neu protein is occasionally observed.[6,7] Cytoplasmic positivity has been regarded as non-specific staining and is excluded from the assessment of membrane staining intensity.[6,8] Although the biological and clinical implications of these cytoplasmic HER2 proteins are largely unknown, recent studies indicate that some variant truncated forms of HER2 proteins play a significant role in the growth of human breast cancer.[9-12] For instance, shedding of the extracellular domain of HER2 is known to affect the binding of HER ligands and Her receptors and to affect the relevant signaling in the cells. In addition, a recent study indicated that the expression of p95 HER2, which is an NH2-terminally truncated fragment, was correlated with the nodal involvement and poor prognosis of patients with primary breast cancer. Therefore, it is important to study the role of these variants of HER2 proteins in more depth.

In this study, we evaluated the cases of cytoplasmic staining in a large series of breast carcinoma specimens using various antibodies to different epitopes of the HER2 protein. To investigate whether such staining is the result of HER2 gene amplification and/or actual reactivity for the HER2 protein, we further applied the samples to FISH analysis and immunoelectron microscopy analysis. In addition, we focused on the relationship between the cytoplasmic staining of HER2 and neuroendocrine differentiation of breast cancer, because we encountered a phenomenon that the cytoplasmic staining of HER2 was often observed in tumors having a phenotype of neuroendocrine differentiation. It is known that neuroendocrine differentiation occurs in some subtypes of mammary carcinomas with granular eosinophilic cytoplasmic features.[13-16] However, these features are not specific and thus the accurate determination of neuroendocrine differentiation often requires additional immunohistochemical staining for neuroendocrine markers, as well as electron microscopic examination for identification of dense-core granules in the cytoplasm.[13,14] Although several studies have reported the cytoplasmic staining of HER2 protein in various types of human cancers such as thyroid neoplasm,[17] pancreatic carcinoma,[18] adrenal tumors,[19] and prostatic cancer[20] as well as breast carcinoma,[6,21,22] little is known about the association of cytoplasmic staining of HER2 with neuroendocrine differentiation.[23,24]

MATERIALS AND METHODS

Tumor specimens and patient characteristics

A total of 1053 breast carcinoma cases that were surgically resected between 2000 and 2004 at the Tokyo Metropolitan Komagome Hospital were examined. The median patient age was 55.2 years (range, 23 - 92 years). Histologic evaluation was performed using hematoxylin and eosin (H&E) staining for all sections and histological type was classified using the World Health Organization criteria.[25] The histological types of the 1053 specimens were as follows: 34 ductal carcinoma *in situ*, 816 invasive ductal carcinoma, 98 invasive lobular carcinoma, 71 mucinous carcinoma, 28 apocrine carcinoma, 4 metaplastic carcinoma, 1 squamous cell carcinoma, and 1 medullary carcinoma. All cases underwent immunohistochemical staining using the HercepTest™ staining kit (DakoCytomation, Glostrup, Denmark). Membrane or cytoplasmic staining was evaluated in the neoplastic cells and quantified and graded as recommended by the manufacturer.

Thirty-four cases showed granular cytoplasmic staining without membranous staining. These 34 cases were then evaluated further by IHC, FISH, and electron microscopy. Relevant clinical and pathological features were reviewed and histological grading was evaluated using the Nottingham histological grading system. Tumors were considered positive for estrogen receptor (ER) and progesterone receptor (PR), if unequivocal nuclear positivity was seen in at least 10% of tumor cells

Immunohistochemistry

Immunohistochemical analysis was carried out on paraffin sections using standard staining methods. Primary antibodies and their dilutions, sources, and pretreatment solutions, are summarized in Table I. To verify whether cytoplasmic staining is a phenomenon limited to the HercepTest, cases selected as cytoplasm-positive were tested by various antibodies against the intracellular domain (CB-11) or extracellular domain (TAB250, SV2-61 γ). Thick paraffin sections (4 μm) mounted on silane-coated glass were dewaxed in xylene, rehydrated through descending concentrations of alcohol, and treated with 0.3% hydrogen peroxide in methanol for 15 min to inhibit endogenous peroxidase activity. Sections were pretreated by heating or enzyme digestion (Table I). Primary antibodies were incubated with tissue sections for 30 min at room temperature. Slides were then processed using the reagent in

Table I. Summary of the primary antibodies used in the study

Antibodies	Reagent/Type	Source	Antigen retrieval	Dilution
1. Antibodies against HER-2				
HercepTest	Poly(rabbit)	DakoCytomation, Glostrup, Denmark	Waterbath at 98℃, 40 min	1:1
CB-11	Mono(mouse)	BioGenex, SanRamon, CA, USA	Microwave, 20 min	1:1
TAB250	Mono(mouse)	Zymed, San Francisco, CA, USA	none	1:1
SV2-61γ	Mono(mouse)	Nichirei, Tokyo, Japan	Protease	1:50
2. Neuroendocrine marker				
Synaptophysin	Poly(rabbit)	DakoCytomation, Glostrup, Denmark	Protease	1:200
Chromogranin A	Poly(rabbit)	DakoCytomation, Glostrup, Denmark	none	1:1000
Neuron-specific enolase (NSE)	BBS/NC/VI-H14, Mono(mouse)	DakoCytomation, Glostrup, Denmark	Microwave, 20 min	1:400
CD56	CD564, Mono(mouse)	Novocastra Laboratories Ltd, UK		1:50
3. Hormone receptor				
Estrogen receptor	1D5, Mono(mouse)	DakoCytomation, Glostrup, Denmark	Autoclave 120℃,15 min	1:100
Progesterone receptor	PgR636, Mono(mouse)	DakoCytomation, Glostrup, Denmark	Autoclave 120℃,15 min	1:2000

the HercepTest kit or the commercial Elite ABC kit (Vectastain, Vector Laboratories, Burlingame, CA, USA). Diaminobenzidine was used as the chromogen and Meyer's hematoxylin was used as a counterstain. Cytoplasmic staining was evaluated in the neoplastic cells and quantified and classified it in four categories, such as more than 50%, 10-50% , less than 10% and negative.

Fluorescence *in situ* hybridization

To detect HER-2/neu gene amplification, dual-color FISH was applied. Before hybridization, tissue microarray slides were deparaffinized and treated according to the paraffin pretreatment reagent kit protocol (Vysis, Downers Grove, IL, USA), followed by proteinase K digestion for 20 to 30 min at 37°C to enhance the access of the DNA probes. The dual-colored probes, Spectrum-Orange-labeled HER-2/neu gene-specific probe and Spectrum-Green-labeled chromosome 17 centromeric (CEP17) probe (Vysis) were used. Slides were hybridized with a mixture of the two probes and hybridization buffer and were counterstained with DAPI II (Vysis). The fluorescent signals were evaluated under a Leica fluorescence microscope (Leica Microsystems Imaging Solutions, Cambridge, UK) equipped with a triple-bandpass filter and ×100 objectives. Hybridization signals were enumerated by the ratio of orange signals for HER-2/neu to CEP-17 signals in morphologically intact and nonoverlapping nuclei. Ratios of at least 2.0 in the tumor cells were considered to indicate HER-2/neu amplification.

Immunoelectron microscopy

Of the 34 cytoplasm-positive cases, four specimens extracted at random were analyzed by electron and immunoelectron microscopy. Pre-embedding and indirect immunoelectron microscopy were performed on formalin-fixed specimens, as described previously.[26] Pretreated formalin-fixed sections were incubated with the HercepTest rabbit polyclonal antibody at 4°C for 18 h and were then washed five times with phosphate-buffered saline (PBS). The second reaction with peroxidase-conjugate anti-rabbit immunoglobulin (DakoCytomation) was carried out overnight at 4°C, followed by washing five times with PBS and the sections were fixed with 1% glutaraldehyde for 15 min at 4°C. After washing with PBS, specimens were immersed in 0.03% diaminobenzidine, 10 mM sodium azide and 1% dimethyl sulfoxide in 50 mM Tris buffer (pH 7.6) for 30 min and the peroxidase reaction was then developed by adding 0.005% H_2O_2 for 5 min. Sections were post-fixed with 1% osmium tetroxide for 1 h, dehydrated in a graded ethanol series, and embedded in Epon 812. Ultrathin sections without lead staining were observed under an electron microscope (H-7000; HITACHI, Ltd., Tokyo, Japan).

