Table 3. Summary of Acute Recovery After Cardiac End Point Trastuzumab Safety Analysis Population Group

Cardiac End Point	No.	%	Median (months)*	Range (months)†
Severe CHF (n = 13)				
Reached acute recovery	9	69.2		
Time to acute recovery			11.6	1.3-28.7
Occurrence of LVEF drop to < 50% after acute recovery	3	33.3		
Time to LVEF drop to < 50% after acute recovery			25.8	3.0-25.8
Symptomatic CHF (n = 32)				
Reached acute recovery	25	78.1		
Time to acute recovery			5.5	0.0-28.7
Occurrence of LVEF drop to < 50% after acute recovery	8	32.0		
Time to LVEF drop to < 50% after acute recovery			27.7	3.0-34.7
Confirmed significant LVEF drop (n = 60)				
Reached acute recovery	50	83.3		
Time to acute recovery			6.3	0.0-33.1
Occurrence of LVEF drop to < 50% after acute recovery	14	28.0		
Time to LVEF drop to < 50% after acute recovery			—	2.5-51.6
Any type of cardiac end point (n = 73)				
Reached acute recovery	59	80.8		
Time to acute recovery			6.4	0.0-33.1
Occurrence of LVEF drop to < 50% after acute recovery	17	28.8		
Time to LVEF drop to < 50% after acute recovery			—	2.5-51.6

Abbreviations: CHF, congestive heart failure; LVEF, left ventricular ejection fraction.

Subsequent LVEF Decrease to Less Than 50% After Acute Recovery and Evidence of Progressive Cardiac Disease

The cumulative proportion of patients who had a subsequent LVEF decrease to less than 50% after reaching acute recovery by time from reaching acute recovery is shown in Figure 2B. It should be noted that Figure 2B is based on the small number of patients with a cardiac end point who reached acute recovery. Among the 59 patients in the trastuzumab group who reached acute recovery, 42 patients had all subsequent LVEF assessments ≥ 50%, and 17 patients had at least one subsequent LVEF decrease to less than 50%. The CAB reviewed the 17 patients with at least one subsequent LVEF decrease and found evidence of progressive cardiac disease in only six patients. The remaining 11 of 17 patients were assessed by the CAB as having a favorable outcome.

The CAB assessment of one patient who reached acute recovery and who had all subsequent LVEF assessments ≥ 50% was undetermined. Fifty-two (88.1%) of the 59 patients who reached acute recovery were assessed by the CAB as having a favorable outcome from the cardiac end point.

CAB Assessment of Outcome From the Cardiac End Point

A flow chart of the CAB assessment for the 73 patients in the trastuzumab group is shown in Figure 3. Among these 73 patients, the CAB assessment was that 57 (78.1%) had a favorable outcome, including five patients who did not reach acute recovery, and that 14 (19.2%) did not have a favorable outcome, including six patients who reached acute recovery. The CAB assessment was undetermined for two patients.

Among the 14 patients who did not reach acute recovery, the CAB assessment was that five had a favorable outcome, eight did not have a favorable outcome, and one was undetermined. For patients in the trastuzumab group, Appendix Figure A1 (online only) illustrates the proportion of patients who had any type of cardiac end point, the proportion with a cardiac end point who reached acute recovery, and the proportion who reached acute recovery assessed by the CAB as having a favorable outcome.

Description of Patient-Related Predictive Factors

We investigated if there was a pattern of chemotherapy treatment or cardiac medication in patients who were assessed by the CAB as not having a favorable outcome from the cardiac end point.

Previous Anthracyclines

Nearly all (94.1%) of the patients enrolled had been treated with anthracyclines. Of the 73 patients in the trastuzumab group with a cardiac end point, 70 had been treated with anthracyclines. Of the 12 patients in the observation group with a cardiac end point, 11 had been treated with anthracyclines.

Patients Who Did Not Have a Favorable Outcome to the Cardiac End Point

Of the 14 patients in the trastuzumab group assessed by the CAB as not having a favorable outcome from the cardiac end point, 13 had been treated with anthracyclines. Cardiac medication was reported for seven of these 14 patients; however, there was no consistent use of a particular type of cardiac medication.

DISCUSSION

The predominant cardiovascular adverse effect of trastuzumab is the induction of cardiac contractile dysfunction, a complication that previously has been associated mainly with anthracycline treatment. In the HERA trial, the incidence of cardiac dysfunction in the

trastuzumab arm at a median follow-up time of 1 year was 0.6% for severe CHF and 7.0% for left ventricular (LV) dysfunction.[10] Results from the HERA trial suggest that trastuzumab-associated cardiac dysfunction has a high rate of reversibility,[10] a characteristic that is fundamentally different from anthracycline-associated cardiac dysfunction. However, several questions remained unanswered: Does the incidence of cardiac end points increase with longer follow-up time? When do the cardiac events predominantly occur? What is the cardiac prognosis of a patient after a cardiac end point? What are the risk factors and outcomes of patients with progressive cardiac dysfunction after trastuzumab treatment? Does trastuzumab treatment worsen anthracycline-associated cardiac dysfunction?

We now show that, after a median follow-up time of 3.6 years, the incidence of severe CHF and LV dysfunction in the trastuzumab group remained low at 0.8% and 9.8%, respectively. Similarly, the rate of discontinuation of trastuzumab as a result of cardiac disorders was low (5.1%). Despite these reassuring results, and because anthracycline cardiac adverse effects typically become manifest 5 to 10 years after the initial exposure, longer follow-up of cardiac safety is still required. Preclinical data suggests that trastuzumab could worsen anthracycline-associated cardiotoxicity.[9]

All occurrences of severe symptomatic CHF failure and 51 (85%) of 60 confirmed significant LVEF decreases in the trastuzumab group occurred during the scheduled trastuzumab treatment period. After a cardiac end point, questions of clinical importance are whether cardiac function recovers and, if so, whether the patient is at risk of a subsequent LVEF decrease. Therefore, we defined acute recovery, and the CAB assessed if the patient had a favorable outcome from the cardiac end point or not on the basis of the patients' LVEF trends. Approximately 80% of patients (57 of 73 patients) in the trastuzumab group with cardiac end points were assessed by the CAB as having a favorable outcome. Among the patients in the trastuzumab group who reached acute recovery from a cardiac end point (59 of 73 patients; 80.8%), most were assessed by the CAB as having a favorable outcome (52 of 59 patients; 88.1%). Among the 59 patients who reached acute recovery, 15 patients in the absence of any additional trastuzumab treatment had a subsequent LVEF drop to 50%, though we do not know the cause of the subsequent LVEF decrease; nine of these 15 patients were assessed by the CAB as having a favorable outcome. Given that the majority of cardiac end points in the trastuzumab group occurred during the scheduled treatment period, reaching acute recovery after a cardiac end point may influence treatment decisions outside clinical trials. In the HERA trial, patients who had a confirmed significant LVEF decrease discontinued trastuzumab treatment.

