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Immunohistochemical Evaluation of Hormone Receptor for Routine Practice of Breast Cancer: Highly Sensitive Procedures Significantly Contribute to the Correlation with Biochemical Assays

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Immunohistochemical evaluation of hormone receptors for breast cancer has been performed parallel to biochemical assays. Recently, immunohistochemistry has tended to substitute the biochemical method in Japan. To clarify the factors concerned and problems to be resolved, we reviewed our evaluation system for hormone receptors by immunohistochemistry from 1990. A total of 861 breast cancer samples were examined by immunohistochemistry and biochemistry. In 3 main periods, phase 1 (1990–1993), phase 2 (1995–1998), and phase 3 (1999–2001), increasing sensitivity of the immunohistochemical method was provided by commercially available staining systems and shown to range from 83.6% (phase 1) to 92.0% (phase 3). The highly sensitive procedures of the antigen retrieval and peroxidase-conjugated polymer method are main contributing factors. The authors examined how these procedures influenced the distribution of positive cell population; concordance rate, including sensitivity and specificity; cutoff points; and evaluation categories. The correlation between biochemistry and immunohistochemistry was extensively studied in the 1980s and 1990s. In reference to the progress achieved in the United States and United Kingdom to control the current situation in Japan, it should be recognized that recently developed, highly sensitive procedures boost the immunoreactivity, which will affect the basic factors for technical validation.
Key Words: Breast cancer—Highly sensitive procedures—Hormone receptor—Immunohistochemical evaluation—Standardization—Technical validation.

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It was in the 19th century that ovariectomy was reported to be effective for breast cancer treatment, which is considered to be the beginning of hormone therapy. Jensen et al. (1) detected the presence of estrogen receptors (ERs) in rat tissue in 1962. The dextran-coated charcoal method was developed in 1970 (2) to measure the amount of ERs, and the sucrose density gradient method was developed for electrophoresis and tissue-slice assay (3). It was reported that breast cancer expressing ERs were regulated hormonally and that ER expression could predict the response to hormone therapy (4,5). A number of studies have supported these findings for ERs in addition to progesterone receptors (PgRs)(6).

Immunohistochemistry is another method to evaluate the hormone receptor (HR) status. Concordance rates between biochemical assays and immunohistochemistry were extensively studied in the 1980s to 1990s and ranged from 72.5% (7) to 95% (8) with frozen sections and from 67% (9) to 94% (10) with paraffin sections. Recently, it has also been generally accepted in Japan to examine HRs by immunohistochemistry and substituting biochemical assays on occasions. When immunohistochemical methods are applied to surgical materials to provide information for treatment, a technically certified examination system is necessary. We reviewed our immunohistochemical evaluation system for HRs from 1990 for surgical materials of breast cancers. We present the factors of concern and provide information that will contribute to establishing a certified evaluation system.

MATERIALS AND METHODS

Samples

A total of 861 samples of breast cancer were immunohistochemically examined for HRs using frozen sections from 1990 to 1993 (n = 215) and paraffin sections after 1995 (n = 646). Because 1994 was a transition period to set up the system for routine examination using paraffin sections lacking serial examination for all samples, the data from 1994 were not included in this study. For the frozen sections, breast cancer tissue measuring 5 × 5 × 3 mm was embedded in OCT compound, rapidly frozen in dry-iced hexane, and cut into 4-μm thick sections. For the paraffin sections, 1 cm^3 breast cancer tissue was fixed in 10% formalin separately for immunohistochemistry until 2000. After 2000, a specimen for routine pathologic examinations was selected for

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immunohistochemistry studies and processed separately for no longer than 48 hours to avoid overfixation.

Antibodies Examined

For the frozen sections, ERICA and PRICA kits (Abbott Laboratories, Chicago, IL) were used. In the case of the paraffin sections, antibodies compared or used for ERs are as follows: clone D5 (Amersham Biosciences Corp., Piscataway, NJ), clone CC4-5 (Novocastra Laboratories Ltd., Newcastle, United Kingdom), clone 1D5 (DAKO Corp., Carpinteria, CA, USA), and clone ER88 (Biogenex, San Ramon, CA). The antibodies compared or used for PgRs are as follows: polyclonal (DAKO Corp.), clone 1A6 (Novocastra Laboratories Ltd.), clone 1A6 (Immunotech SA, Marseille, France), and clone PR88 (Biogenex) (Table 1).

Staining Method

Immunohistochemical study was performed according to standard procedures. For the frozen sections, the procedure was according to the manufacturer's protocol. Briefly, 6-μm thick frozen sections were fixed in 10% phosphate-buffered formalin at room temperature for 30 minutes, soaked in cold methanol for 3 minutes followed by cold acetone for 1 minute, and then subjected to incubation to the primary antibodies at room temperature for 30 minutes. Incubation with secondary antibody and peroxidase–antiperoxidase (PAP) complex followed. For the paraffin sections, the antigen retrieval procedure was used. To determine the most adequate procedure for antigen retrieval, we compared 0.01 M phosphate-buffered saline (pH 7.4) and 0.01 M citrate buffer (pH 6.0) for soaking the sections, and boiling for 10 minutes in a water bath or autoclaving at 121°C for 5 minutes for heating the sections. The sections were then soaked in methanol containing 0.3% H_2O_2 to inhibit endogenous peroxidase activity. We attempted an indirect method, the labeled streptavidin-biotinylated antibody (LSAB) method, and the enzyme polymer enhanced method (Envision System, DAKO Corp.). The primary antibodies were incubated at 4°C overnight for the indirect method and at room temperature for 30 minutes for the ABC and Envision System. Visualization was performed by immersing the sections in 0.05 M Tris-HCl buffer (pH 7.6), containing 30 mg/dL diaminobenzidine, 65 mg/dL sodium azide, and 0.003% H_2O_2.

Biochemical Assays

Biochemical assays for HRs was performed by the dextran-coated charcoal method until September 1998 (Teisin Bioscience Laboratories) and by an enzyme immunoassay (EIA) method thereafter (Sumikin Bioscience, Inc.). The cutoff point was 4.9 fmol/mg wet tissue for both HRs. Fresh tumor samples weighed approximately 500 mg and were rapidly frozen and subjected to the biochemical analysis.

Immunohistochemical Evaluation System for Hormone Receptors

For the immunohistochemical evaluation, we counted positive cells among 500 cancer cells and calculated the percentage. We also needed a more convenient system for practical use in routine surgical pathology. We attempted a semiquantitative system by categorizing into groups such as negative, 1+, and 2+.

Statistical Analysis

Correlation of the data of biochemical assays and positive cell population by immunohistochemistry were analyzed with linear regression analysis. Concordance between the results by biochemistry and immunohistochemistry was statistically analyzed by a chi-square test. Correlation between semiquantitative evaluation system and data of biochemical assays was analyzed using Spearman rank correlation coefficients.

RESULTS

Staining Method

Among the antibodies compared (Table 1), monoclonal antibodies of clone 1D5 for ERs and clone PR88 for PgRs provided better staining results with higher

TABLE 1. *List of antibodies examined*

Antigen	Clone	Source	Applied section
ER related protein	clone D5	Amersham Bioscience Corp.	P
ER	ER ICA kit	Abott Laboratories	FR
	clone CC4-5	Novocastra Laboratories Ltd.	P
	clone 1D5	DAKO	P
	clone ER88	Biogenex	P
PgR	PR ICA kit	Abott Laboratories	FR
	polyclonal	DAKO	P
	clone 1A6	Novocastra Laboratories Ltd.	P
	clone 1A6	Immunotech S. A.	P
	clone PR88	Biogenex	P

ER, estrogen receptor; PgR, progesterone receptor; P, paraffin; FR, frozen section.

specificity and lower background for the paraffin sections. The most suitable staining procedure for these antibodies so far was a combination of the Envision System and heat-induced epitope retrieval performed at 121°C for 5 minutes in 0.01 M citrate buffer (pH 6.0) using autoclaving. We made two significant changes in 1995 and 1998 to achieve the current system. These changes were mainly by improvement of commercially available antibodies or staining kits. The first significant change in 1995 was the use of formalin-fixed, paraffin-embedded sections instead of frozen sections. The second change in 1998 was made for antibodies of PgRs from clone 1A6 to PR88, and staining procedures for ERs and PgRs were changed. These three periods are designated as phase 1 (1990–1993), phase 2 (1995–1998), and phase 3 (1999–2001). The protocols used in these phases are shown in Table 2. Additionally, a minor change was made between 1999 and 2000 of phase 3. Citrate buffer for antigen retrieval was prepared by technicians each time in 1999, but since 2000, commercially available ready-to-use buffer has been used.

Correlation Between Biochemistry and Immunohistochemistry

Estrogen Receptors

The positive cell population among 500 cancer cells was compared with the results of biochemistry. For the analysis, we used the homogeneous data from 1996 to 1998 for phase 2 and from 2000 and 2001 for phase 3 under the stable and standardized condition because of the minor modifications and technical improvements described previously. There were correlations in phase 2 (Fig. 1A) and phase 3 (Fig. 1B). There was a changing pattern of distribution of the positive cell population for immunohistochemistry. During phase 2, the positive cell population was distributed more diffusely, but in phase 3, the distribution showed a tendency to split into two groups: cases with a smaller number of positive cells (less than 30%) and cases with larger number of positive cells (more than 80%).

