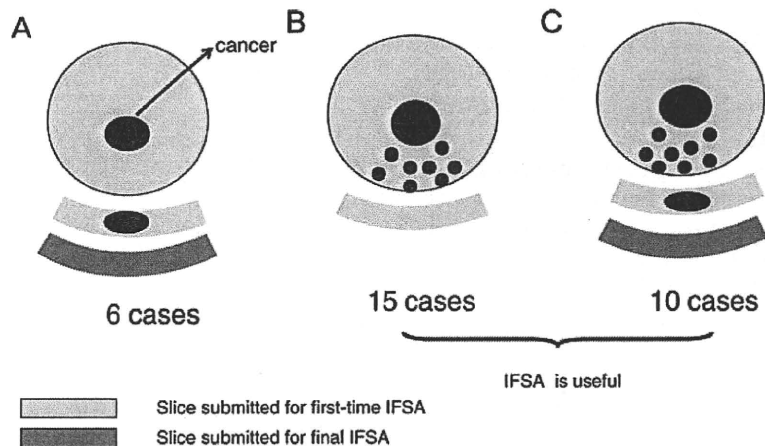

Figure 2. Surgical margin-negative cases shown schematically.

(ii) Although a lesion existed within 5 mm of the excised sample margin, additional cutting of the remnant breast used for IFSA submission secured a sufficient distance between the lesion and the final margin, which was margin-negative. Fifteen cases showed this pattern (Table 2 and Fig. 2B).

(iii) Since IFSA showed margin-positive status, additional excision was performed and final diagnosis was margin-negative. Ten cases showed this pattern (Table 2 and Fig. 2C).

Group in which Margin-Positive Status was Determined Despite IFSA

In 12 cases (10%), final margin status was margin-positive despite the performance of IFSA. Investigation of cases that finally became margin-positive revealed patterns D–H.

(i) Since these cases were IFSA-positive, additional excision was performed until negative margins were obtained. However, in the additionally excised slice, permanent specimens revealed lesions. Two cases showed this pattern (Table 2 and Fig. 3A).

(ii) In two cases, a lesion was confirmed in all of the following: margin of excised sample, IFSA and final margin diagnosis. Of these, a widespread lesion was confirmed in one case even in the additionally excised slice. In that case, total mastectomy was subsequently performed. In the other case, malignancy was also confirmed in the slice submitted for final IFSA, but was limited to just one glandular duct, and the decision was made to achieve control via postoperative radiotherapy. Surgery was therefore considered complete (Table 2 and Fig. 3B).

(iii) Although these cases showed negative margins in IFSA, cancer was revealed in permanent samples from IFSA slices, resulting in a final diagnosis of margin-positive status. Four cases showed this pattern (Table 2 and Fig. 3C).

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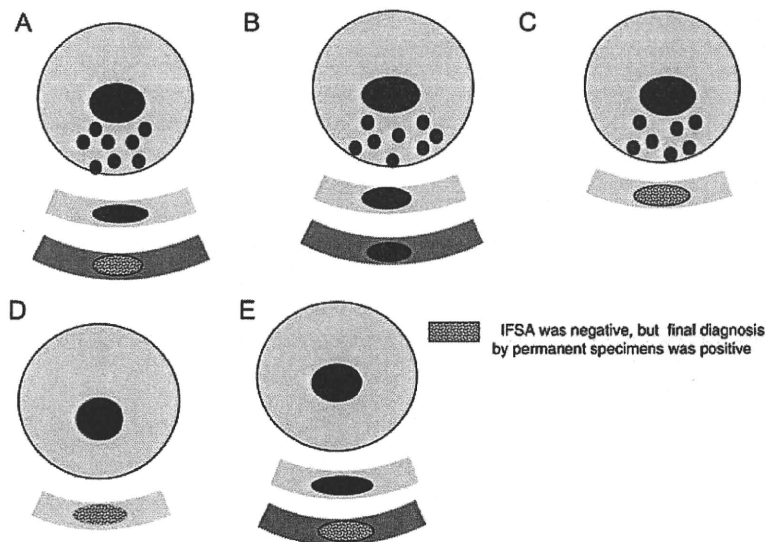

Figure 3. Surgical margin-positive cases shown schematically.

(iv) Although these cases were negative on IFSA, a skip lesion was revealed in the IFSA-slice permanent specimens, and the final diagnosis was thus margin-positive status. Three cases showed this pattern (Table 2 and Fig. 3D).