The relatively good prognosis after a cardiac end point also sheds light on the pathophysiology of trastuzumab-associated cardiac dysfunction. Preclinical data indicate that inhibition of myocardial HER2/ERB2 leads to changes in the tertiary structure of the cardiac contractile apparatus (likely a reversible condition) but does not induce myocardial cell death (likely a progressive condition).[12] This may explain why cardiac contractile dysfunction is predominantly seen during trastuzumab treatment and appears to have a high rate of reversibility. In contrast, anthracyclines can induce myocardial cell death that leads to a maladaptive cardiac remodeling with progressive cardiac dysfunction and heart failure.[13] This suggests that the approximately 80% of patients (57 of 73 patients) in the trastuzumab group with a cardiac end point assessed by the CAB as having a favorable outcome may have primarily trastuzumab-associated cardiac dysfunction. Among the approximately 20% of patients (14 of 73 patients) in the trastuzumab group assessed by the CAB as not having a favorable outcome from the cardiac end point, almost all (13 of 14) had been treated with anthracyclines. In these 13 patients, it is possible that trastuzumab treatment worsened anthracycline-associated cardiac dysfunction.

In the HERA trial, nearly all of the patients enrolled had been treated with anthracyclines. We have insufficient information to assess the influence of cardiac medication on the prognosis of patients in the trastuzumab group with a cardiac end point.

In conclusion, given the benefit in disease-free and overall survivals of 1 year adjuvant trastuzumab in patients with HER2-positive early breast cancer, the low incidence of cardiac end points with longer term follow-up, and the suggestion that trastuzumab-induced cardiac dysfunction may be reversible, adjuvant trastuzumab should be considered as a standard treatment option for patients who fulfill the HERA trial eligibility criteria.

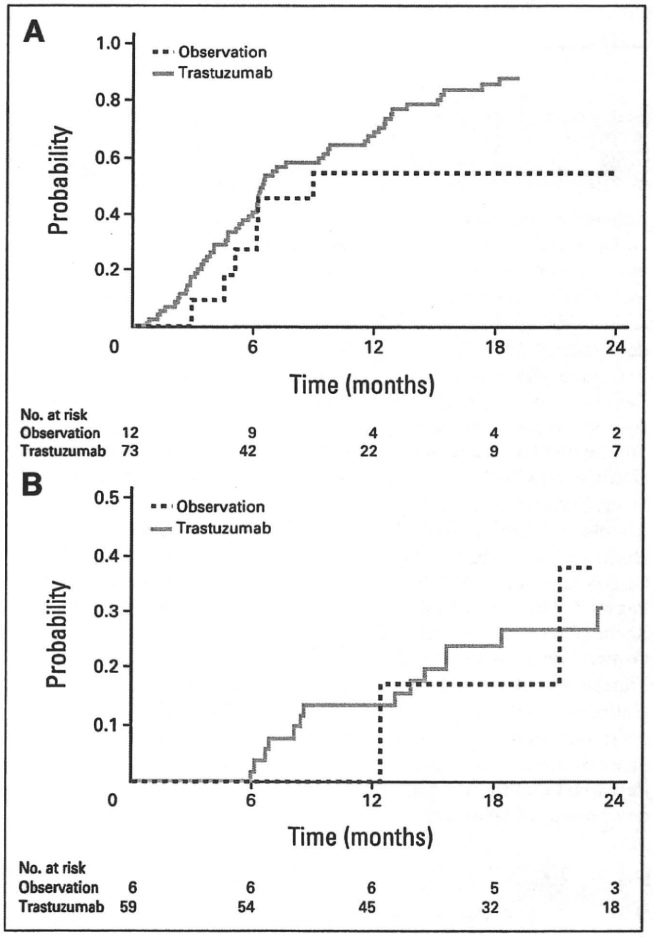

Fig 2. The cumulative incidence by safety analysis population group among patients with any type of cardiac end point of (A) reaching acute recovery and (B) subsequent left ventricular ejection fraction decrease to less than 50% after reaching acute recovery.

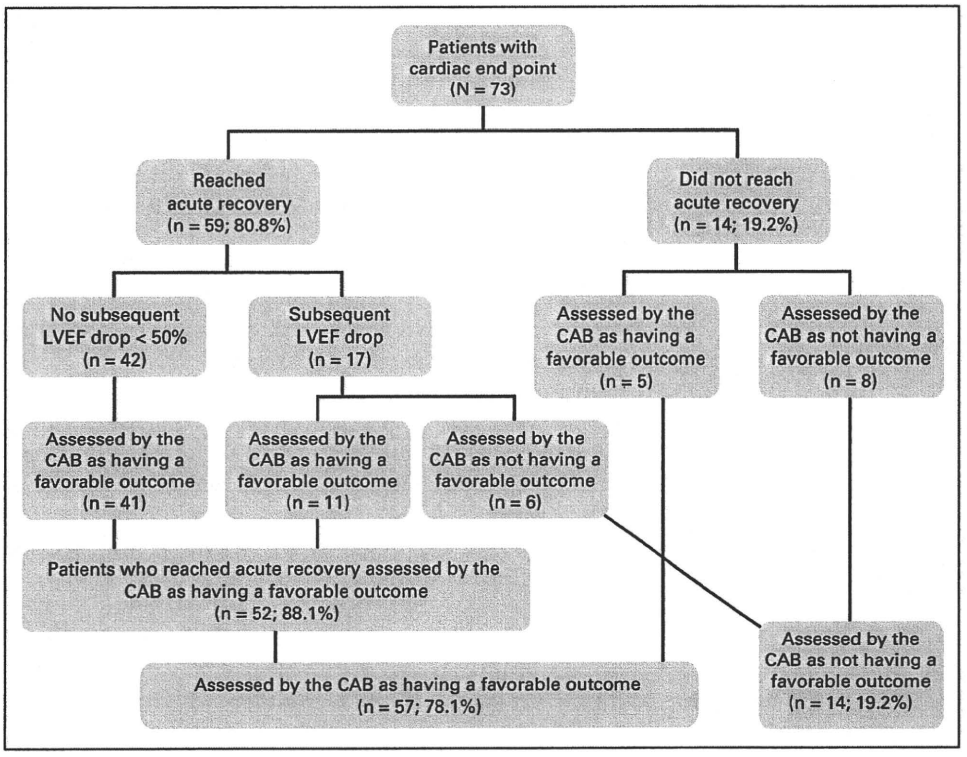

Fig 3. Flow chart of the cardiac advisory board (CAB) assessment for patients with any type of cardiac end point in the trastuzumab group. The CAB assessment was undetermined for two patients. LVEF, left ventricular ejection fraction.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