Progesterone Receptors

There were also correlations between biochemistry and immunohistochemistry for PgRs in phases 2 and 3 (Fig. 2), although a tendency for splitting is not as clear as with ERs.

Concordance Rate, Sensitivity, and Specificity

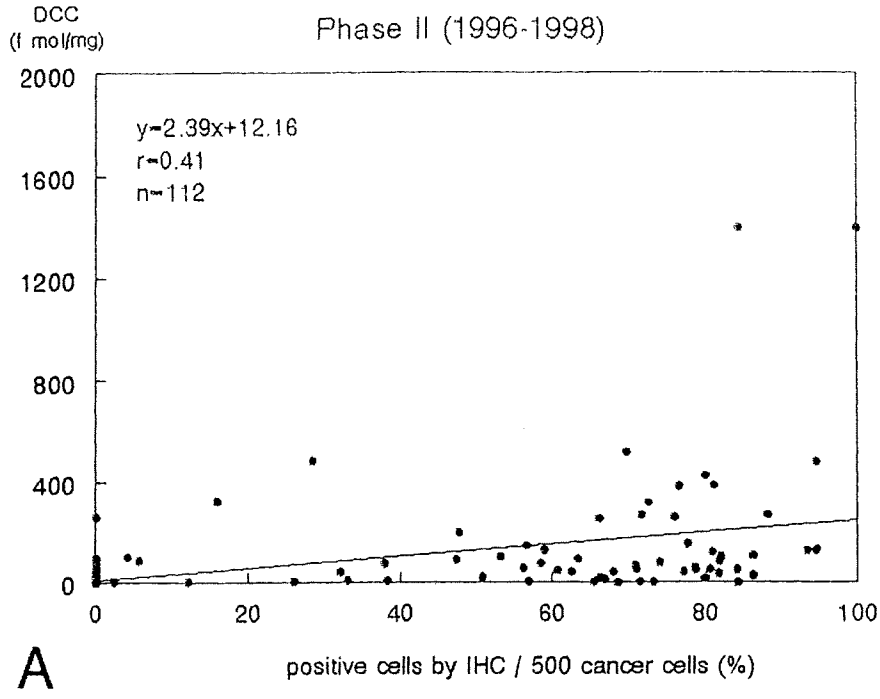
Practically, HRs could be evaluated as positive or negative. When we interpreted the results as positive with any positive cell by immunohistochemistry and set the results of biochemical assay as the gold standard, the correlation of biochemical assay and immunohistochemistry was evident (Table 3).

In comparison with immunohistochemistry using frozen sections (phase 1), paraffin sections (phase 2) showed a rather consistent concordance for ERs and a superior concordance for PgRs (Table 3). The improvement in the staining procedure led to an easier interpretation of the staining results. Comparing the results of phase 2 and phase 3, concordance increased for ERs and PgRs. When we analyzed the results in more detail, however, we must point out that the concordance rate is not the only index of accuracy for the immunohistochemistry examination. In the earlier phase of immunohistochemistry (phases 1 and 2), discordances were mainly brought out by insufficient immunohistochemical staining with a larger number of dextran-coated charcoal–positive, immunohistochemistry-negative cases. After improvement of the staining procedure in phase 3, the number of EIA-positive, immunohistochemistry-negative cases decreased, and inversely, the number of EIA-negative, immunohistochemistry-positive cases increased. The tendency was more obvious in 2000 and 2001, after a minor improvement to the procedure. Figure 3 shows the trends of concordance rate, sensitivity, and specificity. Concordance rate was highest in 1999 for ERs (87.7%) and PgRs (86.0%). In 2000 and 2001, concordance rate decreased to 82.9% for ERs and 74.4% for PgRs, with increased sensitivity and decreased specificity. Figure 3 shows the population of biochemically positive, immunohistochemically negative

TABLE 2. *Protocols for immunohistochemical study*

	Phase I (1990–1993)	Phase II (1995–1998)	Phase III (1999–2001)
Section	Frozen	Paraffin	Paraffin
Fixation	10% phosphate-buffered formalin RT, 30 min	10% formalin overnight	10% formalin overnight
Pretreatment	None	0.01M PBS 100°C, 10 min	0.01M Citrate buffer 121°C, 5 min by autoclave
Inhibition of internal POX	cold MeOH, 3min cold acetone, 1min	0.3% H_2O_2 MeOH	0.3% H_2O_2 MeOH
Primary antibody	ER ICA PR ICA	ER (ID5) PgR (1A6)	ER (ID5) PgR (PR88)
Enhancement method	PAP	LSAB	EnVision

POX, peroxidase; RT, room temperature; PAP, peroxidase-antiperoxidase; LSAB, labeled streptavidin-biotinylated antibody.



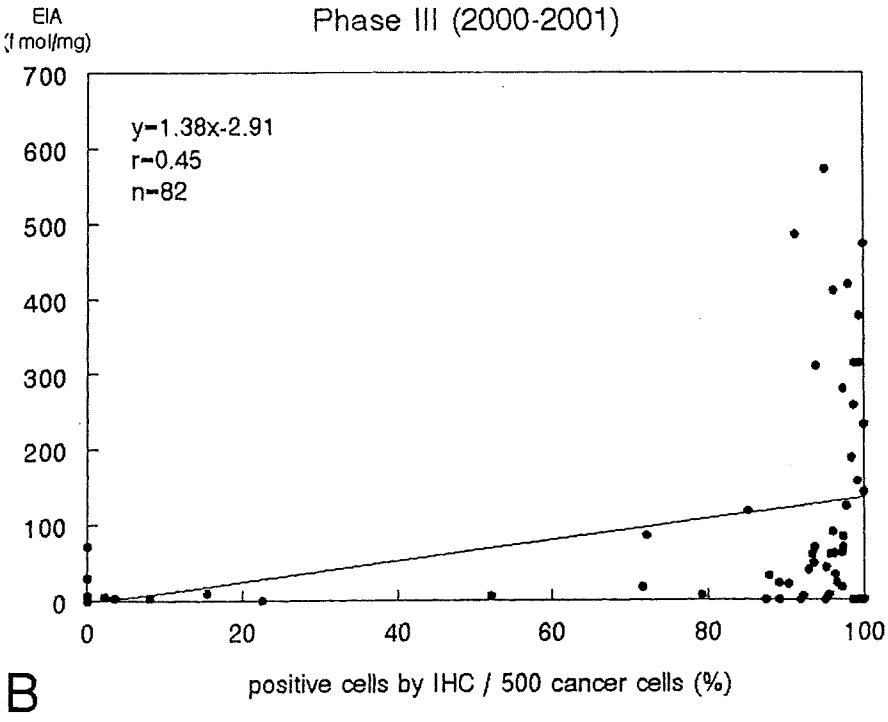


FIG. 1. Correlation between biochemistry and immunohistochemistry for estrogen receptors. Percentages of positive cells in 500 cancer cells were immunohistochemically evaluated. Biochemical assays were performed by the dextran-coated charcoal method from 1996 to 1998 (**A**) and by the enzyme immunoassay method from 2000 to 2001 (**B**). The distribution pattern of positive cell population detected by immunohistochemistry was split into two groups: those with fewer positive cancer cells (less than 30%) and those with more positive cancer cells (more than 70%).

and biochemically negative, immunohistochemically positive cases inversed in 1999.

Cutoff Point

To set the cutoff point for immunohistochemistry, we compared concordance for ER with cutoff points at 0%, 5%, and 10%. The concordance rate for ERs was 80.4% when we set a cutoff point at 0% or 5% in phase 2 (1996–1998), and it was greater than 79.5% with a cutoff point at 10% (Fig. 4). In 2000 and 2001, the concordance rate was 84.1% with a cutoff point at 10% and greater than 82.9% with a cutoff point at 0% or 5%.