RESULTS

Clinicopathological features of cytoplasmic HER2-positive cases

On HercepTest immunostaining, 34 out of 1053 cases had cytoplasmic staining but lacked membranous staining. The clinicopathological data of these 34 cases are

Table Ⅱ. Clinicopathological features of HER2 cytoplasmic-positive cases (n=34)

		No. of cases (%)
Age		32-94 y (Mean 58.9 y)
Menopause		23 (57.0)
Tumor size	Tis	1 (2.9)
	T1	13 (38.2)
	T2	12 (35.2)
	T3	7 (20.6)
Lymph node metastasis	pN0	26 (76.5)
	pN1	8 (23.5)
Distant metastasis	M0	0 (0)
Histological classification	DCIS	1 (2.9)
	Microinvasive carcinoma	3 (8.8)
	Invasive ductal carcinoma	24 (70.6)
	Invasive lobular carcinoma	0 (0)
	Mucinous carcinoma	5 (14.7)
	Solid neuroendocrine carcinoma	1 (2.9)
Histological grade	G1	5 (14.7)
	G2	23 (67.6)
	G3	6 (17.6)
Lymphatic permeation	ly (+)	13 (38.2)
Venous permeation	v (+)	2 (5.9)
Hormone receptor status	ER (+)	33 (97.1)
	PgR (+)	31 (91.2)

summarized in Table Ⅱ. In 5 of 34 cases, neuroendocrine differentiation was seen histologically; these cases included one case of solid neuroendocrine carcinoma detected by light microscopy with H&E staining prior to HER2 immunohistochemical staining. The frequency of positivity was 97.2% for ER and 96.2% for PR.

Immunohistochemistry

Typical appearances of cytoplasmic staining on HercepTest are shown in Figure 1. Tumor cells showed granular cytoplasmic staining without membranous reactivity. The distribution of positive cells often showed an irregular mosaic pattern. Immunohistochemical results using various antibodies against HER2 are summarized in Table Ⅲ. Monoclonal antibody CB-11, which recognizes the intracellular domain of HER2, showed cytoplasmic reactivity in 28 of 34 evaluated specimens. The staining patterns for CB-11 were similar or slightly weaker when compared with the HercepTest results (Fig. 2). In contrast, TAB250 and SV2-61γ, which recognizes the extracellular domain of HER2, showed no cytoplasmic reactivity in any of the 34 specimens (Table Ⅲ).

Immunohistochemical results for the neuroendocrine markers are summarized in Table Ⅳ. In all 34 specimens, synaptophysin expression was detected in addition to other markers, such as chromogranin A, NSE, and CD56. However, the number of positive cells for cytoplasmic HER2 staining and synaptophysin was not always equivalent; the number of positive cells for neuroendocrine markers tended to be more numerous than cytoplasmic HER2-positive cells in each case (Fig. 3).

Fluorescence *in situ* hybridization

None of the cytoplasm-positive cases exhibited amplification of HER2/neu gene copies in the tumor cells, except for one case that showed chromosomal trisomy (Fig. 4).

Immunoelectron microscopy

In all four examined cases, dense-core granules were detected by electron microscopy, although they were not present in all tumor cells. These findings largely

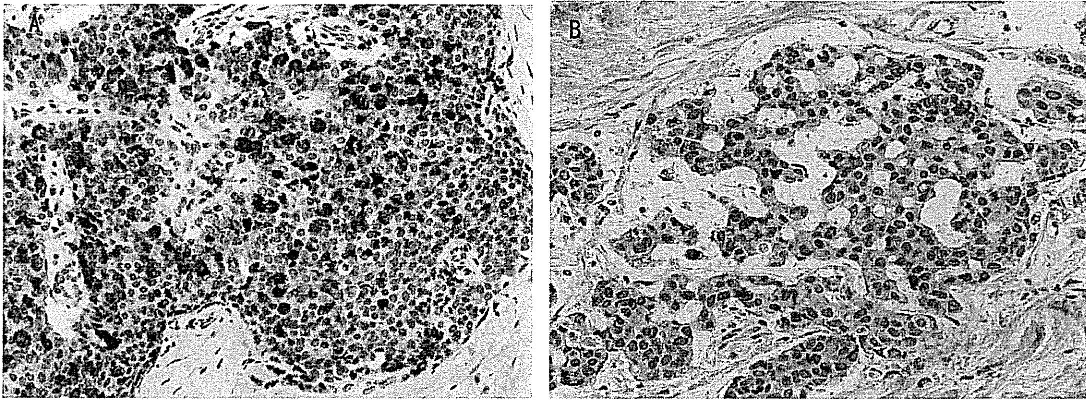

Figure 1 : Immunohistochemistry using a HercepTest staining kit showed a typical cytoplasmic staining pattern. Granular cytoplasmic staining was observed in cases of solid neuroendocrine carcinoma (A) and mucinous carcinoma (B).

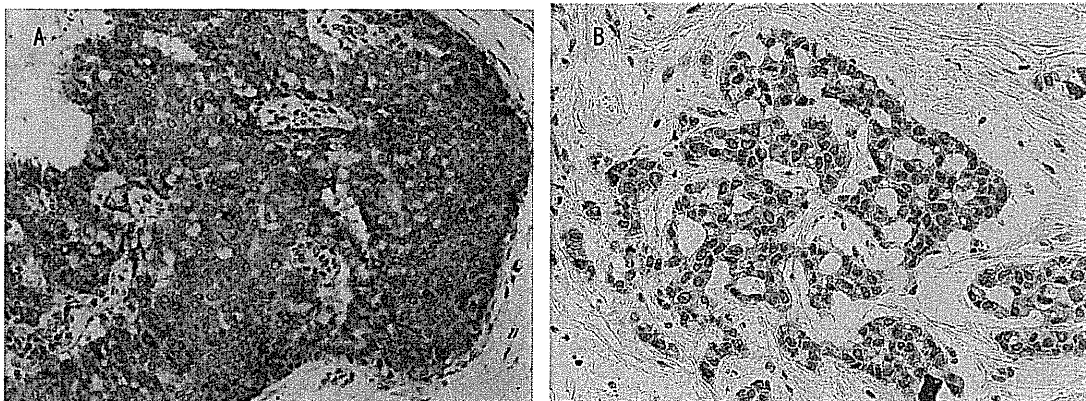

Figure 2 : Immunohistochemistry using CB-11 showed similar or weak cytoplasmic staining in the same cases described in Figure 1A (A) and Figure 1B (B).

Table Ⅲ. Various antibodies for HER2 compared in cytoplasmic positive cases (n=34)

Antibody	No. of Patients (%)			
	More than 50%	10-50%	Less than 10%	negative
HercepTest	2 (5.9)	28 (82.4)	4 (11.8)	0 (0)
CB-11	1(2.9)	19 (55.9)	8 (23.5)	6 (17.6)
TAB250	0 (0)	0 (0)	2 (5.9)	32 (94.1)
SV2-61γ	0 (0)	0 (0)	0 (0)	34 (100.0)

Table Ⅳ. Expression of neuroendocrine (NE) markers in cytoplasmic cases (n=34)

Proportion of	Proportion of cases (%)			
positive cells	Synaptophysin	Chromogranin A	NSE	CD56
Negative	0 (0)	6 (17.6)	5 (14.7)	16 (47.1)
Less than 10%	29 (85.3)	25 (73.5)	25 (73.5)	17 (50.0)
10-50%	3 (8.8)	1 (2.9)	3 (8.8)	0 (0)
More than 50%	2 (5.9)	2 (5.9)	1 (2.9)	1 (2.9)

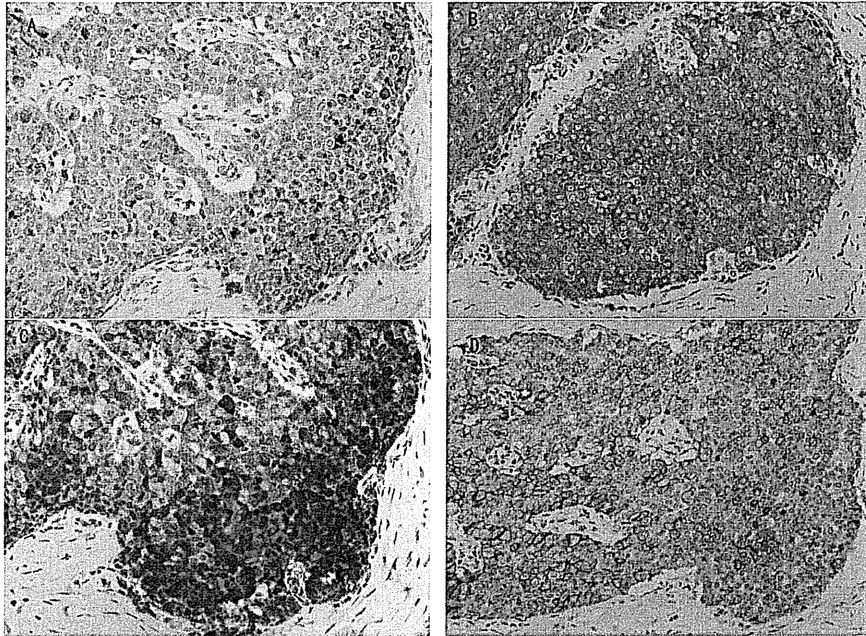

Figure 3 : Immunohistochemistry for neuroendocrine markers. Chromogranin A (A), synaptophysin (B), neuron-specific enolase (NSE) (C), and CD56 (D) were positive in the same cases described in Figure 1A and 2B.