Employment or Leadership Position: Susanne Muehlbauer, F. Hoffmann-La Roche (C) **Consultant or Advisory Role:** Thomas M. Suter, GlaxoSmithKline (U), F. Hoffmann-La Roche (U); Evandro de Azambuja, F. Hoffmann-La Roche (C); Miguel Angel Climent, F. Hoffmann-La Roche (U); David Dodwell, F. Hoffmann-La Roche (C), GlaxoSmithKline (C), AstraZeneca (C), Novartis (C), Aventis (C); Olivia Pagani, F. Hoffmann-La Roche (C); Michael Muschol, F. Hoffmann-La Roche (C); Dirk J. van Veldhuisen, F. Hoffmann-La Roche (U) **Stock Ownership:** Susanne Muehlbauer, F. Hoffmann-La Roche **Honoraria:** Thomas M. Suter, sanofi-aventis; Evandro de Azambuja, F. Hoffmann-La Roche; Miguel Angel Climent, F. Hoffmann-La Roche; Ernst Rechberger, F. Hoffmann-La Roche; Mazakasu Toi, Chugai Pharmaceutical; Martine J. Piccart-Gebhart, F. Hoffmann-La Roche **Research Funding:** Thomas M. Suter, F. Hoffmann-La Roche; Evandro de Azambuja, GlaxoSmithKline; Mazakasu Toi, Chugai Pharmaceutical **Expert Testimony:** None **Other Remuneration:** Ernst Rechberger, F. Hoffmann La-Roche

AUTHOR CONTRIBUTIONS

Conception and design: Marion Procter, Thomas M. Suter, R. Charles Coombes, David Dodwell, Michael Muschol, Martine J. Piccart-Gebhart
Administrative support: Evandro de Azambuja, Veerle van Dooren, Walter Tsang-Wu Liu
Provision of study materials or patients: Miguel Angel Climent, Ernst Rechberger, Walter Tsang-Wu Liu, Mazakasu Toi, R. Charles Coombes, David Dodwell, Olivia Pagani, Jorge Madrid, Marcia Hall, Shin-Cheh Chen, Christian Focan, Martine J. Piccart-Gebhart
Collection and assembly of data: Evandro de Azambuja, Veerle van Dooren, Miguel Angel Climent, Ernst Rechberger, Mazakasu Toi, David Dodwell, Michael Muschol, Dirk J. van Veldhuisen
Data analysis and interpretation: Marion Procter, Thomas M. Suter, Evandro de Azambuja, Urania Dafni, Susanne Muehlbauer, R. Charles Coombes, Michael Muschol, Dirk J. van Veldhuisen
Manuscript writing: Marion Procter, Thomas M. Suter, Evandro de Azambuja, Urania Dafni
Final approval of manuscript: Marion Procter, Thomas M. Suter, Evandro de Azambuja, Urania Dafni, Veerle van Dooren, Susanne Muehlbauer, Miguel Angel Climent, Ernst Rechberger, Walter Tsang-Wu Liu, Mazakasu Toi, R. Charles Coombes, David Dodwell, Olivia Pagani, Jorge Madrid, Marcia Hall, Shin-Cheh Chen, Christian Focan, Michael Muschol, Dirk J. van Veldhuisen, Martine J. Piccart-Gebhart

REFERENCES

1. Smith I, Procter M, Gelber RD, et al: 2-year follow-up of trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer: A randomised controlled trial. Lancet 369:29-36, 2007

2. Romond EH, Perez EA, Bryant J, et al: Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. N Engl J Med 353: 1673-1684, 2005

3. Perez EA, Romond EH, Suman VJ, et al: Updated results of the combined analysis of NCCTG N9831 and NSABP B-31 adjuvant chemotherapy with/without trastuzumab in patients with HER2-positive breast cancer. J Clin Oncol 255:18s, 2007 (suppl; abstr 512)

4. Slamon D, Eiermann W, Robert N, et al: Phase III randomized trial comparing doxorubicin and cyclophosphamide followed by docetaxel (AC-T) with doxorubicin and cyclophosphamide followed by

docetaxel and trastuzumab (AC-TH) with docetaxel, carboplatin and trastuzumab (TCH) in Her2/neu-positive early breast cancer patients: BCIRG 006 study. Cancer Res 69, 2009 (suppl; abstr 62)

5. Cook-Bruns N: Retrospective analysis of the safety of Herceptin immunotherapy in metastatic breast cancer. Oncology 61:58-66, 2001 (suppl 2)

6. Suter TM, Cook-Bruns N, Barton C: Cardiotoxicity associated with trastuzumab (Herceptin) therapy in the treatment of metastatic breast cancer. Breast 13:173-184, 2004

7. Sawyer DB, Zuppinger C, Miller TA, et al: Modulation of anthracycline-induced myofibirillar disarray in rat ventricular myocytes by neuregulin-1beta and anti-erbB2: Potential mechanism for tastuzumab-associated cardiotoxicity. Circulation 105:1551-1554, 2002

8. Zhao YY, Sawyer DR, Baliga RR, et al: Neuregulins promote survival and growth of cardiac myocytes: Persistance of ErbB2 and ErbB4 expression inneonatal and adult ventricular myocytes. J Biol Chem 273: 10261-10269, 1998

9. Timolati F, Ott D, Pentassuglia L, et al: Neuregulin-1 beta attenuates doxorubicin-induced alterations of excitation-contraction coupling and reduces oxidative stress in adult rate cardiomyocytes. J Mol Cell Cardiol 41:845-854, 2006