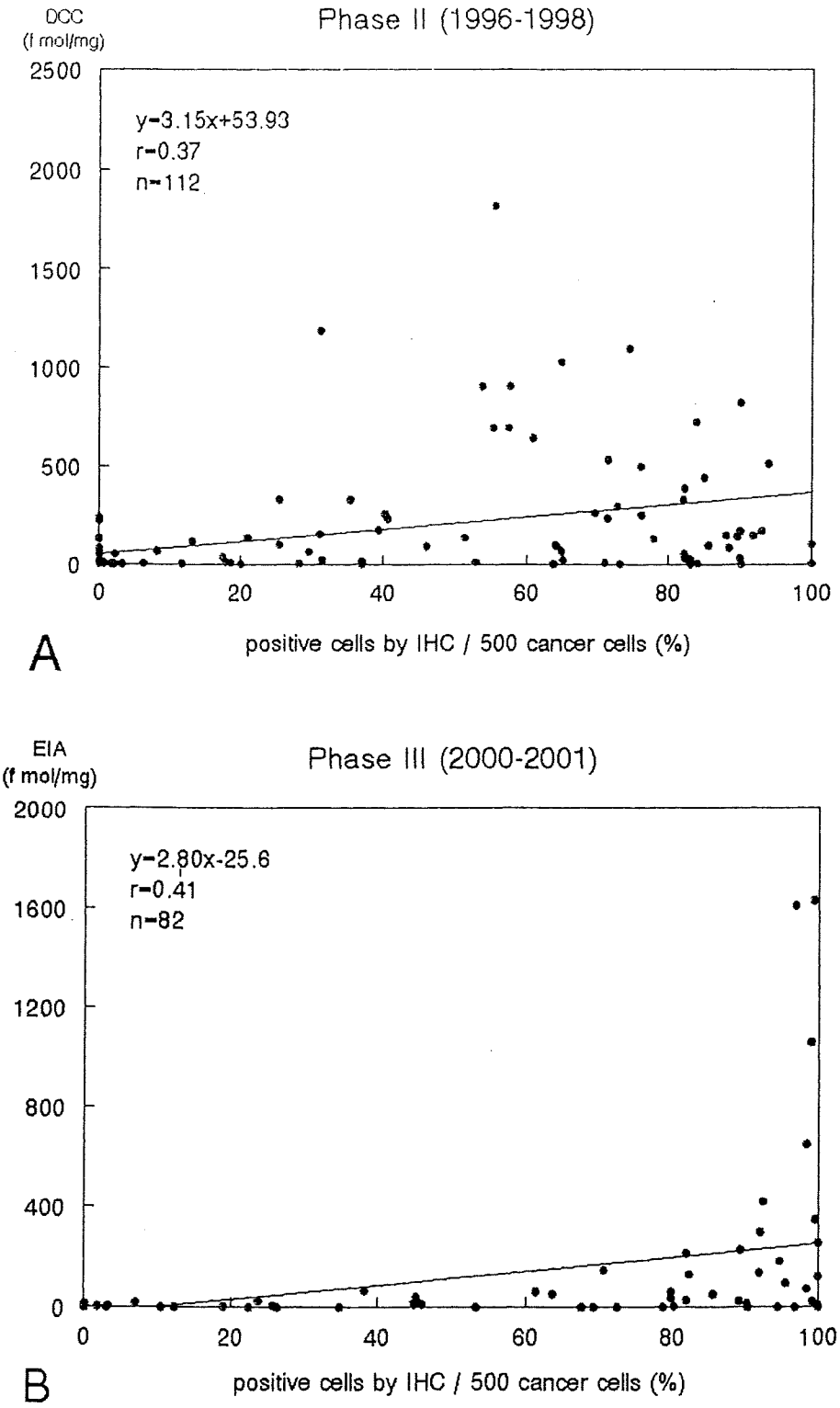


FIG. 2. Correlation between biochemistry and immunohistochemistry for progesterone receptors. The distribution pattern of positive cell population detected by immunohistochemistry changed between phase 2 (**A**) and phase 3 (**B**). A splitting pattern is not clear as with estrogen receptors, but the distribution shifted to an increased number of positive cells as a whole.

Discordant Cases

The causes of discordance between EIA and immunohistochemistry were analyzed. In 2000 and 2001, EIA-negative, immunohistochemistry-positive cases were 11 and 18 for ERs and PgRs, respectively, and EIA-positive, immunohistochemistry-negative cases were 3 and 2 for ERs and PgRs, respectively. Speculated factors contributing to these discordances are summarized in Table 4. EIA-negative, immunohistochemistry-positive results were detected in carcinomas with sparse cancer cells

TABLE 3. *Concordance rate between biochemistry and IHC*

		ER					PgR			
		DCC					DCC			
Phase I	IHC	+	−		CR; 82.6%	IHC	+	−		CR; 66.3%
(1990–1993)	+	65	5	70	SE; 83.6%	+	55	3	58	SE; 64.4%
n=86	−	13	17	30	SP; 78.9%	−	30	12	42	SP; 76.9%
		78	22	100 (%)	p < 0.001		85	15	100 (%)	p < 0.001
		DCC					DCC			
Phase II	IHC	+	−		CR; 80.4%	IHC	+	−		CR; 76.8%
(1995–1998)	+	49	7	56	SE; 79.7%	+	54	14	68	SE; 85.7%
n=112	−	13	31	44	SP; 81.4%	−	9	23	32	SP; 61.9%
		62	38	100 (%)	p < 0.001		63	38	100 (%)	p < 0.001
		EIA					EIA			
Phase III	IHC	+	−		CR; 84.9%	IHC	+	−		CR; 79.1%
(1999–2001)	+	58	10	68	SE; 92.0%	+	50	18	68	SE; 94.5%
n=139	−	5	27	32	SP; 72.5%	−	3	29	32	SP; 62.1%
		63	37	100 (%)	p < 0.001		53	47	100 (%)	p < 0.001

IHC, immunohistochemistry; DCC, dextran-coated charcoal method; EIA, enzyme immunoassay; CR, concordance rate; SE, sensitivity; SP, specificity.

and abundant fibrous stroma, with a small positive cell population by immunohistochemistry (Fig. 5A), with many positive cells with weak intensity (Fig. 5B), and with many positive cells with strong intensity (Fig. 5C). For EIA-positive, immunohistochemistry-negative results, fixation problems, intermixture of nonneoplastic mammary gland (Fig. 5D), and cytoplasmic staining were speculated as the causes.

Immunohistochemical Evaluation System for Hormone Receptors

The evaluation system by counting the positive cell population enables us to observe a correlation with biochemistry. However, it is time-consuming and not always suitable for routine surgical pathology. We used a more convenient evaluation system. Initially, we had five categories for the population of positive cells: 0 (completely negative), 1+ (1–25%), 2+ (26–50%), 3+ (51–75%), and 4+ (76–100%) (Fig. 6A). The distribution of immunoreactive cells has changed since 2000. The clusters of positive cell populations could be divided into two groups: less than 50% and more than 50%. Therefore, we changed to a simpler semiquantitative evaluation system: 0 (completely negative), 1+ (1–50%), and 2+ (51–100%) (Fig. 6B).

DISCUSSION

We reviewed our evaluation system for HRs by immunohistochemistry and presented increasing sensitivity of immunohistochemistry provided by commercially available staining systems. In particular, highly sensitive procedures by heat-induced epitope retrieval and peroxidase-conjugated polymer methods (Envision System) boosted immunoreactivity affecting the basic factors for technical validation, which are concordance rate, sensitivity and specificity, cutoff point, and evaluation categories. We discuss herein the factors that are necessary to establish an adequate immunohistochemical assessment system in Japan.

The concordance rate is not the only benchmark to validate the immunohistochemistry method, but we also must note the sensitivity and specificity. In particular, an application of highly sensitive procedures gave different meanings to the same percentage of concordance rate. As shown in the current study, the discordances were caused by increased numbers of EIA-negative, immunohistochemistry-positive cases. By analyzing the discordant cases in 2000 and 2001, immunohistochemistry could detect a few positive cells that might not be detected by EIA. Intermingled nonneoplastic cells detected by immunohistochemistry led to positive results by EIA. These results reaffirmed the advantages of immunohistochemistry, which are the availability for formalin-fixed and paraffin-embedded tissues, smaller tumors, use of fine-needle aspiration cytology samples and core needle biopsies, possible distinguishing of carcinoma cells from healthy tissue, and possible comparison between HR expression and morphology. We also should point out that the results of EIA are not always absolutely true. We thus faced the contradiction to use the biochemical method as the gold standard for the assessment of immunohistochemistry. It is another subject to be resolved whether breast cancer with a few carcinoma cells expressing HRs is suitable for hormone therapy. The immunohistochemistry method for HRs should be validated clinically, too, such as survival rate or response to hormone therapy. Nonetheless, before discussing the clinical validity, the immunohistochemistry method should be sufficiently validated from the technical point of view.

We can use paraffin sections instead of frozen sections. Practically, immunohistochemistry with frozen