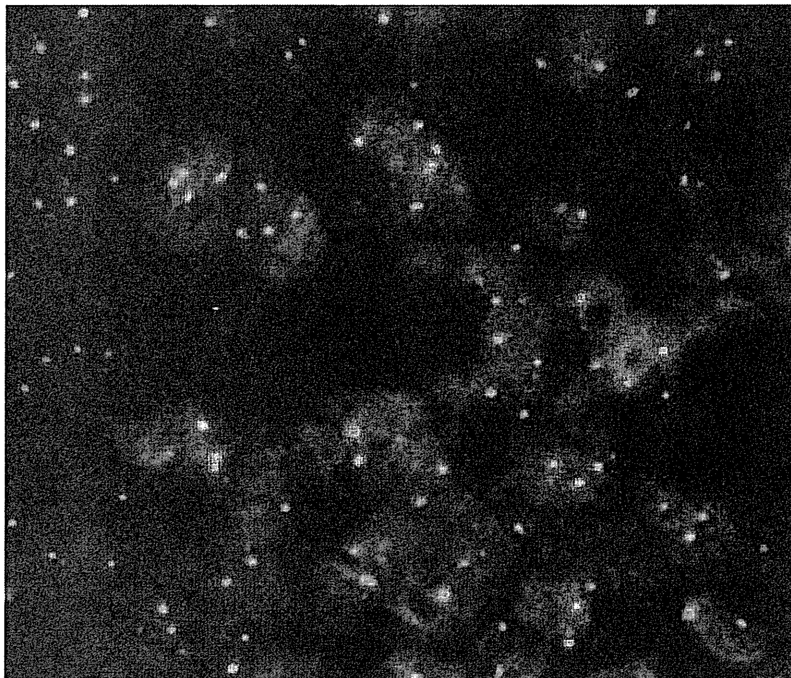

Figure 4 : Fluorescence *in situ* hybridization showed no amplification of the HER2/neu gene.

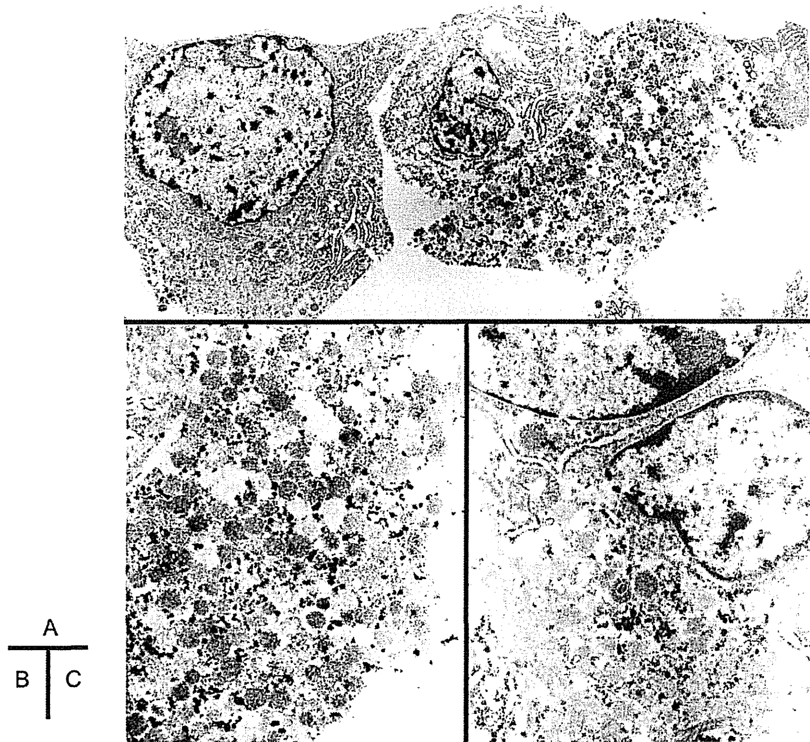

Figure 5 : Immunoelectron microscopy. Dense-core granules were observed in tumor cells (A) and HercepTest antibodies were accumulated in the ribosomes (B) and rough endoplasmic reticulum (C).

corresponded to the immunohistochemical staining pattern of HER2 and neuroendocrine markers.

On immunoelectron microscopy, the polyclonal antibody of the HercepTest was observed in the ribosomes and rough endoplasmic reticulum of tumor cells containing dense-core granules. No direct reactivity for dense-core granules was evident (Fig. 5).

DISCUSSION

In the present study, we found a 3-4% frequency of cytoplasmic HER2 staining. It seems that the cytoplasmic expression of HER2 was not non-specific, because essentially the same tendency in the staining was observed between the assays using two different antibodies (Herceptest® and CB-11) recognizing different HER2 protein epitopes in intercellular domain.Cytoplasmic staining of HER2 protein is frequently observed on routine immunohistochemical examination. There has been much debate as to the significance of cytoplasmic reactivity and its clinicopathological characteristics.[27,28] In a previous report, granular cytoplasmic reactivity with various antibodies for HER2 (TAB-250, CB-11, 3B5, and N3/D10) was described, but the staining pattern did not appear to correlate with the activity of HER2 protein.[27] Also in our study, none of the tumors with the cytoplasmic HER2 staining had gene amplification qualified by FISH analysis, suggesting that these cytoplasmic HER2 expressions could be regulated by a different mechanism from that associated with gene amplification.

The lack of reactivity with antibodies against the extracellular domain of HER2 suggests that the HercepTest and CB-11 antibodies may recognize a shorter-length modified HER2 protein, such as an alternatively processed form or the product of receptor turnover and degradation. A previous study using CB-11 reported that cytoplasmic staining reflects 130- and 150-kDa proteins, which may represent precursor forms of HER2.[29] Cytoplasmic staining with monoclonal antibodies 3B5 and 9G6 has been correlated with a 155-kDa protein on the membranes of mitochondrial cristae.[30] In other types of cancers such as thyroid tumors and transitional cell carcinoma of the bladder, the 130 -140 kDa or 155-kDa shorter-length HER2 protein has been observed by Western blot analysis.[17,31] Even in the preliminary study, we could not detect cytoplasmic HER2 protein in limited formarin fixed materials by

Western blot analysis (data not shown). Immunoelectron microscopy revealed that antibodies against HER2 accumulate in the ribosomes and rough endoplasmic reticulum of tumor cells.

Ninety-seven percent of cancer cells with cytoplasmic HER2 expression showed positive hormone receptor status. A potent positive relationship was seen between the cytoplasmic HER2 staining and ER or PR expression determined by immunohistochemical assay, indicating that the upregulation mechanism of the cytoplasmic HER2 might be unique for hormone-dependent breast cancers. It would be interesting to understand the regulation of cytoplasmic HER2 by hormones such as estradiol. No other clinicopathological parameters showed a significant association with the cytoplasmic HER2 staining.

At present, no data are available on the biological function of cytoplasmic HER2. However, it might not be organ-specific because cytoplasmic HER2 staining was observed in various types of neoplasms such as thyroid neoplasm,[17] pancreatic carcinoma,[18] adrenal tumors,[19] and prostatic cancer. Circumstantially, it seems that tumors arising from endocrine organs are likely to have cytoplasmic HER2 expression. In a study of thyroid cancer, no significant relationship between cytoplasmic HER2 expression and mRNA levels of full-length HER2 was observed.[17]

Neuroendocrine differentiation is classified as a subtype of breast carcinoma.[25] Tumor cells with neuroendocrine differentiation possess typical and characteristic features (granular, eosinophilic) in the cytoplasm. Immunohistochemical evidence of neuroendocrine marker expression has been detected in nearly 20% of breast carcinoma[32]. However, it is often difficult to precisely distinguish these cytological features from conventional breast cancer. This may also be one of the reasons why the significance of neuroendocrine differentiation remains unclear.[33,34] In this study, we found that cytoplasmic HER2 reactivity characterized by granular cytoplasmic staining and mosaic pattern distribution was closely correlated with neuroendocrine differentiation. All 34 tumors with cytoplasmic HER2 staining showed expression of synaptophysin. More than 80% of the tumors having cytoplasmic HER2 staining had either chromogranin A or NSE expression. About 50% of the tumors showed expression of CD56 by immunohistochemical analysis.