10. Suter TM, Procter M, van Veldhuisen DJ, et al: Trastuzumab-associated cardiac adverse effects in the Herceptin Adjuvant Trial. J Clin Oncol 25:3859-3865, 2007

11. Piccart-Gebhart MJ, Procter M, Leyland-Jones B, et al: Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. N Engl J Med 353:1659-1672, 2005

12. Pentassuglia L, Graf M, Lane H, et al: Inhibition of ErbB2 by receptor tyrosine kinase inhibitors causes myofibrillar structural damage without cell death in adult rat cardiomyocytes. Exp Cell Res 315:1302-1312, 2009

13. Cohn JN, Ferrari R, Sharpe N: Cardiac remodeling: Concepts and clinical implications—A consensus paper from an international forum on cardiac remodeling. J Am Coll Cardiol 35:569-582, 2000

J Med Dent Sci 2010; 57: 155–163

Original Article

HER-2/neu cytoplasmic staining is correlated with neuroendocrine differentiation in breast carcinoma

Shin-ichiro Horiguchi[1,2], Tsunekazu Hishima[2], Yukiko Hayashi[2], Yumiko Shiozawa[2], Kazumi Horiguchi[3], Katsumasa Kuroi[3], Masakazu Toi[4], Nobuaki Funata[2] and Yoshinobu Eishi[1]

1) Department of Human Pathology, Tokyo Medical and Dental University Graduate School, Tokyo, Japan
2) Department of Pathology, Cancer and Infectious Diseases Center of Tokyo Metropolitan Komagome Hospital, Tokyo, Japan
3) Department of Surgery, Cancer and Infectious Diseases Center of Tokyo Metropolitan Komagome Hospital, Tokyo, Japan
4) Department of Surgery, Graduate School of Medicine, Kyoto University, Kyoto, Japan

HER2 oncoprotein plays an essential role in breast cancer growth and differentiation. Determination of HER2 status contributes not only to predicting survival but also to selecting the patients for anti-HER2 therapy. HER2 protein expressed in human cancer cells often contains variant forms as well as the full-length wild-type form. In the present study, we investigated the subcellular localization of HER2 protein in 1053 primary breast cancer tissues. HER2 protein was stained by various immunohistochemical methods and studied by immunoelectron microscopy to confirm the intracellular localization.

Thirty-four of 1053 specimens showed cytoplasmic staining of the intracellular domain of HER2 protein by the HercepTest® and CB-11. In contrast, no immunoreactivity to the antibodies against the extracellular domain was observed. None of the 34 specimens showed amplification of the HER2 protein by fluorescence *in situ* hybridization. Subsequently, we studied the association of the cytoplasmic expression of HER2 with neuroendocrine differentiation. Interestingly, all 34 specimens had some positive signals of neuroendocrine markers such as synaptophysin, chromogranin A, neuron-specific enolase, and CD56. Although the result is preliminary, it warrants further study on the role of the cytoplasmic variant form of HER2 in breast cancer growth, particularly in the aspect of neuroendocrine differentiation.

Key words: HER2/neu, cytoplasmic staining, neuroendocrine differentiation, breast cancer, immunohistochemistry.

Corresponding Author: Shin-ichiro Horiguchi, MD
Department of Pathology, Tokyo Metropolitan Komagome Hospital
3-18-22 Hon-komagome, Bunkyo-ku, Tokyo 113-8677, Japan
Fax: +81-3-4463-7561
E-mail: s.horiguchi@cick.jp
Received February 1 ; Accepted March 12, 2010

INTRODUCTION

The HER2/neu (HER2) proto-oncogene, located on chromosome 17, encodes a 185-kDa glycoprotein that acts as a growth factor receptor on the cell surface.[1] In the breast, oncogenic overexpression of the HER2 protein is both a marker for poor prognosis and a target for trastuzumab (Herceptin™; Genentech, Inc., South San Francisco, CA). HER2 gene amplification or protein overexpression can be determined by various reagents and techniques, including fluorescence *in situ* hybridization (FISH), immunohistochemistry (IHC), polymerase chain reaction (PCR), and enzyme-linked immunosorbent assay (ELISA).[2-5] The most frequently employed method is immunohistochemical detection using antibodies against the HER2 protein in paraffin sections; the IHC staining procedure is performed using standard equipment.[4,5]

HER2 status can be classified into four categories based on the degree of positivity in the cell membrane and the percentage of positive tumor cells.[4,5] Cytoplasmic staining for antibodies against HER2/

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 1. 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

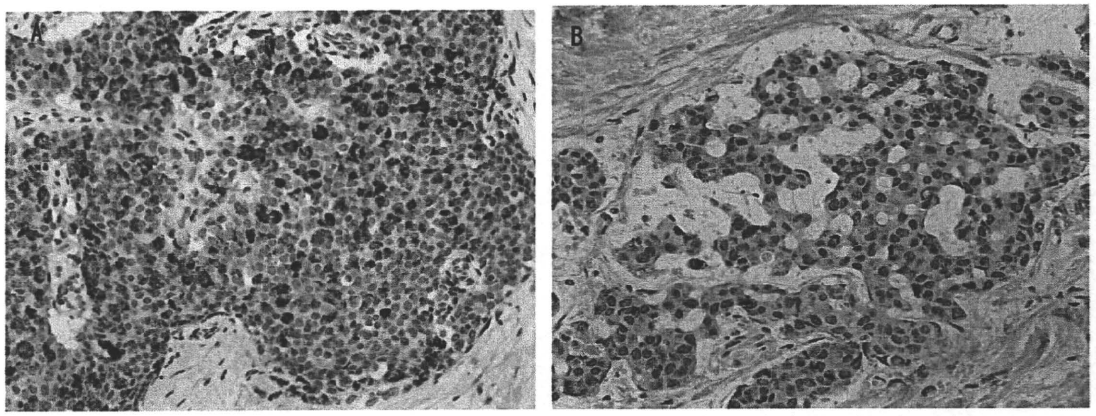

Figure 1 : Immunohistochemistry using a HercepTest staining kit showed a typical cytoplasmic staining pattern. Granular cytoplasmic staining was observed in cases of solid neuroendocrine carcinoma (A) and mucinous carcinoma (B).