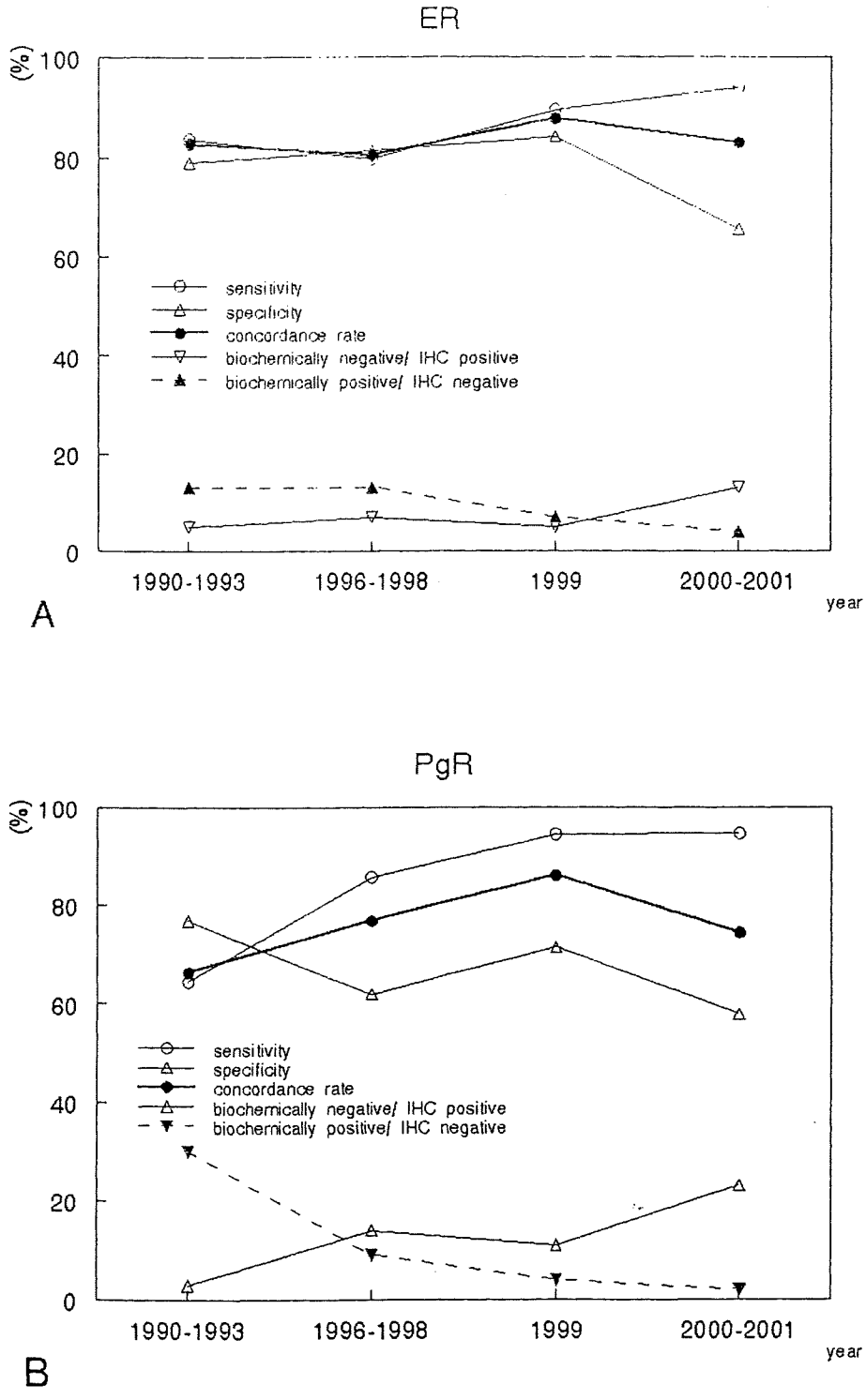
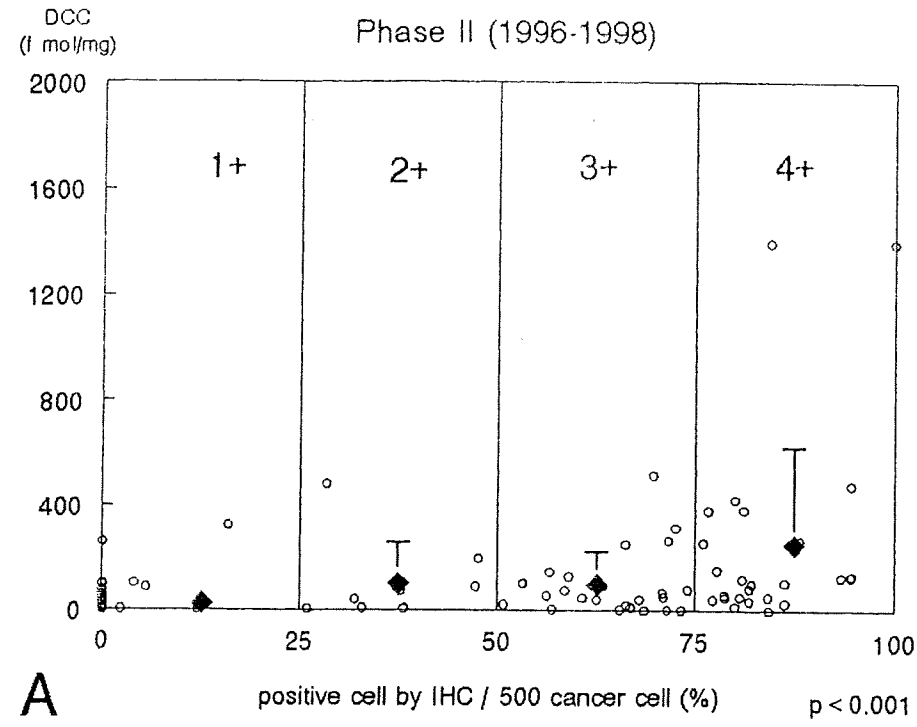


FIG. 3. The trend of the correlation between biochemistry and immunohistochemistry. Concordance rates for estrogen receptors (**A**) and progesterone receptors (**B**) were highest in 1999 and then decreased in 2000 and 2001. Biochemically negative, immunohistochemically positive cases were most frequently observed in 2000 and 2001 with increasing sensitivity.

sections requires much more effort than that with paraffin sections (i.e., sampling of frozen tissue, cutting sections, and evaluation). Therefore, the superior concordance and availability of immunohistochemistry using paraffin sections enables easier evaluation and allows results to be returned to the patient earlier. The site selected for evaluation is also a critical point. We occasionally encounter a strong positive reaction at the peripheral part of the tumor. In contrast, a lesser positive reaction is identified at the central part of the tumor. The possible causes of this discrepancy are the diverse expression of HRs as a biologic characteristic of the cancer cells and the slow permeability of fixatives to the central part of the tumor. It is not recommended to take a sample for biochemistry



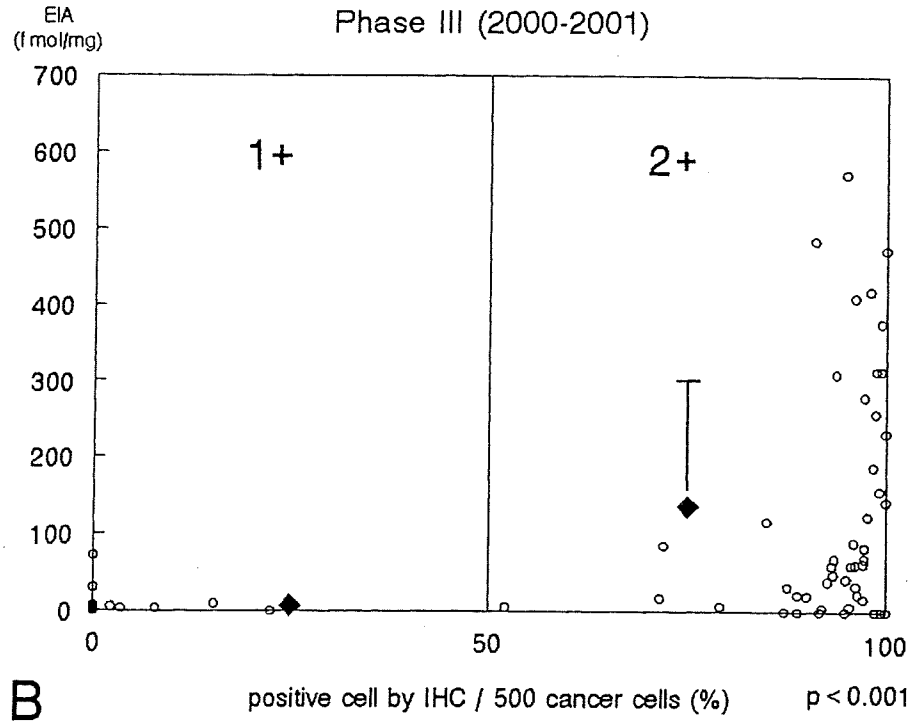


FIG. 6. Semiquantitative evaluation for estrogen receptors by immunohistochemistry. The semiquantitative evaluation system was changed from 5 categories (0, completely negative; 1+, 1–25%; 2+, 26–50%; 3+, 51–75%; and 4+, 76–100%) (**A**) to 3 categories (0, completely negative; 1+, 1–50%; 2+, 51–100%) (**B**).

staining. Maintaining standardized staining conditions (i.e., preparation of antibodies, buffer, and protocol) is critical. It is also significant to check internal and external control sections for quality assurance. Using commercially available staining kits or automated staining machines is another possibility. In other countries, there are attempts in progress to overcome the disadvantages of immunohistochemistry. In the United Kingdom, a national quality assessment scheme for immunocytochemistry has been developed (15,16). A quality control

system for immunocytochemistry for participating institutions including overseas laboratories is noteworthy (16). Large scaled trials in the United States for clinical validation of immunohistochemistry (17-19) have also attracted our attention.