The biological and clinical implications of neuroendocrine differentiation in human breast cancer are still largely unknown. Therefore, it would be interesting to know whether tumors with cytoplasmic HER2 expression have neuroendocrine differentiation frequently. Because cytoplasmic HER2 expression was potently associated with hormone receptor expressions, it is worthy to investigate cytoplasmic HER2 expression from the point of interaction between hormonal regulation and neuroendocrine differentiation in a future study. Furthermore, HER2 status may be examined in daily practice to determine indications for therapy and evaluate prognosis. Therefore, cytoplasmic HER2 staining might be useful for detecting neuroendocrine differentiation.

In conclusion, we investigated the cytoplasmic HER2 expression in human breast cancer. The frequency of cytoplasmic HER2 staining was around 3-4%. The expression of cytoplasmic HER2 was recognized by two antibodies for different protein epitopes. All the tumors with cytoplasmic HER2 expression were FISH-negative. There was a significant correlation between cytoplasmic HER2 expression and hormone receptor expression. Intriguingly, it was newly discovered that all of these tumors with cytoplasmic HER2 expression had a phenotype of neuroendocrine differentiation determined by imunocytochemical assay using multiple marker antibodies. Although confirmatory analyses are required, a significant association between cytoplasmic HER2 expression and neuroendocrine differentiation has been suggested. Further investigation into the biological role of cytoplasmic HER2 expression and its clinical implication in breast cancer is warranted. Particularly, the engagement of neuroendocrine differentiation in cytoplasmic HER2-expressing tumors will be a new aspect of study.

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The Breast Journal

LETTER TO THE EDITOR

Alternation of Estrogen Receptor and Progesterone Receptor Expression in Primary Breast Cancer Patients Treated with Neoadjuvant Chemotherapy

To the Editor:

Since the benefits of post-operative adjuvant therapy were established in the Early Breast Cancer Trialist's Collaborative Group (EBCTCG) overview (1), many clinical trials have been conducted in order to improve the prognosis. In parallel, to increase the application of breast-conserving surgery (BCS), neoadjuvant chemotherapy (NAC) was developed, with a pathological complete response (pCR) used as a surrogate marker for a favorable prognosis. If the patient achieves pCR after NAC, the risk of recurrence is very low. However, therapeutic strategies after NAC have not yet been established. There are some clinical questions regarding whether additional chemotherapy is needed for non-pCR cases after NAC, and whether additional treatment is required for pCR cases.

When evaluating the profiles of tumor cells, we often observe that the features of post-operative tissue do not always correspond with those of pre-operative biopsy specimens. In particular, the alteration of hormone receptor (HR) expression after NAC is very important for decision-making regarding postoperative endocrine therapy. However, it remains controversial whether endocrine therapy should be conducted in cases of HR-negative conversion.

To further clarify this issue, we evaluated the HR status before and after NAC among operable breast cancer patients, and attempted to validate the alteration of the HR status. Patients enrolled in this study were women with primary invasive breast cancer, confirmed by core needle biopsy (CNB) or incisional biopsy, between January 2000 and February 2006 at the Tokyo Metropolitan Cancer and Infectious Disease Center, Komagome Hospital. One hundred and sixty-five cases with operable early breast cancer (cT1-3, cN0-2, and cM0) received NAC. The NAC regimen was FEC (5-fluorouracil: 500 mg/m^2, epirubicine: 100 mg/m^2, and cyclophosphamide: 500 mg/m^2) every 3 weeks for four cycles followed by docetaxel (75 mg/m^2) every 3 weeks for four cycles (2). In evaluating the HR status, pre-treatment and surgical specimens were stained with mouse monoclonal anti-human ERα antibody (1D5; DAKO, Glostrup, Denmark) and anti-human PgR antibodies (PgR636, DAKO). Hormone receptor expression was scored by assigning proportion and intensity scores, according to Allred's procedure (3). Scores of 0–2 were designated as negative, while 3–8 were considered positive.

Except for patients with bilateral breast cancer or who achieved a pCR, a total of 107 patients among 165 cases were evaluated for receptor status conversion. The characteristics of these patients were not significantly different. The median age was 51 years (range, 23–71 years). Fifty-nine patients were aged less than 50 years and 48 patients were aged 50 or more.

The pretreatment ER/PgR status in CNB or excisional biopsy was classified into four groups: ER+/PgR+, ER+/PgR−, ER−/PgR+, and ER−/PgR−, with 63 (58.9%), 24 (22.4%), 2 (1.9%) and 18 (16.8%) cases, respectively. Table 1 shows the conversion of HR after NAC. On preoperative evaluation, there were 89 HR+ cases (83.2%) and 18 HR− cases (16.8%). Negative conversion of HR was seen in five cases (4.7%), and no positive conversions were noted. The HR status in almost all patients was not changed by NAC. In the conversion of the ER and PgR status after NAC, there were 87 pre-treatment ER+ cases, and 83 were unchanged after treatment. In primary ER− cases, positive conversion was observed in only one case. This result indicates that the ER status was also not affected by NAC in most cases (95.4%). However, 19 (17.8%) cases exhibited the negative conversion of the PgR status, and positive conversion from PgR− comprised 6.5% (7/107) (Table 2). PgR+ cases were then divided into two groups: negative conversions were noted in

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Table 1. Conversion of Hormone Receptor Status After Neo-adjuvant Chemotherapy

NAC		
Pre	Post	Alteration rate (n = 107)
HR+	HR+	84 (78.5%)
HR−	HR−	18 (16.8%)
HR+	HR−	5 (4.7%)
HR−	HR+	0

HR+, hormone receptor positive; HR−, hormone receptor negative.

Table 2. Alteration of ER and PgR Status After NAC

	Alteration of status after NAC (n = 107)		
	Change		
	Pos	Neg	No change
ER	1 (0.9%)	4 (3.7%)	102 (95.3%)
PgR	7 (6.5%)	19 (17.8%)	81 (75.7%)

ER, estrogen receptor; PgR, progesterone receptor; Pos, positive conversion; Neg, negative conversion.

46.9% (15/32) of patients aged <50 years and 18.2% (6/33) of patients aged 50 or more years (p = 0.014).

In 102 cases (95.3%), the HR status did not change after NAC. This indicates that NAC does not influence the selection of adjuvant endocrine therapy in most cases. However, we were particularly interested in the alteration of the PgR status. Cases showing a negative conversion of PgR were more frequently observed than those with a positive conversion. Jain et al. reported a PgR-negative conversion rate of 22.2% (4 of 18 cases), and an ER-negative conversion rate of 5.6% (1 of 18 cases) (4). These results were similar to those in our study.

When patients were classified into two groups based on age (50 or more years and under 50 years), negative conversion of PgR-positive cases were more frequently observed in patients under 50 years (46.9%, p = 0.014). As the average menopausal age of Japanese women is around 50 years, we speculate that chemotherapy induced amenorrhea, causing the negative conversion of PgR expression. In general, it is thought that PgR gene expression depends on estrogen initiating ER-mediated transactivation. Anderson et al. suggested that the frequency of ER+/PgR− breast cancer increases with age (5). It was assumed that the overexpression of PgR occurs in an estrogen-rich environment, as a declining ovarian function causes a decline in blood estrogen levels, and the expression of PgR is reduced. Thus, the negative conversion of PgR in our study may have been caused by chemotherapy-induced amenorrhea.

Since the chemotherapy-induced amenorrhea was observed in all premenopausal women in our study, we speculate that there are two types of HR-positive premenopausal breast cancer. One depends on estrogen derived only from ovaries, and the other utilizes estrogen from aromatase after chemotherapy-induced amenorrhea. If this is the case, patients experiencing the negative conversion of PgR by NAC could be administered tamoxifen, whereas non-negative conversion cases could be treated with aromatase inhibitor, particularly when they experience amenorrhea after NAC. Although we cannot exclude the possibility that non-estrogen-dependent PgR induction contributes to the continuous expression of PgR after chemotherapy-induced amenorrhea, this notion may lead to tailor-made endocrine therapy for premenopausal breast cancer.

