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Original Article

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

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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.

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/

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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, SV2-61γ). 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

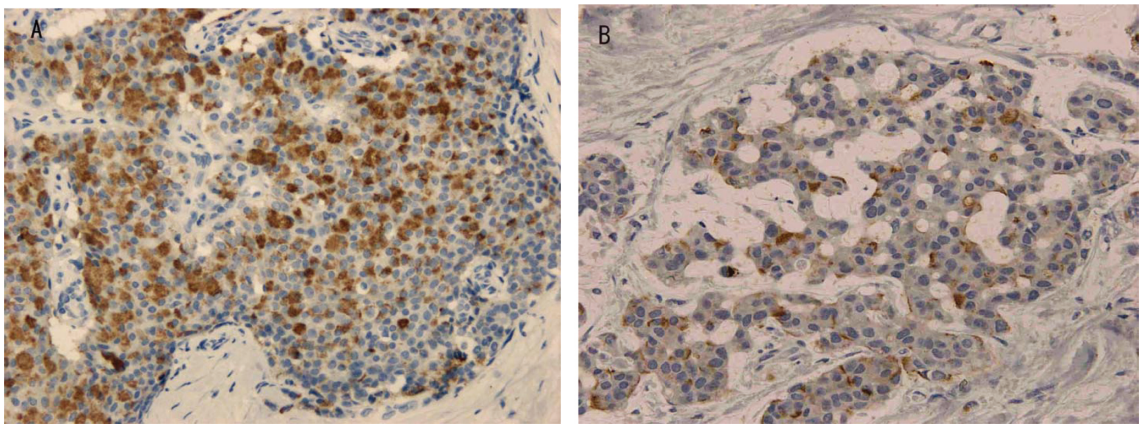

Figure 1 : Immunohistochemistry using a HercepTest staining kit showed a typical cytoplasmic staining pattern. Granular cytoplasmic staining was observed in cases of solid neuroendocrine carcinoma (A) and mucinous carcinoma (B).

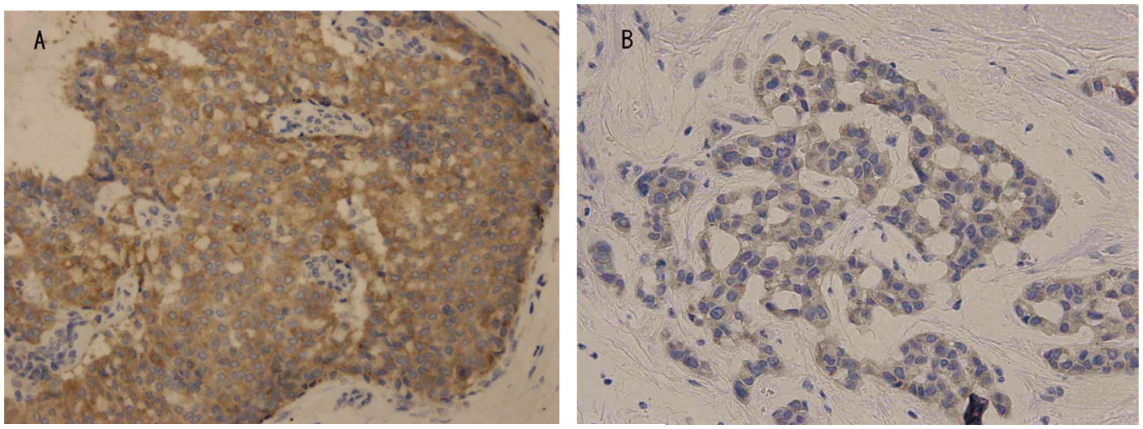

Figure 2 : Immunohistochemistry using CB-11 showed similar or weak cytoplasmic staining in the same cases described in Figure 1A (A) and Figure 1B (B).

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 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)

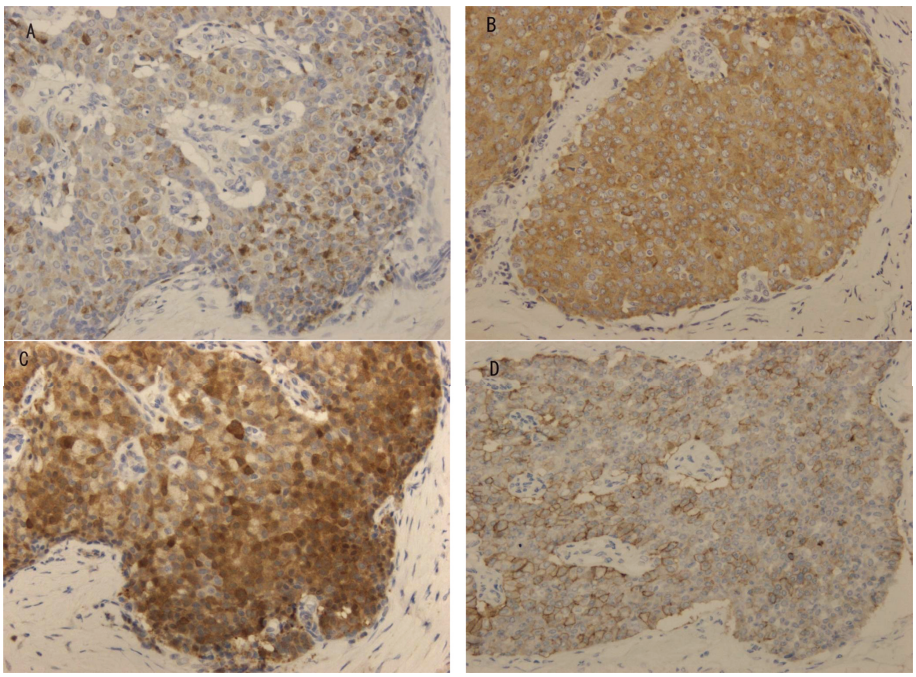

Figure 3 : Immunohistochemistry for neuroendocrine markers. Chromogranin A (A), synaptophysin (B), neuron-specific enolase (NSE) (C), and CD56 (D) were positive in the same cases described in Figure 1A and 2B.