In conclusion, we report how changing methodologies for immunohistochemistry of HRs influenced the correlation to biochemical assays. There are many studies for the correlation between biochemistry and immunohistochemistry in the 1980s and 1990s, and there are many noteworthy and large studies about interinstitutional analyses, but it is not well recognized that recently developed highly sensitive procedures significantly affect the basic factors for technical validation. We presented these procedures that provided increased sensitivity with EIA-negative, immunohistochemistry-positive cases, a change of distribution of positive cell population, and higher cutoff points by review of the system developed in an institution. It must be emphasized to understand how the methodology affects the correlation between biochemistry and immunohistochemistry for the certified evaluation system. □

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Heterogeneous gene alterations in primary breast cancer contribute to discordance between primary and asynchronous metastatic/recurrent sites: HER2 gene amplification and p53 mutation

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Abstract. The aim of the present study was to clarify differences in genetic events between primary breast cancers and asynchronous metastatic/recurrent lesions, by examining HER2 gene amplification and p53 mutation. The subjects were 44 breast cancer patients with asynchronous metastasis or recurrence. Synchronous metastases were excluded. HER2 overexpression and gene amplification were examined using immunohistochemistry and fluorescent *in situ* hybridization (FISH). P53 point mutation was examined by immunohistochemistry, laser-captured microdissection, PCR-single-strand conformation polymorphism, and a direct sequencing method. Immunohistochemistry showed that, for HER2, p53, ER and PgR, discordance rates between primary and recurrent tumor were 2 (4.5%), 1 (2.3%), 7 (15.9%) and 10 (22.7%), respectively. Two primary tumors with discordant HER2 overexpression were composed of at least two populations of carcinoma cells, with and without HER2 gene amplification. Distribution of HER2 gene amplification was consistent with protein overexpression. Corresponding recurrent tumors consisted of carcinoma cells without HER2 gene amplification. Of 6 recurrent tumors in which the primary carcinoma had a p53 point mutation, 3 tumors had identical mutations, 1 tumor had a different point mutation, and 2 tumors had no mutation. It was suspected that the latter 3 recurrent tumors comprised a minor component of the primary tumor.

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Key words: breast cancer, human epidermal growth factor receptor 2, p53 mutation, heterogeneity, asynchronous metastasis/recurrence, fluorescent *in situ* hybridization

In the present study, we examined a large series of asynchronous recurrent tumors. A limited number of these tumors showed discordance between primary and recurrent tumors. Detailed observations revealed that cell populations present in recurrent tumors were also present in the primary tumors, although they comprised a minor component of the primary tumor. Heterogeneity of the primary tumor apparently contributed to discordance.

Introduction

Human epidermal growth factor receptor 2 (HER2) is a proto-oncogene located on chromosome 17 (17q12-21.32), and encodes a 185-kDa transmembrane tyrosine kinase receptor for an unknown growth factor (1-6). HER1 (EGFR), HER2, HER3 and HER4 are homologue proteins that comprise the HER family (4-6). Studies show that 20 to 30% of primary breast cancers show HER2 overexpression (7,8), which predicts poor prognosis (7,9). It has also been reported that HER2 overexpression can predict therapeutic response (10,11). A humanized anti-HER2 monoclonal antibody (trastuzumab) has been produced by genetic recombination; 5% of trastuzumab (the region that recognizes HER2) is derived from the mouse monoclonal antibody 4D5, and 95% is derived from human IgG (12). Administration of trastuzumab with chemotherapeutics has been shown to produce longer time to progression, higher rate of objective response, longer survival and other clinical benefits (13-15). Selection of patients who would benefit from trabtuzumab requires examination of HER2 gene amplification or HER2 protein overexpression. Although it has generally been assumed that the HER2 gene status of recurrent or metastatic carcinoma is the same as that of primary carcinoma, there is increasing evidence that, in a limited percentage of cases, there are differences in HER2 gene status between primary and metastatic carcinoma (16-18). However, most studies of HER2 gene status have involved comparison between primary and synchronous metastatic lesions, or have not distinguished between synchronous and asynchronous lesions. The aim of the present study was to clarify differences

in genetic events between primary breast cancers and asynchronous metastatic/recurrent lesions, by examining HER2 gene amplification and p53 mutation. We examined the details of discordant cases, using techniques that enabled us to compare histopathological characteristics and genetic alterations. We demonstrated that cell populations present in recurrent tumors are also present among the heterogeneous carcinoma cells of the primary tumor.

Materials and methods

Patients. We compared 44 asynchronous metastatic/recurrent breast cancer tumors (diagnosed and treated at Tokai University Hospital from January 1997 to March 2000) with the 44 corresponding primary tumors. Age at surgery for the primary tumor ranged from 28 to 74 years, with an average of 50.7 years. Histologically, all 44 patients had invasive ductal carcinoma. The following cases were excluded: cases with bilateral breast cancers; cases with multiple cancers at other sites (because of the possibility of metastasis from another site); and cases with bone metastasis insufficiently processed due to decalcification. The tumor samples were fixed within 48 h, and 4-μm formalin-fixed, paraffin-embedded sections were prepared.

Immunohistochemistry. For p53, ER and PgR, we performed heat-induced epitope retrieval by autoclaving at 121°C for 5 min in citrate buffer (pH 6.0). The monoclonal antibodies used were anti-ER antibody (clone 1D5, dilution 1:40, Dako Cytomation Denmark A/S, Glostrup, Denmark), anti-PgR antibody (clone PR88, dilution 1:50, BioGenex, San Ramon, CA, USA) and anti-p53 antibody (clone DO7, dilution 1:50, Novocastra Laboratories Ltd., Newcastle, UK). Anti-mouse and anti-rabbit envision polymer/HRP (Dako Cytomation Denmark A/S) was used as a secondary agent. Results for p53, ER and PgR were considered positive if more than 10% of cancer cells showed immunoreactivity. For immunohistochemical evaluation of HER2 overexpression, we used HercepTest® kits (Dako Cytomation Denmark A/S). Immunohistochemical staining was performed according to the manufacturer's protocol. HER2 immunoreactivity was evaluated as a score of 0, 1+, 2+ or 3+, using standardized criteria (Table I). A score of 2+ or 3+ was interpreted as positive, and a score of 0 or 1+ as negative.

Fluorescent in situ hybridization. Cases with discordant results for HER2 overexpression were examined by FISH. Serially cut 4-μm-thick paraffin sections were used for the HecepTest and FISH. The procedure was performed according to the manufacturer's protocol [PathVysion™ HER2 DNA Probe Kits (Vysis, Inc. Downers Grove, IL, USA)]. Briefly, sections were deparaffinized with d-limonen (HEMO-De), dehydrated with 100% EtOH, digested with 0.2 N HCl for 20 min, and then rinsed in DW and 'wash buffer' (prepared by Vysis, Inc.). Sections were incubated with 'pretreatment buffer' (prepared by Vysis, Inc.) at 80°C for 30 min, and rinsed in DW and 'wash buffer'. They were then re-fixed in 4% formaldehyde-PBS, rinsed in 'wash buffer', and denatured in 2X SSC/70% formamide (pH 7.4) at 72°C for 5 min. After heating at 45-50°C for 5 min, sections were hybridized with DNA probes [combination of HER2/neu and CEP 17 (α satellite DNA located at the centromere of chromosome 17)] for 18 h at 37°C. After hybridization, the sections were washed with 2X SSC containing 0.3% NP-40, and then submerged in 2X SSC containing 0.3% NP-40 at 72°C for 2 min. After 10 μl of DAPI was applied to each section, they were observed using an 'Axioskop 2 plus' fluorescent microscope (Carl Zeiss). Signal numbers of HER2 gene (labeled with Spectrum Orange) and CEP17 (labeled with Spectrum Green) were counted in 60 tumor cells from each site, and the HER2/CEP17 signal number ratio was calculated. An HER2/CEP17 signal ratio greater than 2.0 was interpreted as positive.

Table I. Scoring system for HER2 protein.

Score	Staining pattern
0:	No staining, or membrane staining is detected in less than 10% of tumor cells
1+:	Faint or barely positive staining is detected in more than 10% of tumor cells
2+:	Moderate and/or incompletely circumscribed staining is detected in more than 10% of tumor cells
3+:	Strong and completely circumscribed membrane staining is detected in more than 10% of tumor cells

Laser-captured microdissection and PCR single-strand conformation polymorphism (PCR-SSCP) for p53. Six cases with strong p53 immunoreactivity were examined for p53 mutation. Point mutations in exons 5-8 of p53 were screened by SSCP. Two primer sets were used for each exon (Table II). To selectively obtain DNA from specific cells, we used laser-captured microdissection (19,20). Briefly, 10-μm thick paraffin sections were serially mounted on silane-coated and uncoated glass slides for p53 immunostaining and H&E staining, respectively. Strongly positive cells were laser-captured onto a thermoplastic polymer-filmcoated cap, using a PixCell® II LCM system (Arcuturus Engineering Inc., Mountain View, CA, USA). The cells on the polymer film were digested with 40 μg/ml proteinase K in 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA and 1% Tween 20, at 37°C for 16 h, and then heated at 95°C for 8 min to stop digestion. After denaturation at 94°C for 10 min, PCR was performed with 30 cycles (92°C for 1 min, 58°C for 1 min, 72°C for 2 min) in 50 μl of a reaction mixture containing 10 mM Tris-HCl, 0.1% Triton X-100, 2 mM $MgCl_2$, 0.2 mM each dNTP, 0.05 μM each primer, and 5 μl digested DNA sample. A 1-μl aliquot of PCR product was added to 50 μl of a PCR reaction mixture with the same composition as the above mixture for nested-PCR, with the exception of primer concentration (0.5 μM each primer). Using nested-PCR products, SSCP was performed. Briefly, 10 μl of formamide denaturing

Table II. Primer sets used for nested-PCR of p53 exon 5-8.