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Changes in microRNA expression levels correlate with clinicopathological features and prognoses in endometrial serous adenocarcinomas

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This study aimed to determine the expression profiles of microRNAs (miRNAs) in endometrial serous adenocarcinoma and to examine the association between miRNA expression and clinical outcomes. Twenty-one patients diagnosed with endometrial serous adenocarcinoma between January 2001 and December 2006 were enrolled. miRNA expression profiles were examined using miRNA microarray and qRT-PCR. miRNA expression levels were correlated with clinicopathological variables and survival rates. A total of 120 miRNAs were differentially expressed in endometrial serous adenocarcinoma compared to normal endometria. Of these, 54 miRNAs were down-regulated (>2-fold), including miR-101, miR-10b*, miR-152, and miR-29b, and the remainder were up-regulated (>2-fold), including miR-200a, miR-200b, and miR-205. Decreased expression of miR-10b*, miR-29b, and miR-455-5p was correlated with vascular invasion ($P = 0.048$, $P = 0.013$, and $P = 0.032$, respectively). Univariate analysis revealed that lower expression of miR-101, miR-10b*, miR-139-5p, miR-152, miR-29b, and miR-455-5p was significantly correlated with poor overall survival ($P < 0.05$), and reduced expression of miR-152, miR-29b, and miR-455-5p was significantly correlated with poor disease-free survival ($P < 0.05$). Multivariate analysis demonstrated that decreased expression of miR-152 ($P = 0.021$) was a statistically independent risk factor for overall survival, and decreased expression levels of miR-101 ($P = 0.016$) and miR-152 ($P = 0.010$) were statistically independent risk factors for disease-free survival. In addition, transfection of miR-101 or miR-152 precursors into an endometrial serous carcinoma cell line inhibited cell growth ($P < 0.0001$ and $P = 0.01$, respectively). Moreover, strong positive immunoreactivity of cyclooxygenase-2 (COX-2) was significantly correlated with down-regulation of miR-101 ($P = 0.035$). These findings suggest that the dysregulation of miRNAs is associated with the poor prognosis in endometrial serous adenocarcinoma patients. (*Cancer Sci* 2010; 101: 241–249)

Serous adenocarcinoma of the endometrium was first identified as a distinct clinical entity by Hendrickson *et al.* in 1982.[1] This disease accounts for 10% of all endometrial cancers and generally occurs in postmenopausal women.[2] Serous adenocarcinoma is considered to be an aggressive tumor with a high relapse rate, early and deep myometrial invasion, and frequent lymphovascular space involvement.[1,2] Patients without any myometrial invasion are as likely to have extrauterine disease as those with deeply invasive tumors.[3] The 5-year survival rate for stage I serous adenocarcinomas varies from 15 to 51%.[4] Thus, the prognosis of these patients is similar to or worse than that of patients with grade 3 endometrial carcinomas confined to the uterus.[5,6] The identification of new prognostic factors may facilitate the development of novel treatments, thereby leading to an improved clinical outcome for this uncommon, highly aggressive tumor.

MicroRNAs (miRNAs) are noncoding, single-stranded RNAs of 18 to 24 nucleotides in length that constitute a novel class of gene regulators. miRNAs function as guide molecules by base-pairing with the mRNAs that are partially complementary to the miRNAs in miRNA-associated effector complexes.[7] The binding of miRNAs to their target mRNAs leads to translational repression or decreases the stability of the mRNA molecule.[7] miRNAs have a profound impact on many processes that are frequently disrupted during malignant transformation, including cell proliferation,[8] apoptosis, stress responses, maintenance of stem cell potency, and metabolism.

Several miRNAs have emerged as candidate components of oncogene and tumor suppressor gene networks. The miR-17-92 cluster, as well as miR-155 and miR-372-373, have been identified as proto-oncogenes in B-cell lymphomas and testicular tumors.[9,10] The miR-17-92 cluster accelerates c-Myc–induced oncogenesis in a mouse model of B-cell lymphoma.[11] In contrast, some miRNAs act as tumor suppressors by suppressing expression of oncogenes. For instance, miR-15a and miR-16, which can induce apoptosis by targeting the anti-apoptotic gene *Bcl-2*, are frequently lost in B-cell chronic lymphocytic leukemia (CLL) due to chromosomal deletions.[12,13] The let-7 family, which is down-regulated in lung cancers in which RAS is frequently mutated,[14] negatively regulates RAS.[15] Moreover, several reports have identified metastasis-mediating miRNAs.[16] These findings suggest that aberrant miRNA expression contributes not only to tumorigenesis but also to metastasis. Under normal physiological conditions, individual miRNAs show strict tissue- and developmental-stage-specific expression patterns. In contrast, miRNAs display unique expression patterns depending on clinical features in several cancers, including CLL,[17] breast cancer,[18] and lung cancer,[19] suggesting that some miRNAs could be used as diagnostic and/or prognostic markers.

In this study, we investigated miRNA expression profiles in specimens from patients with serous adenocarcinoma of the endometrium and attempted to identify miRNAs capable of predicting clinical prognosis as novel clinical biomarkers.

Materials and Methods

Cell lines and tissue samples. The established human endometrial serous carcinoma cell line SPAC-1-L was provided by the laboratory of Dr. Hirai from the Department of Gynecology, Cancer Institute Hospital (Tokyo, Japan).[20] This cell line was cultured in the appropriate medium and passed at confluence to a 10-cm^2 dish (Becton Dickinson, Lincoln Park, NJ, USA). The dishes were incubated at 37°C in a CO_2 incubator supplied with

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humidified 5% CO_2 and 95% air. The medium was changed twice a week.

After obtaining informed consent, 21 serous adenocarcinoma tissues and seven normal endometrial tissues (four proliferative phase, three secretory phase) were retrieved from surgical pathology files at Tohoku University Hospital (Sendai, Japan). The clinical data and patient information are shown in Table 1. The research protocol was approved by the Ethics Committee at Tohoku University Graduate School of Medicine (Sendai, Japan). All specimens were obtained from surgery that was performed from January 2001 to December 2006 at Tohoku University Hospital (Sendai, Japan). We also obtained non-pathologic endometrial tissue as a normal control from hysterectomy specimens performed due to non-endometrial carcinomas. All endometrial carcinoma specimens were obtained during hysterectomy. No patient had received preoperative irradiation or chemotherapy. Information about age, performance status on admission, histology including the percentage of the tumor comprised of serous adenocarcinoma, stage, degree of myometrial invasion, degree of lymphovascular space invasion, degree of lymph node invasion, and overall survival was retrieved from patient charts. The median follow-up time of patients examined in this study was 23 months (range, 3–64 months). Disease-free survival and overall survival were calculated from the time of initial surgery to recurrence and/or death or the date of last contact. Survival times of patients still alive or lost to follow-up were censored in September 2008. The standard primary treatment for endometrial carcinoma at Tohoku University Hospital was surgery consisting of total abdominal hysterectomy, salpingo-oophorectomy, pelvic and/or para-aortic lymphadenectomy, and cytologic analysis of peritoneal washings. If the patient was diagnosed with serous adenocarcinoma by curettage before surgical treatment, omentectomy was also performed. Of the 21 patients, 19 patients received platinum-based chemotherapy and one patient underwent postoperative radiotherapy. The lesions were classified according to the histological typing of female genital tract tumors by the World Health Organization and staged according to the International Federation of Gynecology and Obstetrics system.[21,22] This study only enrolled patients whose endometrial carcinoma was comprised of pure serous adenocarcinoma and did not have any other histological components. These specimens were processed in 10% formalin, fixed for 24–48 h, paraffin embedded, and thin (3 μm) sectioned. All of these archival specimens were embedded immediately in OCT compound (Sakura Finetechnical, Tokyo, Japan) and stored at −80°C until further use. Only sections containing a minimum of 90% carcinoma by examination with hematoxylin–eosin staining were used for total RNA preparation.

Table 1. Patient and clinical data

	Total (n = 21)	%
Median age (years)	64.9 (54–87)	
Death	11	52.4
Stage		
I	8	38.1
II	2	9.5
III	3	14.3
IV	8	38.1
Myometrial invasion		
Absent	8	38.1
<1/2	7	33.3
<<1/2	6	28.6
Lymph node metastasis†		
Absent	13	61.9
Present	2	9.5
Vascular invasion		
Absent	16	76.2
Present	5	23.8
COX-2 immunoreactivity		
Weakly positive	10	47.6
Strongly positive	11	52.4

†Six patients did not receive lymphadenectomy. COX-2, cyclooxygenase 2.

Immunohistochemistry. Immunohistochemical analysis was performed employing the streptavidin–biotin amplification method using a Histofine kit (Nichirei, Tokyo, Japan). Goat polyclonal antibody for cyclooxygenase (COX)-2 (C-20) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). For immunostaining of COX-2, the slides were heated in an autoclave at 120°C for 5 min in 0.01M citric acid buffer (2 mmol/L citric acid and 9 mmol/L trisodium citrate dehydrate [pH 6.0]) following deparaffinization for antigen retrieval. The primary antibody was diluted 1:500. The antigen–antibody complex was visualized with 3,3′-diaminobenzidine solution (1 mmol/L 3,3′-diaminobenzidine, 50 mmol/L Tris-HCl buffer [pH 7.6], and 0.006% H_2O_2) and counterstained with hematoxylin. Tissue sections of kidney were used as positive controls. For COX-2 expression, the distribution and intensity was scored according to the method which has been previously described:[23] negative, weakly positive, and strongly positive. The immunohistochemical expression was independently reviewed by two of the authors (E. H. and J. A.).