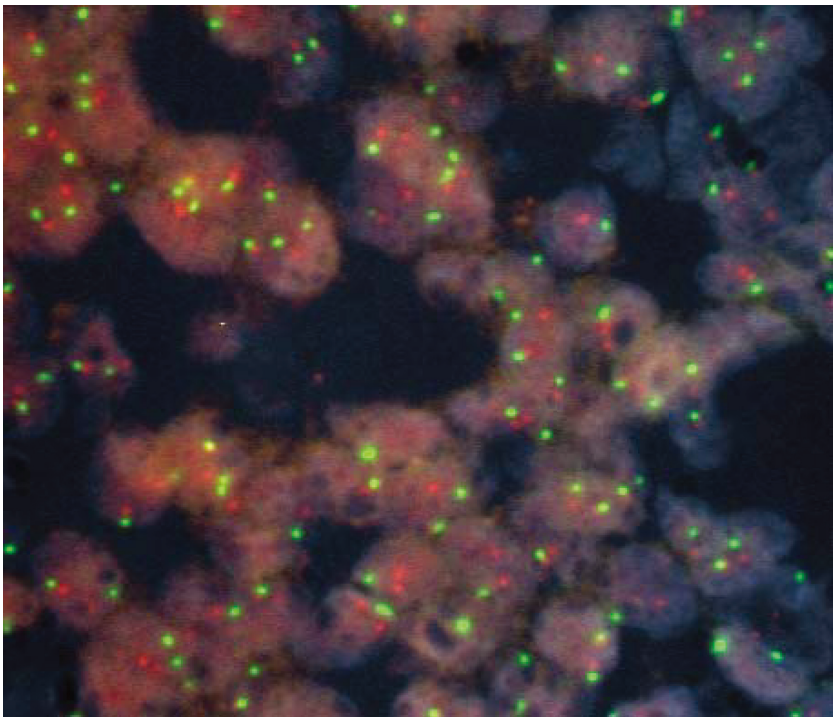

Figure 4 : Fluorescence *in situ* hybridization showed no amplification of the HER2/neu gene.

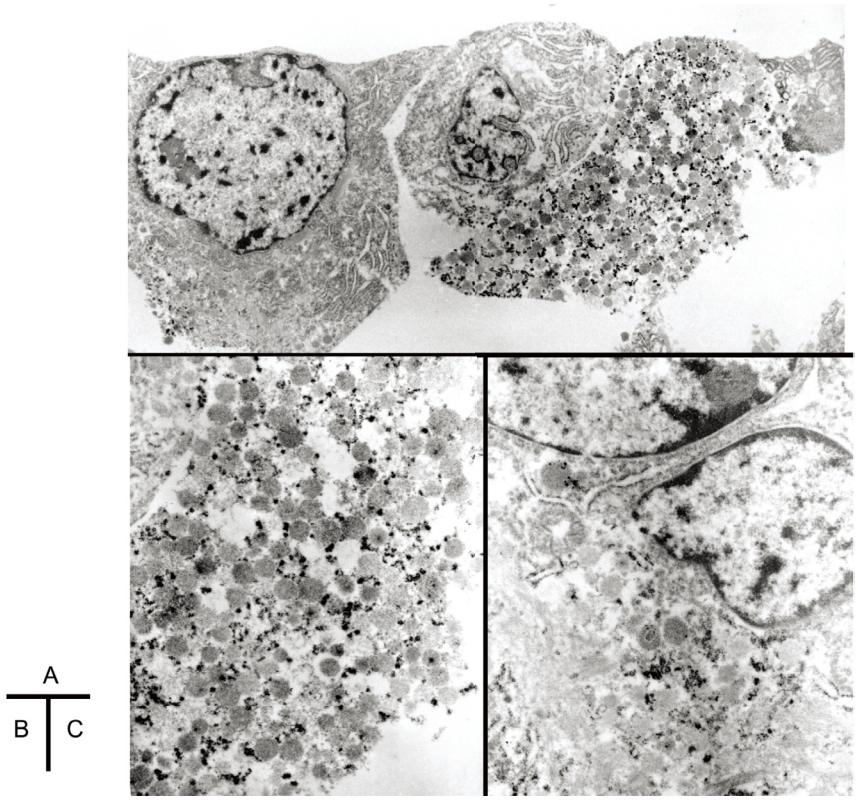

Figure 5 : Immunoelectron microscopy. Dense-core granules were observed in tumor cells (A) and HercepTest antibodies were accumulated in the ribosomes (B) and rough endoplasmic reticulum (C).

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.

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