Out		
Exon 5	Sense	5'-ttcctcttcctgcagtactcc-3'
	Anti-sense	5'-gccccagctgctcaccatcg-3'
Exon 6	Sense	5'-cactgattgctcttaggtctg-3'
	Anti-sense	5'-agttgcaaaccagacctcagg-3'
Exon 7	Sense	5'-gtgttgtctcctaggttggc-3'
	Anti-sense	5'-caagtggctcctgacctggag-3'
Exon 8	Sense	5'-cctatcctgagtagtggtaat-3'
	Anti-sense	5'-gtcctgcttgcttacctcgc-3'
In		
Exon 5	Sense	5'-tgcagtactcccctgccctc-3'
	Anti-sense	5'-ctcaccatcgctatctgagc-3'
Exon 6	Sense	5'-tgctcttaggtctggcccct-3'
	Anti-sense	5'-accagacctcaggcggctca-3'
Exon 7	Sense	5'-ctaggttggctctgactgta-3'
	Anti-sense	5'-ctgacctggagtcttccagt-3'
Exon 8	Sense	5'-gtagtggtaatctactggga-3'
	Anti-sense	5'-cttacctcgcttagtgctcc-3'

buffer (glycerol:formamide:2X Tris-glicine SDS sample buffer = 1:18:5) was added to 5 µl of PCR product for each exon, followed by denaturing by heating at 95°C for 10 min. Denatured PCR product was applied to 20% TBE acrylamide gel, and electrophoresed at 180 V for 210 min at 8°C in 1X TBE running buffer. Gels were stained in 0.5 µg/ml ethidium bromide solution.

For sequencing of PCR product, DNA was extracted from gels and analyzed as follows: PCR product was applied to 2% TBE agarose gel, and electrophoresed at 100 V for 35 min with a 50-bp DNA step ladder (Promega Inc.) as a marker. Gels were stained in 0.5 µg/ml ethidium bromide solution, and the target bands were trimmed and collected onto GenElute Minus EtBr spin columns. Columns were centrifuged for 10 min at 14,500 rpm at 4°C, followed by addition of 50 µl of 1X TE buffer and further centrifugation for 10 min at 14,500 rpm at 4°C. DNA was extracted from 100-µl DNA samples using 10 µl of 3 M sodium acetate, 275 µl of cold EtOH and 1.5 µl of 20 µg/µl glycogen. Samples were centrifuged at 4°C for 20 min at 14,500 rpm, followed by addition of 500 µl of 70% cold EtOH, centrifugation at 4°C for 10 min at 14,500 rpm, and drying. Then, 10 µl of 1X TE buffer was mixed with purified DNA product, which was then analyzed using a Dye terminator ABI PRISM™ 3100 Genetic Analyzer (Perkin-Elmer Corp., Wilton, CT).

Results

Immunohistochemical assay for HER2, p53 and hormone receptors. For HER2, p53, ER and PgR, the number of positive primary tumors was 12 (27%), 13 (30%), 33 (75%) and 24 (55%), respectively (Table III). Two cases (4.5%, cases 26 and 40) showed discordance of HER2 expression results between primary and metastatic carcinoma (Tables III and IV). The primary tumors of these 2 cases had HER2 immunoreactivity scores of 2 to 3+ (interpreted as positive), whereas their recurrent tumors showed weak HER2 expression (1+, interpreted as negative). One case (case 33) showed equivocal discordance, changing from heterogeneous positive (2+) in the primary tumor to strongly positive (3+) in the recurrent tumor. Heterogeneous HER2 immunoreactivity in the primary tumor was found in 3 cases. The details of immunoreactivity and comparison between findings of IHC and FISH are described below. In 1 (2.3%) of the 13 cases in which the primary tumor was p53-positive, the recurrent tumor was p53-negative. There were no cases in which p53 immunoreactivity changed from negative to positive. For ER and PgR, discordance was detected in 7 tumors (15.9%) and 10 tumors (22.7%), respectively. In 6 cases, ER immunoreactivity changed from positive to negative. In 1 case, ER immunoreactivity changed from negative to positive. In 8 cases, PgR immunoreactivity changed from positive to negative. In 2 cases, PgR immunoreactivity changed from negative to positive.

Heterogeneous HER2 gene amplification and expression in primary and recurrent tumors. In case 26, most invasive components of the primary tumor showed no HER2 expression, whereas intraductal components and some invasive components showed 3+ HER2 expression (Fig. 1A). There was sharp contrast between HER2-positive and HER2-negative areas. The recurrent tumor was scored as 1+ (HER2-negative) (Fig. 2A). Results of FISH were consistent with HER2

Table III. Immunohistochemical results for HER2, p53, ER and PgR.

	Positive cases (%)			Changed to	
	Primary	Metastatic/recurrent	Discordant cases (%)	Negative	Positive
HER2	12 (27)	10 (23)	2 (4.5)	2	0
p53	13 (30)	12 (27)	1 (2.3)	1	0
ER	33 (75)	28 (64)	7 (15.9)	6	1
PgR	24 (55)	18 (41)	10 (22.7)	8	2

Table IV. Summary of clinical and histological features of cases with discordant results between primary and recurrent tumors.

Case no.	Histological type	Site of recurrence	Duration until recurrence (months)	HER2	P53	ER	PgR
40	IDC	Chest wall	27	-	NC	NC	+
26	IDC	Chest wall	64	-	NC	NC	NC
33	IDC	Chest wall	94	+	NC	NC	NC
11	IDC	Skin	53	NC	-	+	+
3	IDC	Lung	36	NC	NC	-	-
8	IDC	Skin	29	NC	NC	-	-
25	IDC	Local recurrence	51	NC	NC	-	-
42	IDC	Lymph node	28	NC	NC	-	-
6	IDC	Lymph node	85	NC	NC	-	NC
29	IDC	Lymph node	14	NC	NC	-	NC
10	IDC	Lymph node	97	NC	NC	NC	-
13	IDC	Skin	34	NC	NC	NC	-
32	IDC	Chest wall	94	NC	NC	NC	-
34	IDC	Chest wall	127	NC	NC	NC	-

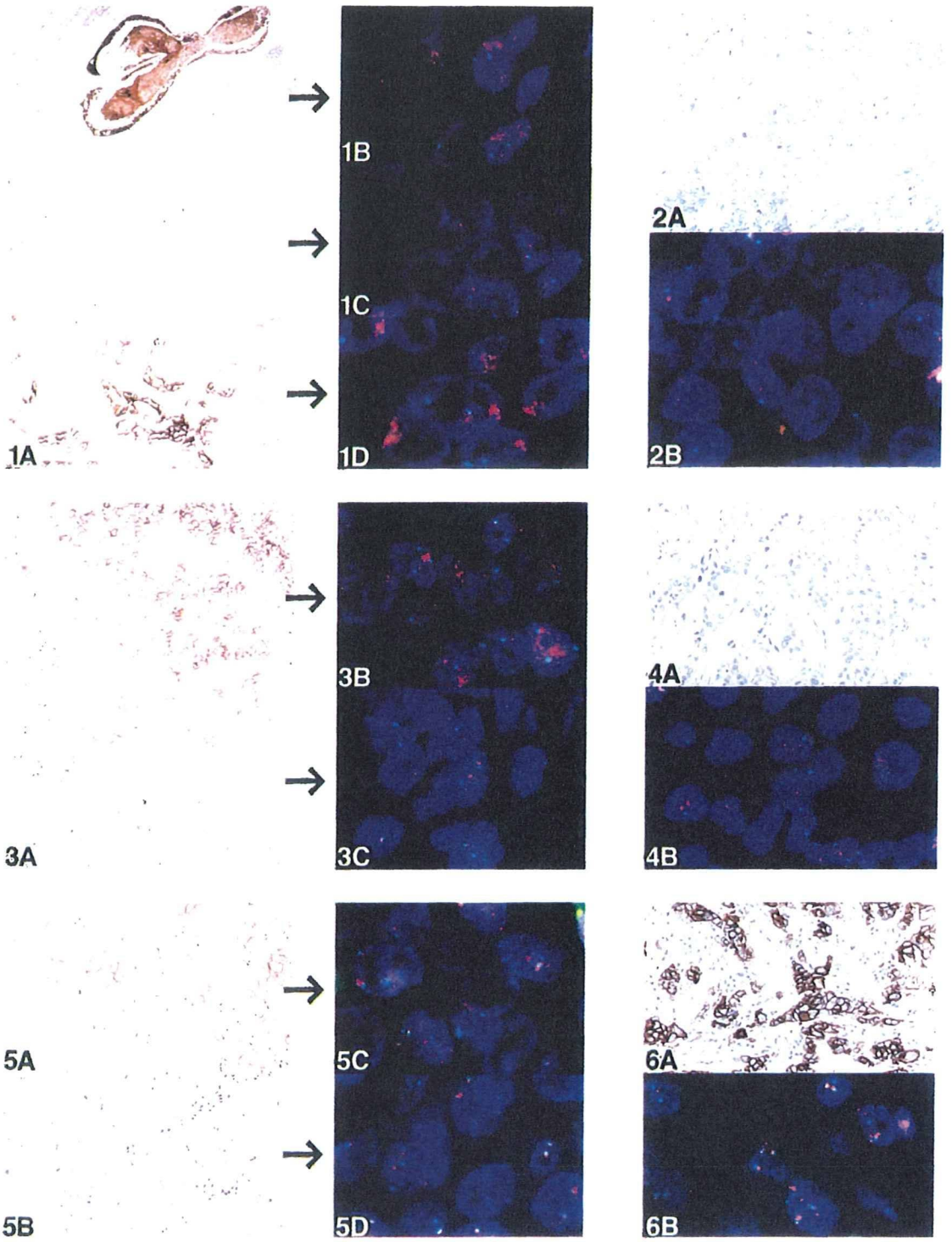
IDC, invasive ductal carcinoma; -, changed to negative; +, changed to positive; NC, no change.