(v) Although this case was negative in the excised sample margin, a skip lesion was found in the IFSA slice, and despite additional excision, cancer was revealed in permanent samples from IFSA slices. One case showed this pattern (Table 2 and Fig. 3E).

Among the cases finally determined as margin-positive, with the exception of the two type E cases, all were cases in which the lesion was revealed when sections were made from permanent specimens.

Summarizing the above findings, even if IFSA had not been performed, margin evaluation of the excised sample margin itself would have become the final diagnostic evaluation. That is, in the present investigation of 122 cases, 33 cases (27%; Groups shown in Figs 2B and C, and 3A–C) were diagnosed as margin-positive (Table 2). However, use of IFSA reduced the number of margin-positive cases to 12 (9.8%; Table 2). Final margin-positive rate was thus significantly reduced ($P < 0.001$).

Investigation of Margin-Positive Sites

With regard to 33 cases that became margin-positive within the first excised sample margin (Table 2), investigation was made of the direction in which margin-positive results were identified. In these cases, tumour cells were identified at the nipple-side margin in 10 cases, the distal-side margin in 11 cases, and lateral-side margins in 23 cases (with some overlap; Fig. 4). Among lateral-side margin-positive cases, 14 cases were diagnosed as positive except for the side nearest to the tumour.

Investigation of the Accuracy of Intraoperative FSA

For the estimation of correct diagnosis rate, sensitivity, specificity, false-positive rate and false-negative rate, positive cases were defined as IFSA-positive cases showing positive results on permanent samples from IFSA slices. Likewise, negative cases were defined as IFSA-negative cases showing negative results on permanent samples from IFSA slices. IFSA made in this study showed a sensitivity of 78.6%, a specificity of 100%, and a correct diagnosis rate of 95.1%, and a positive predict value of 100%, and a negative predict value of 94.0%. Cases determined as negative on IFSA, but in which a cancer was afterwards revealed by slicing in the permanent sample, were determined as final margin-positive, and included as 'false-negative' cases.

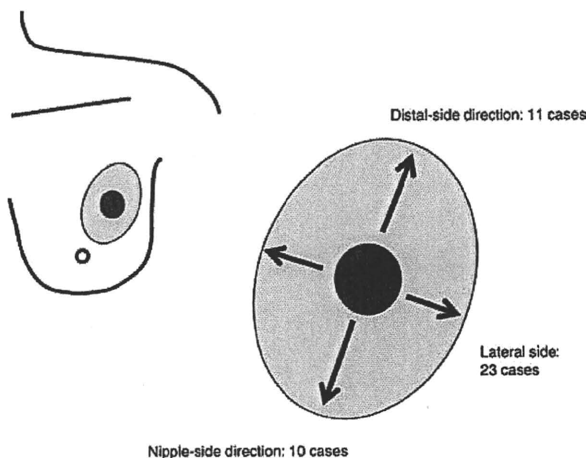

Figure 4. The direction determined as margin-positive in IFSA.

Table 3. Histological characteristics of final margin-positive cases and IFSA-negative cases

	Final margin-positive cases, no. (%)	*P*-value	IFSA-negative cases, no. (%)[a]	*P*-value
Histology		0.14		0.67
DCIS	2 (16.7)		25 (26.6)	
IDC	6 (50.0)		63 (67.0)	
ILC	1 (8.3)		1 (1.1)	
Others	3 (25.0)		5 (5.3)	
EIC		0.07		0.36
Positive	8 (80.0)		31 (45.6)	
Negative	2 (20.0)		37 (54.4)	
Lymphovascular invasion		0.41		0.67
Positive	5 (50.0)		30 (44.1)	
Negative	5 (50.0)		38 (55.9)	

DCIS, ductal carcinoma *in situ*; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; EIC, extensive intraductal component.
[a]These cases included only cases in which met requirements of final margin-negative and IFSA-negative.

Histological Characteristics

Histological characteristics in the cases with final margin-positive and IFSA-negative are shown in Table 3. The group of IFSA-negative cases included only those which met the requirements of final margin-negative and IFSA-negative. The frequencies of pathological factors compared the group of final margin-positive cases with the other cases. In a similar way, the group of IFSA-negative cases were compared. Patients who had extensive intraductal component (EIC) were more likely to have final margin-positive compared with patients without this characteristic, although there was no significant difference.

DISCUSSION

Many reports have examined relationships between status of surgical margins and IBTR in BCS, and a high rate of IBTR has been seen in cases with positive surgical margins (1–5).

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Surgical margins must be taken into consideration when BCS is to be performed, and IFSA is now being performed to ensure negative margins.