MicroRNA isolation and microarray analysis. For microRNA microarray and further studies, total RNA, including miRNA, was isolated from frozen pure and typical serous adenocarcinoma tissues and normal endometrial tissues using the miRNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Frozen tissues were homogenized in QIAzol Lysis reagent (Qiagen). RNA purity and concentration were confirmed by spectrophotometry using the NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA) and the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

miRNA microarrays were manufactured by Agilent Technologies and contained 20–40 features targeting each of the 470 human miRNAs. The labeling and hybridization of total RNA samples were performed according to the manufacturer's protocol. Total RNA (100 ng) was dephosphorylated with calf intestine alkaline phosphatase (Takara Biomedicals, Tokyo, Japan), denatured with dimethyl sulfoxide, and labeled with pCp-Cy3 using T4 RNA ligase (Ambion, Austin, TX, USA). After hybridization and washing, the arrays were scanned with an Agilent microarray scanner using high dynamic range settings as specified by the manufacturer. Microarray results were extracted using Agilent Feature Extraction software version 9.5.3.1 (Agilent Technologies) and analyzed using Gene Spring GX 7.3.1 software (Agilent Technologies) to obtain gene expression ratios.

Quantitative real-time RT-PCR. Quantitative RT-PCR analysis was performed using Taqman MicroRNA Assays (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocol. cDNA was made from 5 ng total RNA from each sample using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) and miRNA specific primers (Applied Biosystems) for eight down-regulated (miR-101, miR-10b*, miR-133a, miR-133b, miR-152, miR-29b, miR-34b, and miR-411) and three up-regulated (miR-200a, miR-200b, and miR-205) miRNA species. Reactions were performed on an ABI 7500 thermocycler (Applied Biosystems), with cycle threshold values determined using the manufacturer's software. Negative control reactions without RNA and without reverse transcriptase were also performed. U6 snRNA (RNU6B; Applied Biosystems) served as an endogenous control. The fold-change for each

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miRNA, relative to RNU6B, was calculated using the $2^{-\Delta\Delta Ct}$ method.[24] Two independent RT-PCR reactions were performed.

Transfection of precursor miRNA and cell proliferation assay. SPAC-1-L cells (1×10^5) were transfected with 100 pmol of Pre-miR miRNA Precursor Molecules (Applied Biosystems) or Pre-miR miRNA Molecules Negative Control 1 (Applied Biosystems) using a Lipofectamine RNAi MAX (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. For biological assays, cells were used 72 h after transfection. All experiments were performed in duplicate, and each experiment was repeated independently in triplicate. The cell number was evaluated using a Cell counting kit-8 (Dojindo, Kumamoto, Japan) according the manufacturer's instructions.

Statistical analysis. Raw microarray data were normalized and analyzed using the Gene Spring GX 7.3.1 software (Agilent Technologies). Expression data were median centered. Statistical analysis was performed using StatView software version 5.0 (SAS Institute, Cary, NC, USA). miRNAs that had a >2-fold change were considered to have significant differential expression compared to normal endometrium. The Mann–Whitney *U*-test was performed to identify miRNAs that demonstrated statistically significant differential expression between normal endometrium and carcinoma tissues, and to evaluate differences between miRNA expression and patient characteristics. For survival analysis and generation of Kaplan–Meier survival curves, miRNA levels measured on miRNA chips were converted into discrete variables by splitting the samples into two classes (high and low expression), using the respective median level of miRNA expression levels as a threshold. Survival curves were compared by log-rank analysis. Significance was accepted with 95% confidence. Multivariate analysis was performed using the Cox proportional hazards model. Results were expressed as means ± SD and analyzed by one-way ANOVA and the Bonferroni test for cell proliferation assays. $P < 0.05$ was considered to be statistically significant.

Results

Distinct microRNA signatures in endometrial serous adenocarcinoma compared to normal endometrial tissues. miRNA microarray analysis was used to identify miRNAs that were differentially expressed between endometrial serous carcinoma and normal endometrial tissue (Fig. 1). Table 2 lists the differentially expressed miRNAs with at least a 2-fold change in expression. A total of 66 miRNAs were up-regulated and 54 were down-regulated in endometrial serous carcinoma. Of these miRNAs, miR-205 exhibited the largest degree of

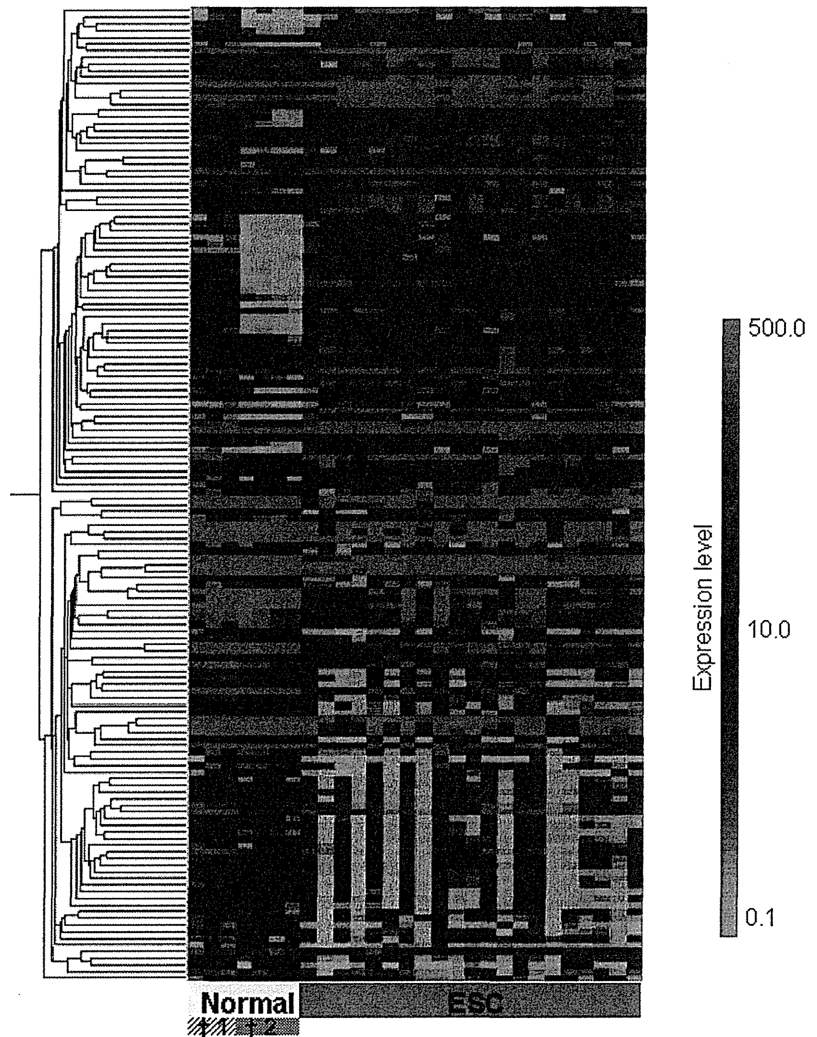

Fig. 1. Unsupervised hierarchical clustering analysis of microRNAs (miRNAs) that exhibited a >2-fold increase or decrease in expression in endometrial serous adenocarcinoma. The level of miRNA expression is color-coded as follows: red, higher miRNA expression; green, lower miRNA expression; black, no difference. The bar at the bottom indicates the group of cancer samples (blue) or normal endometrial tissues (yellow). Normal, normal endometrial tissues; ESC, endometrial serous adenocarcinoma. *1, secretory phase; *2; proliferative phase.