Table V. Comparison of FISH with immunohistochemistry.

	HER2/CEP17 signal ratio	Amplified/not amplified	CEP17[a]
Case 26			
Primary tumor			
HER2-immunoreactive	4.19 (6.50)[b]	Amplified	1.57
HER2-non-immunoreactive	1.08	Not amplified	1.60
Recurrent tumor	1.02	Not amplified	1.62
Case 40			
Primary tumor			
HER2-immunoreactive	3.46	Amplified	1.68
HER2-non-immunoreactive	1.11	Not amplified	1.61
Recurrent tumor	1.32	Not amplified	1.41
Case 33			
Primary tumor			
HER2-immunoreactive	1.46	Not amplified	2.45
HER2-non-immunoreactive	1.17	Not amplified	3.18
Recurrent tumor	2.17	Amplified (low)	2.01

[a]Average numbers of CEP17 signals in 60 cancer cells of invasive components. [b]Signal ratio of intraductal components showing strong immunoreactivity for HER2.

expression (Fig. 2B). For the primary tumor, HER2/CEP17 signal ratio was 6.50 and 4.19 (amplified, Fig. 1B and D) in intraductal components and invasive components with 3+ HER2 expression, respectively (Table V). No HER2 amplification was detected in most invasive components of the primary tumor or in the recurrent tumor; these had HER2/CEP17 signal ratios of 1.08 and 1.02, respectively (not amplified, Figs. 1C and 2B).



Figures 1-6. HER2 overexpression and gene amplification of case 26 (Figs. 1 and 2), case 40 (Figs. 3 and 4), and case 33 (Figs. 5 and 6). Primary tumors (Figs. 1, 3 and 5) and recurrent tumors (Figs. 2, 4 and 6) are shown. Primary tumor of case 26 shows mixed HER2 immunohistochemical staining (Fig. 1A). HER2 gene amplification corresponding to HER2 expression is labeled in red: Fig. 1B, intraductal components (3+, HER2/CEP17=6.50); Fig. 1C, invasive components (3+, HER2/CEP17=4.19); Fig. 1D, invasive components (0, HER2/CEP17=1.08). Recurrent tumor shows weak HER2 expression (Fig. 2A), and no HER2 amplification (HER2/CEP17=1.02). Primary tumor of case 40 shows mixed HER2 expression (Fig. 3A). HER2 gene amplification for areas with HER2 overexpression (Fig. 3B) (2+, HER2/CEP17=3.46) and without overexpression (Fig. 3C) (HER2/CEP17=1.11) is shown. Recurrent tumor shows weak HER2 expression (Fig. 4A), and no HER2 amplification (HER2/CEP17=1.32). Primary tumor of case 33 shows focal HER2 overexpression (Fig. 5B), although there is an extensive area of weak HER2 staining (Fig. 5A). HER2 gene amplification was not detected in the areas corresponding to Fig. 5A (2+) and Fig. 5B (1+): Fig. 5C (HER2/CEP17=1.46) and Fig. 5D (HER2/CEP17=1.17), respectively. Recurrent tumor shows HER2 overexpression (Fig. 6A, 3+) and possible gene amplification (Fig. 6B, HER2/CEP17=2.01).

In case 40, areas of HER2 expression scored as 1+ and 2+ were intermixed in the primary tumor (Fig. 3A), which was determined to be positive for HER2 expression (Table I). In the recurrent tumor, there were diffuse areas that were scored as 1+. HER2/CEP17 signal ratio (FISH) was 3.46 (amplified, Fig. 3B) in areas of the primary tumor with 2+ HER2

Table VI. p53 mutation found in primary and recurrent carcinoma.

Case no.	PCR-SSCP	Mutation	
		Primary tumor	Recurrent tumor
3	Exon 8, P=R	Codon 273 CGT→CAT	NC
17	Exon 7, P=R	Codon 245 GGC→AGC	NC
26	Exon 6, P=R	Codon 194 CTT→CGT	NC
1	Exon 7, P≠R	Codon 241 TCC→TTC	Codon 249 AGG→TGG
11	Exon 7, P≠R	Codon 238 TGT→TAT Codon 244 GGC→AGC	No mutation
25	Exon 8, P≠R	Codon 275 TGT→TAT	No mutation

P, primary; R, recurrent; NC, no change.

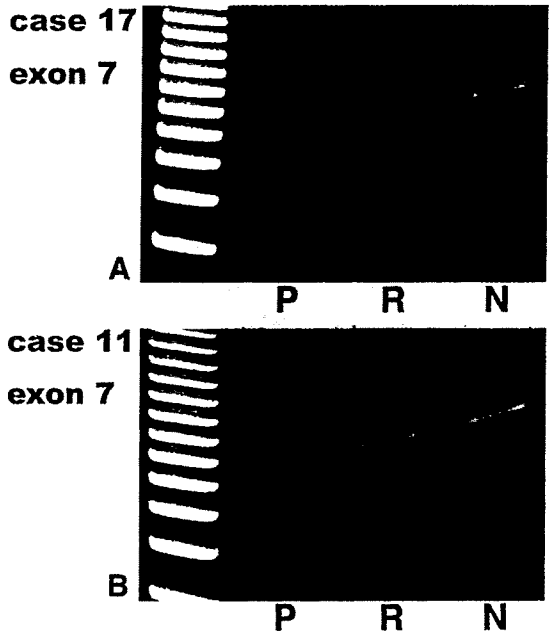


Figure 7. PCR-SSCP for case 17 (A) and case 11 (B). (A), Banding patterns of PCR products for exon 7 of primary tumor (P) and recurrent tumor (R) was similarly deviated from those of the non-neoplastic lymph node (N). (B), Banding patterns of PCR products obtained from the recurrent tumor (R) differed from those of the primary tumor of case 11 (P), although it was the same as those of the lymph node.

expression, and was 1.11 and 1.32 (not amplified, Figs. 3C and 4B) in areas of the primary and recurrent tumor with 1+ HER2 expression, respectively.

In case 33, the primary lesion primarily showed 1+ HER2 expression (Fig. 5B), although areas with 2+ expression comprised more than 10% of the tumor (Fig. 5A). The recurrent tumor showed strong HER2 expression (3+) (Fig. 6A). HER2/CEP17 signal ratio was 1.46 and 1.17 (not amplified, Fig. 5C and D) for areas of the primary tumor with scores of 2+ and 1+, respectively. Signal ratio was 2.17 for the recurrent tumor (Fig. 6B).

Average numbers of CEP 17 signals ranged from 1.4 to 1.7 (disomy) in cases 26 and 40, and ranged from 2.01 to 3.18 (aneusomy) in case 33 (Table V).

p53 mutation in primary and recurrent tumors. Nested PCR detected exons 5-8 of p53 in all 6 cases in which primary tumor cells showed diffuse, strong p53 immunoreactivity. Three cases (cases 3, 17 and 26) had the same banding pattern by SSCP between the primary tumors and the recurrent tumors, whereas the remaining 3 cases (cases 1, 11 and 25) had different patterns (Table VI, Fig. 7). In all 6 cases, the primary tumor had point mutations distributed among exons 6, 7 and 8 (Table VI). Cases 3, 17 and 26 had identical point mutations in their recurrent tumors. A different point mutation was detected in the recurrent tumor of case 1. No mutation was detected in the recurrent tumor of cases 11 and 25.

Discussion

In the present study, there was discordance in the status of HER2 gene amplification and p53 mutation between primary and asynchronous metastatic/recurrent tumors in a small population of breast cancers: 4.5% for HER2, and 2.3% for p53. HER2 gene amplification was not homogeneous throughout the tumor, and some recurrent tumors contained populations present in the primary tumor. Furthermore, not all carcinoma cells with aggressive HER2 gene amplification or p53 mutation were found in metastatic/recurrent lesions, and some carcinoma cells without these genetic alterations metastasized. In a limited number of breast cancers, HER2 expression has been observed to change from the primary tumor to metastatic tumors.