In several previous studies, risk factors have been investigated to identify those patients with a high likelihood of having positive margin during BCS (5,8–11). Histological characteristics including ductal carcinoma *in situ* (DCIS), presence of EIC, presence of lymphovascular invasion, multifocality, lobular carcinoma, have all been associated with positive margins. In this study, our result was similar to the previous studies of cases with EIC. It has been shown that the case with EIC has increased the risk of IBTR, particularly severe positive-margin cases. Therefore, these cases must be followed carefully. However, there was not so much of difference about other histological characteristics. The case with multifocal lesions was only two. One was final margin-positive and the other was margin-negative case. There were a few cases of final positive margin in our study. If there were more final margin-positive cases in our study, we might also have been obtaining the data similar to previous studies.

No clear standard currently exists that defines 'margin-positive' within surgical specimens, with a variety of standards existing for different institutions. In the 'Breast Conservation Therapy Guidelines' used in Japan, a determination of margin-positive status is considered reasonable in cases when a cancer is confirmed within 5 mm. Other countries show a broad range of definitions, with some institutions defining 'margin-positive' only when a cancer is exposed at the excision margin, and others diagnosing 'margin-positive', when a cancer has been confirmed within 2 mm or 5 mm (12–18). The accuracy (i.e. correct diagnosis rates) of IFSA thus cannot be determined simply by comparing values from existing reports, and no meaningful discussion can be made regarding the superiority or inferiority of accuracy rates.

In the present investigation, results were obtained under an extremely strict standard compared with those of previously existing reports. As stated earlier, our results regarding the accuracy of margin diagnoses were as follows: sensitivity, 78.6%; specificity, 100%; correct diagnosis rate (diagnostic accuracy rate), 95.1%; positive predictive value, 100%; and negative predictive value, 94.0%. The following rates have been reported previously: sensitivity, 65.0–96.0%; specificity, 84.0–100%; correct diagnosis rate, 83.8–98.0%; positive predictive value, 81.4–97.1%; and negative predictive value, 81.0–100% (12–23). Our values for IFSA were calculated under extremely strict standards of a definition stating 'existence of cancerous cells within 5 mm' and 'cancer identified by slicing with optical microtome from a permanent sample previously determined as negative on IFSA shall be included as a 'false-negative' case'. In previous investigations, no mention has been made of reinvestigation of permanent samples; that is, of slices that had been used for IFSA evaluation after unfreezing. Despite this, accuracy of our IFSA was comparable to results described elsewhere (Table 4). In this study, if margin-positive status was defined as the existence of cancer cells within 2 mm of the final margin, only six margin-positive cases were identified (4.9%). Accuracy rates were as follows: sensitivity, 95.7%; specificity, 100%; correct diagnosis rate, 99.2%; positive predictive value, 100%; and negative predictive value, 99.0%. These values were superior to results described elsewhere.

Furthermore, the majority of cases finally identified as margin-positive were cases in which the lesion first appeared when a reinvestigation was made in the FSA-slice permanent sample. Mammary gland tissue is surrounded by adipose tissue, and thin slices of such adipose tissue are difficult to obtain from frozen samples. As a result, not all gland tissue may show up in the evaluated surface. This is an unavoidable phenomenon characteristic of IFSA, and has been considered as one of the key limitations of IFSA.

Conversely, even in cases where a cancer appears on evaluated surface, frozen specimens are inferior in quality to formalin-fixed permanent samples, and this is thought to have a major impact on the diagnosis. Singletary et al. have stated that since evaluation of structural atypia and nuclear atypia is difficult owing to artefacts from the freezing operation, low-grade DCIS and atypical ductal hyperplasia is

Table 4. Comparison of IFSA accuracy of previous reports and our study

Author	No.	Positive[a]	Positive rate (%)[b]	Accuracy (%)	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
Olson et al. (13)	290	[c]	4.1	98.0	73.0	99.5	91.9	98.3
Cabioglu et al. (12)	154	<2 mm	20.0	87.4	77.8	91.7	88.9	86.1
Cendan et al. (14)	97	exposure	22.7	96.0	65.0	100	94.0	100
Weber et al. (15,20)	80	<1 mm	12.5	83.8	80	87.5	81.4	86.5
Ikeda et al. (21)	56	exposure	32.1	91.1	94.4	89.5	97.1	81.0
This study	122	<5 mm	9.8	95.1	78.6	100	100	94.0

[a]Blanks in table represent data not described in that report.
[b]Definition of positive margin.
[c]Margin-positive rate for final margin diagnosis.

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