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Table 2. Differentially expressed miRNAs with >2-fold change in endometrial serous adenocarcinoma *vs* normal endometrial tissues

miRNA	*P*-values	Fold change
Down		
miR-1	0.002	15.7
miR-101	0.016	3.59
miR-10b*	0.028	2.27
miR-127-3p	0.036	4.5
miR-132*	0.003	2.04
miR-133a	0.001	142.1
miR-133b	0.003	27.8
miR-136	<0.001	11.6
miR-136*	0.002	21.3
miR-139-5p	0.009	9.42
miR-140-3p	0.021	2.83
miR-140-5p	0.002	3.86
miR-142-3p	0.005	3.18
miR-142-5p	0.01	2.69
miR-143	0.002	8.6
miR-143*	0.001	15.5
miR-145	0.005	7.15
miR-145*	0.005	6.31
miR-152	<0.001	4.09
miR-195	<0.001	10.5
miR-196b	0.004	6.16
miR-199a-5p	0.009	5.92
miR-199b-3p	0.01	4.98
miR-199b-5p	0.012	7.08
miR-214	0.009	5.13
miR-214*	0.009	13.6
miR-23b	0.014	3.8
miR-24-1*	0.001	4.6
miR-27b	0.016	3.82
miR-299-3p	0.004	9.43
miR-299-5p	0.002	26.9
miR-29b	0.032	2.25
miR-33a	0.002	6.1
miR-337-5p	0.005	17.1
miR-34b	0.014	40.2
miR-34b*	0.016	2.97
miR-34c-5p	0.014	6.17
miR-376a	0.024	8.29
miR-376c	0.018	6.74
miR-377	0.001	27.2
miR-379	0.005	6.53
miR-381	0.01	9.43
miR-410	0.028	6.38
miR-411	0.016	22.2
miR-424	<0.001	24.8
miR-450a	<0.001	8.45
miR-455-3p	0.024	2.57
miR-455-5p	0.032	2.41
miR-497	<0.001	11.3
miR-503	<0.001	15.1
miR-542-3p	<0.001	16.7
miR-542-5p	0.005	8.878
miR-654-3p	0.028	14.3
miR-873	0.001	17.6
Up		
miR-106a	0.047	2.08
miR-10a	0.01	3.43
miR-10a*	0.004	9.51
miR-1224-5p	0.001	3.7
miR-1225-5p	0.003	3.11
miR-1226*	0.001	14.8
miR-125a-3p	0.001	2.62
miR-134	0.016	2.71
miR-135a*	<0.001	50.8
miR-135b	<0.001	10.4
miR-150*	0.001	4.32
miR-17	0.014	2.32
miR-182	0.003	72.9
miR-183	0.001	8.49
miR-188-5p	<0.001	4.24
miR-18a	0.002	3.98
miR-18b	0.012	2.84
miR-193a-5p	0.006	2.56
miR-198	<0.001	54.2
miR-200a	0.012	2.89
miR-200a*	<0.001	29.5
miR-200b	0.004	3.82
miR-200b*	0.001	6.93
miR-200c	0.012	2.68
miR-202	0.002	24.9
miR-203	0.001	8.95
miR-205	<0.001	267.8
miR-210	0.002	3.17
miR-223	0.01	4.45
miR-224	0.047	2.82
mIR-23a*	<0.001	56.6
miR-30c-2*	0.002	4.36
miR-31	0.041	2.45
miR-330-3p	0.021	8.17
miR-33b*	<0.001	7.57
miR-371-5p	<0.001	10.8
miR-425	0.005	3.14
miR-429	0.001	4.61
miR-483-5p	0.001	4.64
miR-494	0.001	4.36
miR-501-5p	0.028	2.98
miR-505*	0.047	2.36
miR-513a-5p	0.001	31.3
miR-513b	0.002	6.56
miR-518c*	<0.001	128
miR-557	0.003	3.36
miR-564	0.021	2.39
miR-575	0.002	5.2
miR-601	0.001	5.21
miR-622	<0.001	70.2
miR-623	0.001	9.81
miR-629*	0.002	120.8
miR-630	<0.001	13.8
miR-652	0.018	2.21
miR-663	<0.001	13.7
miR-7	0.001	3.64
miR-760	0.012	3.53
miR-765	<0.001	48.7
miR-768-5p	0.014	2.89
miR-801	<0.001	4.28
miR-877	<0.001	10.4
miR-892b	0.004	4.86
miR-923	0.004	3.16
miR-939	0.002	2.42
miR-95	0.014	2.93
miR-96	0.005	3.65

P-values <0.05 were considered significant.

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up-regulation (267.8-fold), and miR-133a displayed the smallest degree of down-regulation (142.1-fold) (Table 2).

Validation of microarray results. To validate the microarray results, quantitative RT-PCR analysis of the differentially expressed miRNAs was independently performed. The following miRNAs that were differentially expressed between normal endometrial tissue and endometrial serous carcinoma tissue were selected: miR-101, miR-10b*, miR-133a, miR-133b, miR-152, miR-29b, miR-34b, miR-411 miR-200a, miR-200b, and miR-205 (Fig. 2). In agreement with the microarray results, miR-101, miR-10b*, miR-133a, miR-133b, miR-152, miR-29b, miR-34b, miR-411 were down-regulated, whereas miR-205 was up-regulated in endometrial serous carcinoma. miR-200a and miR-200b also appeared to be up-regulated, but not to a statistically significant degree (data not shown).

Correlation between microRNA expression and clinicopathological parameters in patients with endometrial serous adenocarcinoma. We next analyzed results from miRNA expression profiles in endometrial serous carcinoma to evaluate whether a correlation existed with various clinicopathological features associated with tumor specimens. We analyzed endometrial serous carcinomas of different clinical stages (stage I, II/III, and IV), degree of myometrial invasion (pT1-2/pT3), degree of lymph node metastasis, and degree of vascular invasion. While no statistically significant associations were observed between miRNA expression and clinical stage, myometrial invasion, or lymph node metastasis (data not shown), a statistically significant association with vascular invasion was noted ($P < 0.05$) (Table 3). Specifically, expression of miR-10b*, miR-29b, and miR-455-5p was lower in specimens with vascular invasion ($P = 0.048$, $P = 0.013$, and $P = 0.032$, respectively).

Immunostaining for COX-2 was detected in the cytoplasm of normal glandular cells and tumor cells (Fig. 3a,b). All tumor cases were positive for COX-2: 10 out of 21 tumor cases (47.6%) were scored as weakly positive and the remainder (52.4%) as strongly positive. In contrast, all of normal cases were weakly positive. A more intense reactivity was noted in the infiltrating cells at the tumor periphery. Strong, positive COX-2 immunoreactivity was significantly correlated with down-regulation of miR-101 ($P = 0.035$) (Fig. 3c).

MicroRNA signatures are associated with the prognosis of patients with endometrial serous adenocarcinoma. We next investigated the correlation between miRNA expression level and survival. The association between median survival as calculated by Kaplan–Meier analysis and miRNA expression is shown in Fig. 4. Lower expression of miR-101, miR-10b*, miR-139-5p, miR-152, miR-29b, and miR-455-5p was significantly