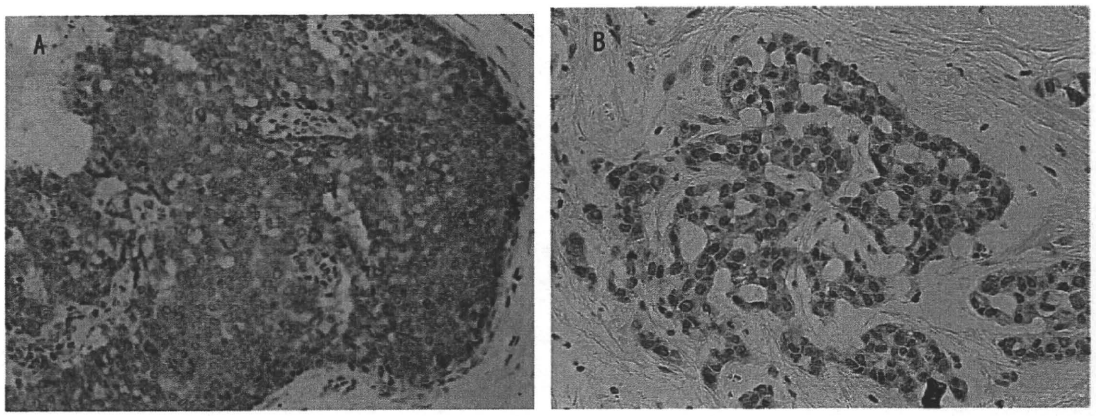

Figure 2 : Immunohistochemistry using CB-11 showed similar or weak cytoplasmic staining in the same cases described in Figure 1A (A) and Figure 1B (B).

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 positive cells	Proportion of cases (%)			
	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)

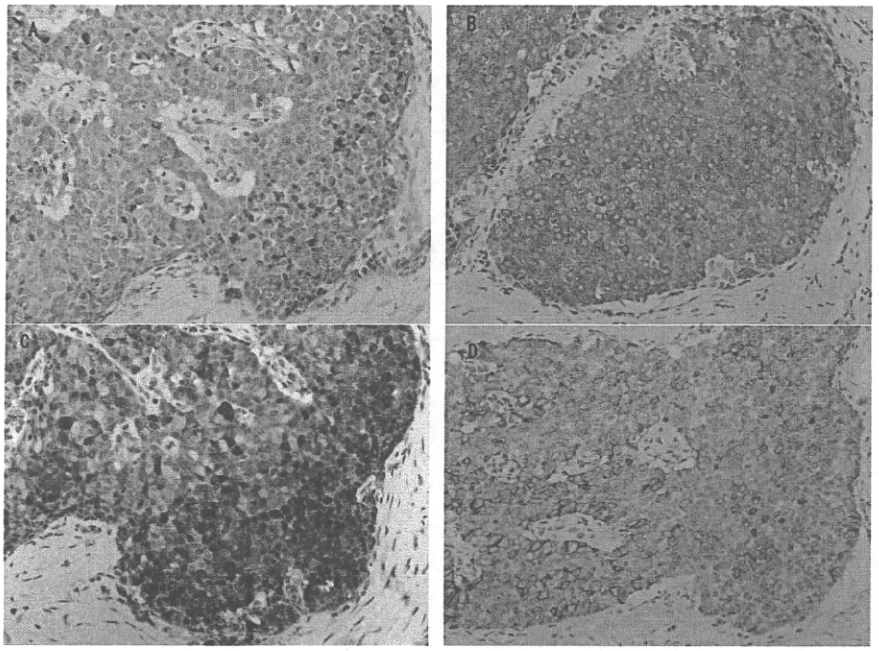

Figure 3 : Immunohistochemistry for neuroendocrine markers. Chromogranin A (A), synaptophysin (B), neuron-specific enolase (NSE) (C), and CD56 (D) were positive in the same cases described in Figure 1A and 2B.

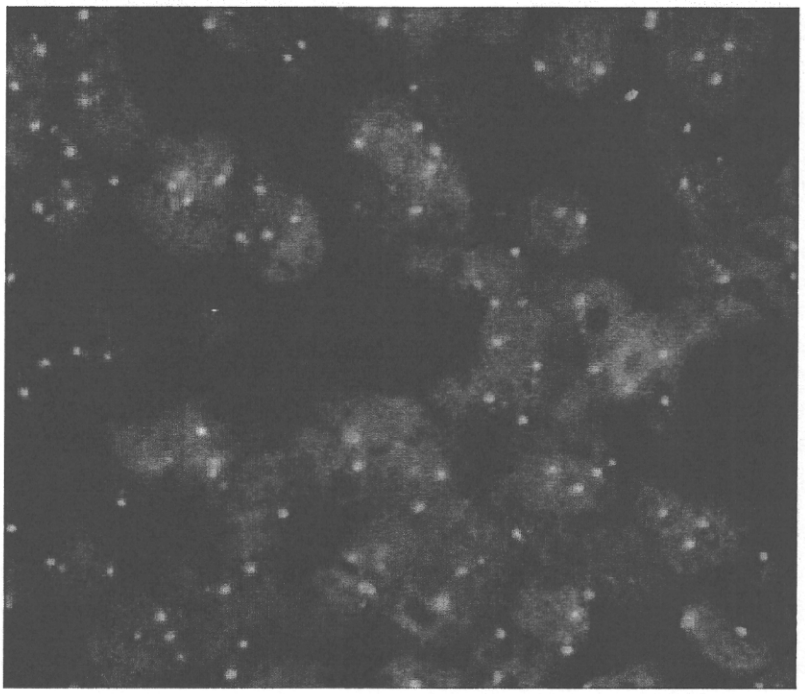

Figure 4 : Fluorescence *in situ* hybridization showed no amplification of the HER2/neu gene.