Shimizu *et al* (21) reported that all 21 of the cases they examined showed immunohistochemical concordance between primary and asynchronous recurrent/metastatic tumors, although there was a trend toward loss of hormone receptors in recurrent breast cancers (22). Xu *et al* (23) reported no difference in amplification ratios was identified between 12 primary cancers and synchronous axillary metastases. On

the other hands, discordant immunohistochemical results between primary tumors and synchronous metastases to lymph nodes were found in 2% (16) and 9.8% (17) of the breast cancer cases in other studies. With regard to p53, Shimizu *et al* (21) and Davidoff *et al* (24) reported p53 mutation in the primary breast cancer conserved in the recurrent/metastatic lesions, whereas Cardoso *et al* (16) show 6% discordant cases. Using DNA ploidy analysis, Symmans *et al* (25) found that all 17 of the primary breast cancers they examined were composed of multiple distinct populations, and that major populations in approximately 50% of regional metastases were, unexpectedly, not major populations in the primary tumors. However, there are no previous reports of such heterogeneity for specific genes such as HER2 or p53, in intraductal and invasive components of breast cancer. In the present study, using detailed observation of HER2-FISH preparations, we compared distribution of HER2 gene amplification with histologic characteristics (invasive or intraductal) and HER2 protein expression (detected by IHC). We calculated discordance rates between primary and asynchronous metastatic/recurrent cancer using a very large series. Also, we found that, within some individual cases, cell populations corresponding to specific gene alterations were present in both the recurrent and original tumor.

Several studies (26-28) have found strong HER2 immunoreactivity and/or HER2 gene amplification in intraductal components, and little or no HER2 expression in invasive components. There are several possible explanations for the findings. First, carcinoma cells may retain HER2 gene amplification in invasive areas where there is no HER2 expression. Disruption of post-transcriptional protein synthesis or transportation to the cytoplasmic membrane may cause the lack of HER2 expression. Second, HER2 gene amplification may be lost when carcinoma cells invade the stroma. Third, it may be the case that, although 2 cell populations (with and without HER2 gene amplification) are originally mixed, 1 of the 2 populations later becomes predominant. In case 26 in the present study, HER2 gene amplification was detected in ductal components and some of the invasive components (which also showed HER2 overexpression), but no amplification was detected in the majority of invasive areas. This suggests that the first explanation is unlikely, although we cannot exclude post-transcriptional inhibitory mechanisms. The problem with the second explanation is that there is no known mechanism for repair or loss of genetic alterations during the invasive process. The third explanation appears to be the most likely. In case 26 of the present study, the majority of ductal component carcinoma cells had HER2 gene amplification, but detailed observation revealed the presence of a few carcinoma cells that lacked HER2 overexpression. The results of the present examination of p53 suggest the possibility that only 1 of multiple clones survives to comprise the recurrent tumor. No mutation was detected in the recurrent tumor of case 11 or 25, although point mutations were observed in the primary tumor. In case 1, the primary and metastatic carcinomas had different point mutations.

Another interesting issue (28) is the biological significance of HER2 protein expression in relation to aneusomy of chromosome 17. In case 33, cancer cells showed aneusomy with CEP17 signal numbers (2.01-3.18) that were above the range of each cancer component of cases 26 and 40 (1.4-1.7). HER2/CEP17 signal ratio was 1.46 for the primary tumor, and 2.17 for the recurrent tumor. It seems unlikely that carcinoma cells in the recurrent tumor would undergo HER2 gene-specific amplification. Moderate HER2 expression (2+) is generally considered to indicate that humanized anti-HER2 antibody therapy will benefit the patient. However, concordance rates between IHC and FISH in tumors with 2+ HER2 expression are not particularly high [18.5% (26) and 36% (29)]. On the other hand, Bose *et al* (28) reported that 17 of 44 cases with equivocal (1+) to moderate (2+) HER2 immunoreactivity showed polysomy of chromosome 17. Thus, low-grade HER2 expression (1+ to 2+) could be caused by increased protein production due to either aneusomy or HER2 gene-specific amplification. It has not been determined whether trastuzumab is effective for cancers with 1+ to 2+ HER2 expression due to aneusomy. In HER2 evaluation, HER2 expression due to aneusomy should be distinguished from HER2 gene amplification.

In conclusion, although there is great similarity in HER2 and p53 status between asynchronous lesions and primary tumors, the present results indicate that one of the heterogeneous components of a primary tumor could be a major component of the corresponding recurrent tumor.

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Some organotin compounds enhance histone acetyltransferase activity

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Abstract

Eukaryotic DNA is packaged into chromatin, whose basic subunit is the nucleosome, which consists of DNA and a core histone octamer. Histone acetylation is important for the regulation of gene expression and is catalyzed by histone acetyltransferase (HAT). We observed the effects of suspected endocrine-disrupting chemicals (EDCs) on HAT activity. We showed that some organotin compounds – tributyltin (TBT) and triphenyltin (TPT) – enhanced HAT activity of core histones in a dose-dependent way and other EDCs did not affect HAT activity. Organotin compounds have various influences on physical function including the hormone and immune systems, embryogenesis, and development. Dibutyltin and diphenyltin, metabolites of TBT and TPT, respectively, also promoted HAT activity, but monobutyltin, monophenyltin, and inorganic tin had no effect. Further, TBT and TPT enhanced HAT activity when nucleosomal histones were used as substrates. These data indicate that the organotin compounds have unique effects on HATs independent of their EDC activities and suggest that the varied toxicities of the organotin compounds may be caused by aberrant gene expression following altered histone acetylation.

Keywords: Histone acetyltransferase; Organotin; Tributyltin; Triphenyltin; Endocrine-disrupting chemical

1. Introduction

Nuclear eukaryotic DNA is packaged into chromatin, which has a major impact on levels of gene transcription. The basic unit of chromatin is the nucleosome core particle, which consists of 146 bp of DNA wrapped around a histone octamer. This octamer con-

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sists of two each of the core histones H2A, H2B, H3, and H4, all of which have a basic, unstructured amino terminal tail. These basic proteins are susceptible to a variety of posttranslational modifications, e.g., acetylation (Howe et al., 1999; Wu et al., 1986). One of the well-characterized modifications is acetylation of specific lysine residues, which is reversibly catalyzed by histone acetyltransferase (HAT) and histone deacetylase (HDAC).

The GCN5-related *N*-acetyltransferase family includes GCN5 and PCAF, which share a remarkable degree of homology throughout their sequences and are present in a multisubunit complex consisting of more than 20 distinct polypeptides (Ogryzko et al., 1998). GCN5 and PCAF are transcriptional coactivators with intrinsic HAT activity; they contribute to transcriptional activation by acetylating chromatin (Sterner and Berger, 2000). Disruption of *Gcn5* and *Pcaf* genes revealed that they play distinct but functionally overlapping roles during embryogenesis (Yamauchi et al., 2000). Other well-characterized coactivators possessing HAT activity are CBP and P300, which are ubiquitously expressed global transcriptional coactivators that have critical roles in a wide variety of cellular processes, including development (Giles et al., 1998; Giordano and Avantaggiati, 1999; Yao et al., 1998).

Recent studies have demonstrated that some environmental pollutants affect the hormonal system and produce adverse effects on animals and probably also humans (Colborn et al., 1996; Van der Kraak et al., 1992). These pollutants are referred to as endocrine-disrupting chemicals (EDCs). The major targets of EDCs are nuclear hormone receptors, which bind steroid hormones and regulate transcription of their target genes (Nishihara et al., 2000; Nishikawa et al., 1999). For ligand-dependent gene activation, nuclear hormone receptors require coactivators that link the basal transcriptional machinery with the hormone receptors (Chen, 2000). Recent studies have shown that the nuclear hormone receptor coactivators possess HAT activity and recruit two other types of HATs, CBP and PCAF (Chen et al., 1997; Spencer et al., 1997). Hormone-dependent gene activation mediated by nuclear receptors involves the mutual recruitment of at least three classes of HATs.

These observations raise the possibility that HATs may be the targets of EDCs, and we tested the effects of suspected EDCs on HAT activity. Interestingly tributyltin (TBT) and triphenyltin (TPT) enhanced HAT activity, but other EDCs did not. These organotin chemicals have been used in such applications as wood preservation and as antifouling agents in marine paints, and are ubiquitous in the environment. TBT and TPT have been found to induce imposex (the superimposition of male sex organs in female gastropods) in the rock shell *Thais clavigera* and are known EDCs in marine species (Horiguchi et al., 1997). These compounds are also reported to affect not only the hormone system but also embryogenesis in mammals (Harazono et al., 1998; Nakanishi et al., 2002). Organotins caused behavioral and neurological symptoms and pancreatic and hepatic toxicities in rodents (Brown et al., 1979; Merkord et al., 2001). In the immune system, at low doses TBT inhibits immature thymocyte proliferation, whereas at higher doses in particular TBT induces apoptotic cell death (Gennari et al., 2002). However, the biological mechanism of the effects of organotin compounds on marine species and mammals awaits further characterization. The present study showed that some organotin compounds enhanced HAT activity when both core and nucleosomal histones were used as substrates. These data suggest that the varied toxicities of the organotin compounds may be caused by aberrant gene expression following altered histone acetylation.

2. Materials and methods

2.1. Chemicals

All chemicals were dissolved in dimethyl sulfoxide (DMSO) (Wako Pure Chemicals, Osaka, Japan). Organotin and related chemicals tested are listed in Table 1.

2.2. Preparation of rat liver nuclear extracts and HAT fraction

All animal care and handling procedures were approved by the animal care and use committee of Osaka University. Rat liver nuclear extracts (RLNE) were prepared as described previously (Osada et al., 1995). For binding RLNE to Ni^{2+}-NTA agarose (Qiagen, Hilden, Germany), nuclei were suspended in a nuclear lysis buffer (10 mM HEPES, pH 7.6, 100 mM KCl, 10% glycerol, 3 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and