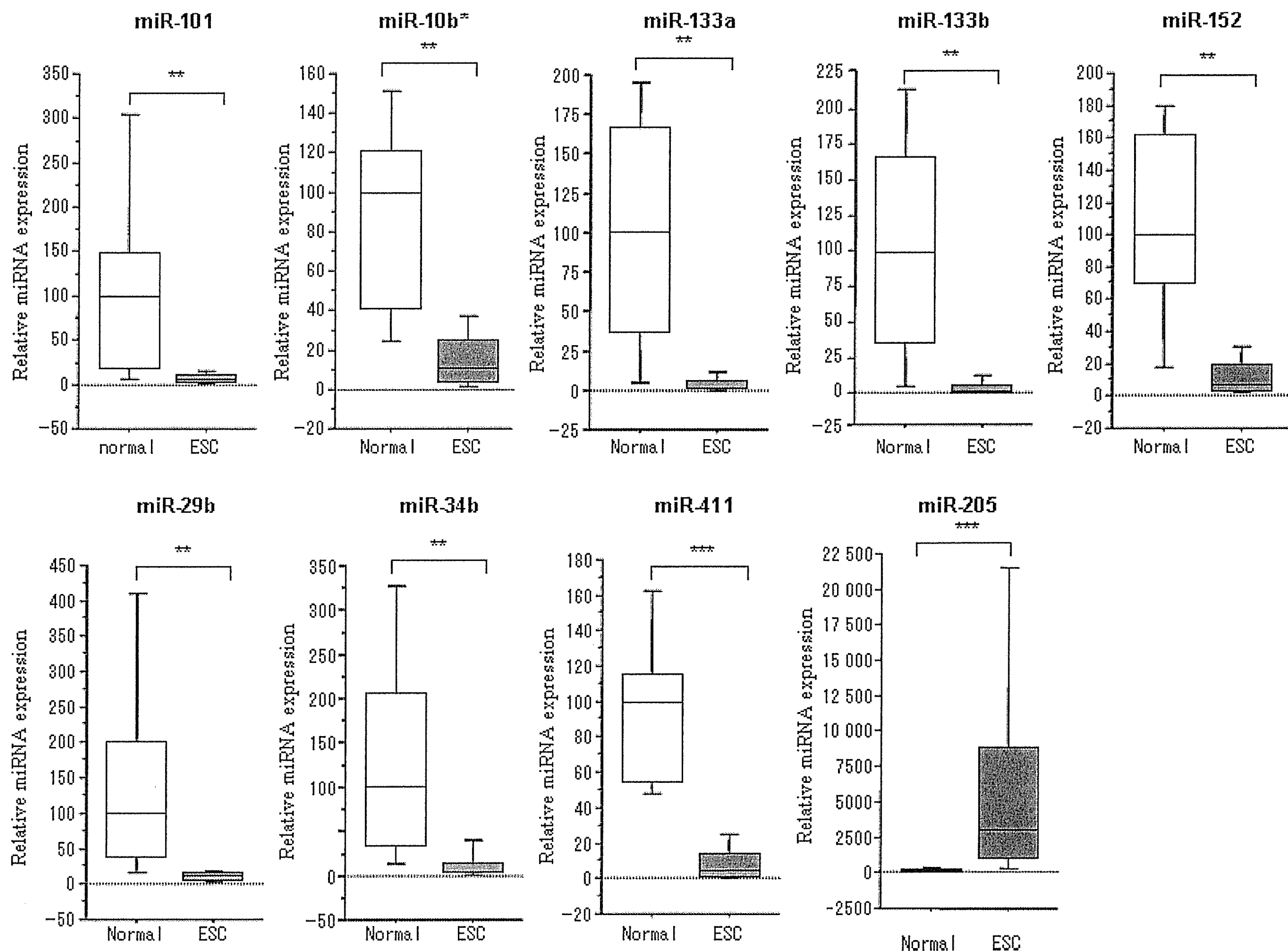

Fig. 2. Quantitative RT-PCR of nine different miRNA species (miR-101, miR-10b*, miR-133a, miR-133b, miR-152, miR-29b, miR-34b, miR-411, and miR-205). Expression of miR-101, miR-10b*, miR-133a, miR-133b, miR-152, miR-29b, miR-34b, and miR-411 was down-regulated, while miR-205 expression was up-regulated in endometrial serous adenocarcinoma. Normal, normal endometrial tissues; ESC, endometrial serous adenocarcinoma. **$P < 0.01$; ***$P < 0.001$.

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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.

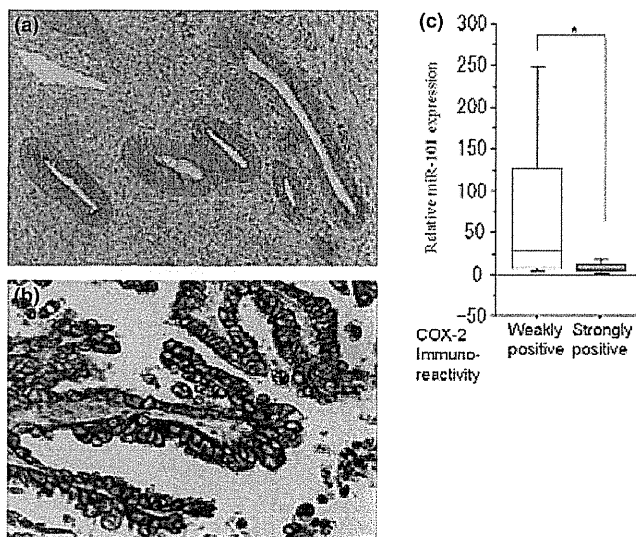

Fig. 3. Immunohistochemistry for cyclooxygenase (COX)-2 expression in endometrial tissues. (a) Immunoreactivity of COX-2 was weakly detected in the cytoplasm of normal endometrium glandular cells. (b) Strong immunoreactivity was detected in the cytoplasm of carcinoma cells. Original magnification, ×200 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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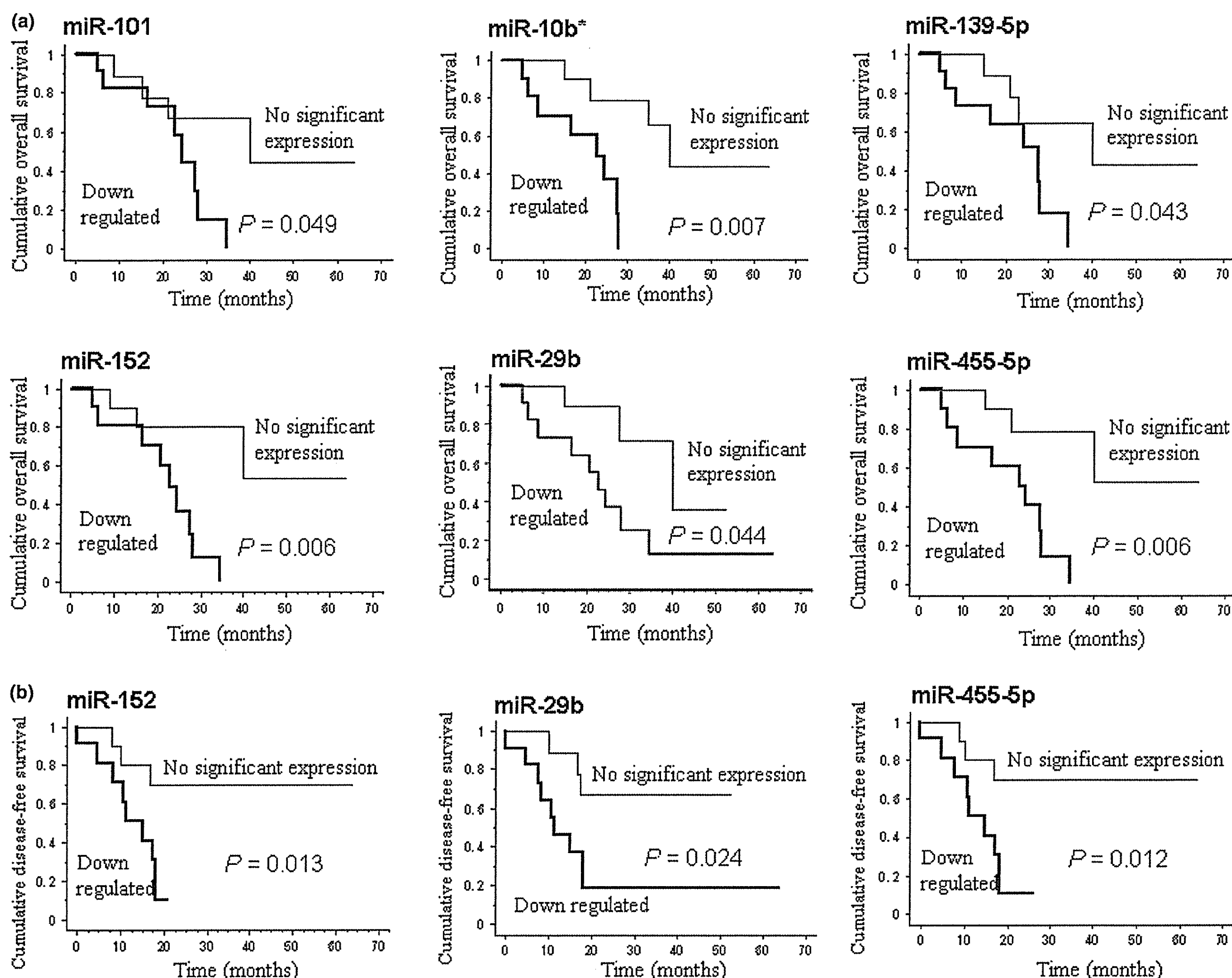

Fig. 4. Kaplan–Meier overall survival curve for patients with endometrial serous adenocarcinoma of based on miRNA expression. (a) Reduced expression of miRNA-101, miR-10b*, miR-139-5p, miR-152, miR-29b, and miR-455-5p was significantly correlated with shorter overall survival. (b) Decreased expression 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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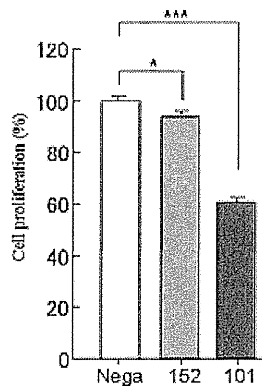

Fig. 5. Cell proliferation assay in SPAC-1-L cells. SPAC-1-L cells were transfected with pre-miR miR-101, pre-miR-152 precursor molecules, or a negative control for 72 h. Nega, transfected with negative control; 152, transfected with pre-miR-152 molecules; 101, transfected with pre-miR-101 molecules. 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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