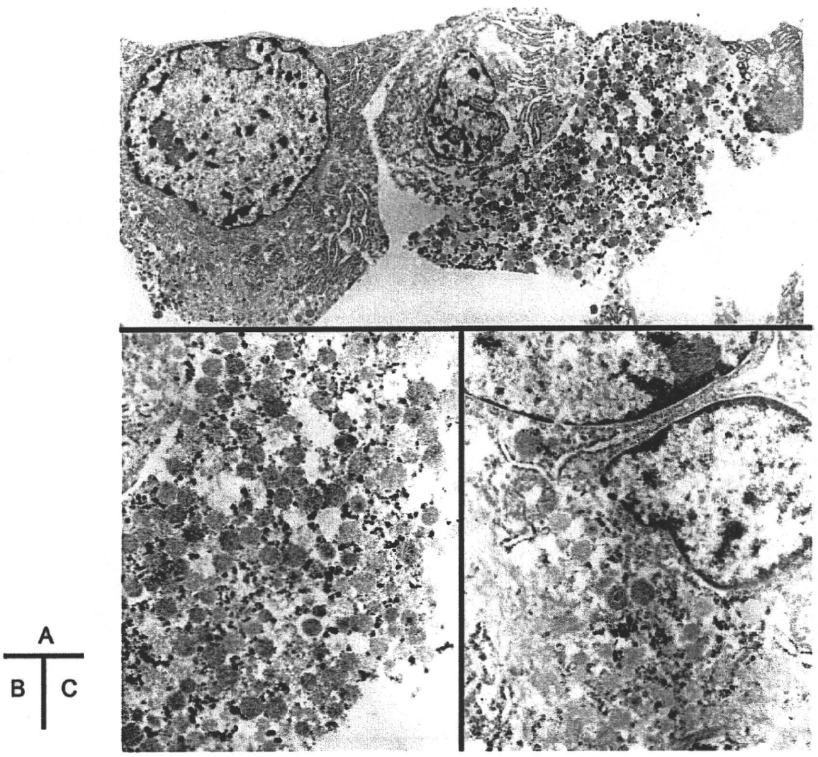

Figure 5 : Immunoelectron microscopy. Dense-core granules were observed in tumor cells (A) and HercepTest antibodies were accumulated in the ribosomes (B) and rough endoplasmic reticulum (C).

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.

References

1. Roskoski R, Jr. The ErbB/HER receptor protein-tyrosine kinases and cancer. Biochem Biophys Res Commun 2004; 319: 1-11.
2. Kay EW, Walsh CJ, Cassidy M, et al. C-erbB-2 immunostaining: problems with interpretation. J Clin Pathol 1994; 47: 816-22.
3. Keshgegian AA. ErbB-2 oncoprotein overexpression in breast carcinoma: inverse correlation with biochemically and immunohistochemically determined hormone receptors. Breast Cancer Res Treat 1995; 35: 201-10.
4. Bartlett JM, Going JJ, Mallon EA, et al. Evaluating HER2 amplification and overexpression in breast cancer. J Pathol 2001; 195: 422-8.
5. Hanna W. Testing for HER2 status. Oncology 2001; 61 Suppl 2: 22-30.
6. Keshgegian AA, Cnaan A. erbB-2 oncoprotein expression in breast carcinoma. Poor prognosis associated with high degree of cytoplasmic positivity using CB-11 antibody.

Am J Clin Pathol 1997; 108: 456-63.
7. Hanna W, Kahn HJ, Andrulis I, et al. Distribution and patterns of staining of Neu oncogene product in benign and malignant breast diseases. Mod Pathol 1990; 3: 455-61.
8. Press MF, Cordon-Cardo C, Slamon DJ. Expression of the HER-2/neu proto-oncogene in normal human adult and fetal tissues. Oncogene 1990; 5: 953-62.
9. Scott GK, Robles R, Park JW, et al. A truncated intracellular HER2/neu receptor produced by alternative RNA processing affects growth of human carcinoma cells. Mol Cell Biol. 1993 Apr;13(4):2247-57.
10. Egeblad M, Mortensen OH, Jäättelä M. Truncated ErbB2 receptor enhances ErbB1 signaling and induces reversible, ERK-independent loss of epithelial morphology. Int J Cancer 2001 Oct 15;94(2):185-91.
11. Xia W, Liu LH, Ho P, et al. Truncated ErbB2 receptor (p95ErbB2) is regulated by heregulin through heterodimer formation with ErbB3 yet remains sensitive to the dual EGFR/ErbB2 kinase inhibitor GW572016._Oncogene. 2004 Jan 22;23(3):646-53.
12. Sáez R, Molina MA, Ramsey EE, et al. p95HER-2 predicts worse outcome in patients with HER-2-positive breast cancer. Clin Cancer Res. 2006 Jan 15;12(2):424-31.
13. Miremadi A, Pinder SE, Lee AH, et al. Neuroendocrine differentiation and prognosis in breast adenocarcinoma. Histopathology 2002; 40: 215-22.
14. Maluf HM, Koerner FC. Carcinomas of the breast with endocrine differentiation: a review. Virchows Arch 1994; 425: 449-57.
15. Sapino A, Righi L, Cassoni P, et al. Expression of apocrine differentiation markers in neuroendocrine breast carcinomas of aged women. Mod Pathol 2001; 14: 768-76.
16. Tse GM, Ma TK, Chu WC, et al. Neuroendocrine differentiation in pure type mammary mucinous carcinoma is associated with favorable histologic and immunohistochemical parameters. Mod Pathol 2004; 17: 568-72.
17. Sugg SL, Ezzat S, Zheng L, et al. Cytoplasmic staining of erbB-2 but not mRNA levels correlates with differentiation in human thyroid neoplasia. Clin Endocrinol (Oxf) 1998; 49: 629-37.
18. Ueda S, Ogata S, Tsuda H, et al. The correlation between cytoplasmic overexpression of epidermal growth factor receptor and tumor aggressiveness: poor prognosis in patients with pancreatic ductal adenocarcinoma. Pancreas 2004; 29: e1-8.
19. Saeger W, Fassnacht M, Reincke M, et al. Expression of HER-2/neu receptor protein in adrenal tumors. Pathol Res Pract 2002; 198: 445-8.
20. Myers RB, Srivastava S, Oelschlager DK, et al. Expression of p160erbB-3 and p185erbB-2 in prostatic intraepithelial neoplasia and prostatic adenocarcinoma. J Natl Cancer Inst 1994; 86: 1140-5.
21. Taylor SL, Platt-Higgins A, Rudland PS, et al. Cytoplasmic staining of c-erbB-2 is not associated with the presence of detectable c-erbB-2 mRNA in breast cancer specimens. Int J Cancer 1998; 76: 459-63.
22. Taylor SL, Rudland PS, Barraclough R. C-erbB-2 mRNA in breast cancer specimens that exhibit membrane or cytoplasmic immunoreactivity for c-erbB-2. Oncol Res 1999; 11: 311-7.
23. Lilleng R, Hagmar BM, Nesland JM. C-erbB-2 protein and neuroendocrine expression in intraductal carcinomas of the breast. Mod Pathol 1992; 5: 41-7.
24. Nesland JM, Ottestad L, Heikilla R, et al. C-erbB-2 protein and neuroendocrine expression in breast carcinomas. Anticancer Res 1991; 11: 161-7.
25. Tavassoli FA, Devillee P, editors. Pathology and Genetics of Tumours of the Breast and Female Genital Organs. IARCPress: Lyon; 2002.432 pp
26. Hayashi Y, Fukayama M, Shiozawa Y, et al. Incorporation of human chorionic gonadotropin alpha-subunit into different types of granule in stomach endocrine cells. An immunoelectron microscopic study. Acta Pathol Jpn 1989; 39: 737-42.
27. Singleton TP, Niehans GA, Gu F, et al. Detection of c-erbB-2 activation in paraffin-embedded tissue by immunohistochemistry. Hum Pathol 1992; 23: 1141-50.
28. Tetu B, Brisson J. Prognostic significance of HER-2/neu oncoprotein expression in node-positive breast cancer. The influence of the pattern of immunostaining and adjuvant therapy. Cancer 1994; 73: 2359-65.
29. Corbett IP, Henry JA, Angus B, et al. NCL-CB11, a new monoclonal antibody recognizing the internal domain of the c-erbB-2 oncogene protein effective for use on formalin-fixed, paraffin-embedded tissue. J Pathol 1990; 161: 15-25
30. De Potter CR, Quatacker J, Maertens G, et al. The subcellular localization of the neu protein in human normal and neoplastic cells. Int J Cancer 1989; 44: 969-74.
31. Coombs LM, Pigott DA, Sweeney E, et al. Amplification and over-expression of c-erbB-2 in transitional cell carcinoma of the urinary bladder. Br J Cancer 1991; 63: 601-8.
32. Makretsov N, Gilks CB, Coldman AJ, et al. Tissue microarray analysis of neuroendocrine differentiation and its prognostic significance in breast cancer. Hum Pathol. 2003; Oct;34(10):1001-8.
33. Sapino A, Papotti M, Righi L, et al. Clinical significance of neuroendocrine carcinoma of the breast. Ann Oncol 2001; 12 Suppl 2: S115-7.
34. Sapino A, Bussolati G. Is detection of endocrine cells in breast adenocarcinoma of diagnostic and clinical significance? Histopathology 2002; 40: 211-4.

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

Address correspondence and reprint requests to: Shigehira Saji, MD, PhD, Department of Breast Surgery, Division of Clinical Trials and Research, Tokyo Metropolitan Cancer and Infectious Disease Center, Komagome Hospital, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8677, Japan, or e-mail: shige@cick.jp.

DOI: 10.1111/j.1524-4741.2010.00943.x

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.

Dai Kitagawa, MD*
Shigehira Saji, MD, PhD*,†,‡
Shin-ichiro Horiguchi, MD§
Yu Satoh, MD*
Kazumi Horiguchi, MD*
Masakazu Toi, MD, PhD¶
Nobuaki Funata, MD, PhD§
Katsumasa Kuroi, MD, PhD*,†

*Department of Breast Surgery; †Division of Clinical Trials and Research; ‡Department of Medical Oncology, Saitama Medical University, International Medical Center, Saitama city, Japan; §Department of Pathology, Tokyo Metropolitan Cancer and Infectious Disease Center Komagome Hospital, Tokyo, Japan; and ¶Department of Surgery (Breast Surgery) Graduate School of Medicine, Kyoto University, Kyoto, Japan

REFERENCES

1. Early Breast Cancer Trialists' Collaborative Group (EBCTCG). Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomized trials. *Lancet* 2005;365:1687–717.

2. Toi M, Nakamura S, Kuroi K, *et al.* Phase II study of preoperative sequential FEC and docetaxel predicts of pathological and disease free survival. *Breast Cancer Res Treat* 2008;110:531–9.

3. Allred DC, Harvey JM, Berardo M, *et al.* Prognostic and predictive factors in breast cancer by immunohistochemical analysis. *Mod Pathol* 1998;11:155–68.

4. Jain V, Landry M, Levine EA. The stability of estrogen and progesterone receptors in patients receiving preoperative chemotherapy for locally advanced breast carcinoma. *Am Surg* 1996;62:162–5.

5. Anderson WF, Chu KC, Chatterje N, *et al.* Tumor variants by hormone receptor expression in white patients with node-negative breast cancer from the surveillance epidemiology, and results database. *J Clin Oncol* 2001;19:18–27.

Cancer Science
The official journal of the Japanese Cancer Association

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

Eri Hiroki,[1,3] Jun-ichi Akahira,[2] Fumihiko Suzuki,[1] Satoru Nagase,[1] Kiyoshi Ito,[1] Takashi Suzuki,[2] Hironobu Sasano[2] and Nobuo Yaegashi[1]

[1]Department of Obstetrics and Gynecology and [2]Pathology, Tohoku University Graduate School of Medicine, Sendai, Japan

(Received June 29, 2009/Revised August 30, 2009; September 7, 2009/Accepted September 15, 2009/Online publication November 4, 2009)

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

[3]To whom correspondence should be addressed.
E-mail: e-hiroki@mail.tains.tohoku.ac.jp

doi: 10.1111/j.1349-7006.2009.01385.x

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

doi: 10.1111/j.1349-7006.2009.01385.x

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

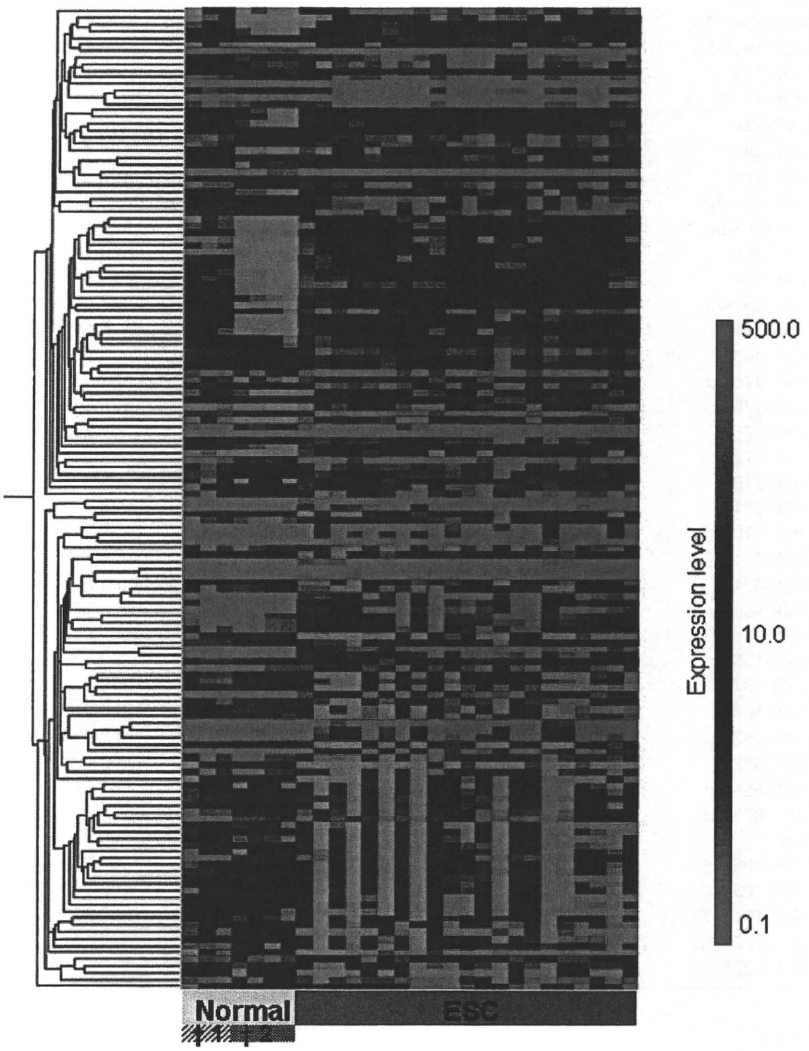

Fig. 1. Unsupervised hierarchical clustering analysis of microRNAs (miRNAs) that exhibited a >2-fold increase or decrease in expression in endometrial serous adenocarcinoma. The level of miRNA expression is color-coded as follows: red, higher miRNA expression; green, lower miRNA expression; black, no 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.

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

Table 2. (continued)

miRNA	*P*-values	Fold change
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.

doi: 10.1111/j.1349-7006.2009.01385.x

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

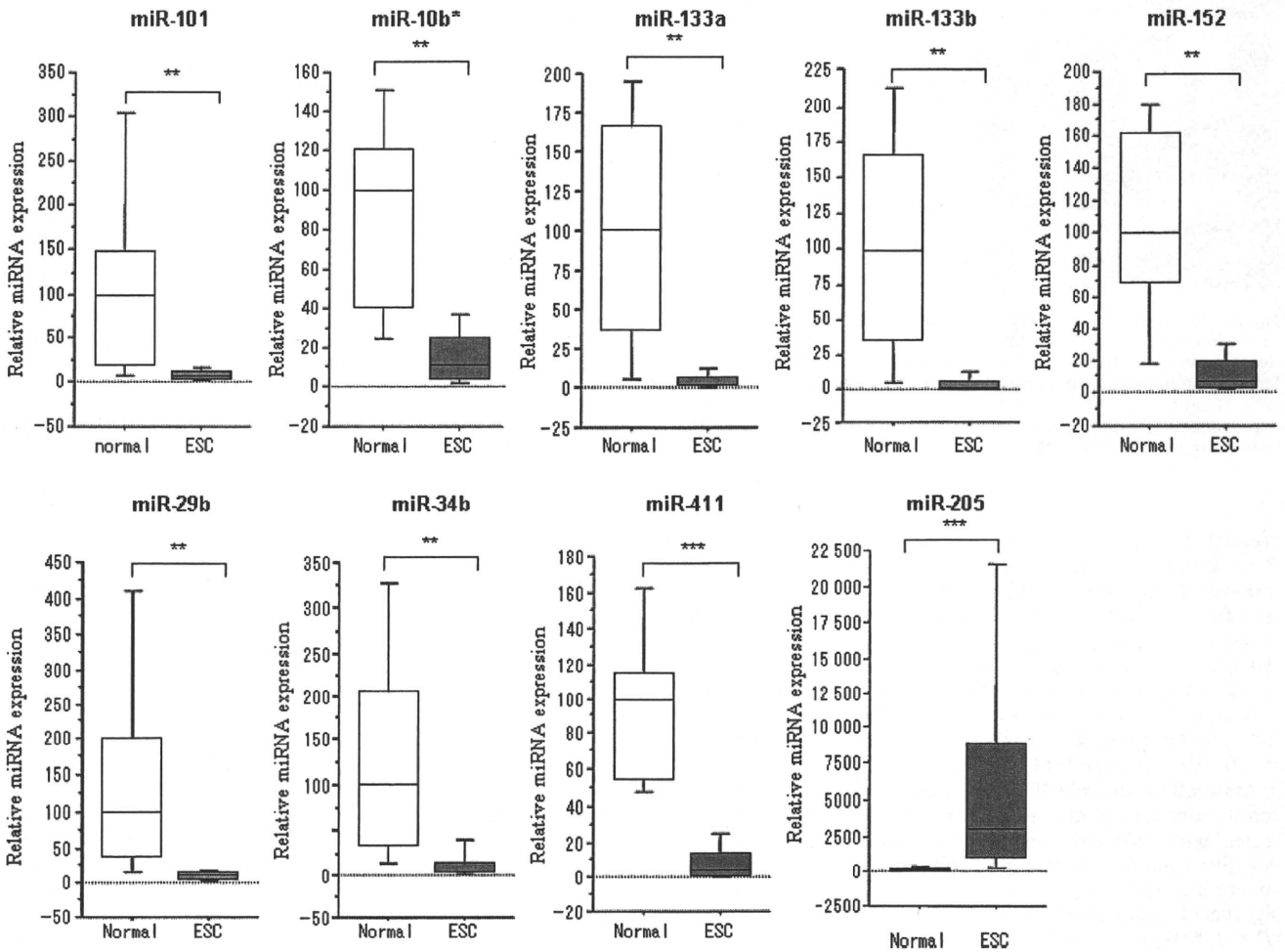

Fig. 2. Quantitative RT-PCR of nine different miRNA species (miR-101, miR-10b*, miR-133a, miR-133b, miR-152, miR-29b, miR-34b, miR-411, and miR-205). Expression of miR-101, miR-10b*, miR-133a, miR-133b, miR-152, miR-29b, miR-34b, and miR-411 was down-regulated, while miR-205 expression was up-regulated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