THE SIGNIFICANCE OF HER-2/neu/c-erbB-2 GENE AMPLIFICATION IN BENIGN AND MALIGNANT BREAST DISEASE

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Abstract. This study was carried out to investigate the amplification of HER-2/neu oncogene in 66 patients with primary breast cancer and 90 samples from benign breast disease (BBD). The amplification of HER-2/neu oncogene in the DNA of paraffin-embedded specimens was determined by differential PCR. Nineteen out of 66 (28.8%) breast cancer patients showed amplification of the gene. No gene amplification was found in benign breast disease. There was no significant correlation of HER-2/neu amplification with, age, menopausal status, the number of positive nodes, tumor size, estrogen receptor, however, amplification of HER-2/neu gene was strongly correlated with nodal status ($p = 0.0049$). In node positive patients, the incidence of HER-2/neu amplification was high (43%). These findings indicate that the amplification of HER-2/neu gene may be of pathogenetic significance in breast cancer and may have a poor prognosis in node positive breast cancer patients while no gene amplification in benign breast disease suggests that HER-2/neu amplification is a late molecular alteration event in the pathogenesis of breast cancer.

INTRODUCTION

The c-erbB-2 oncogene or neu or HER-2 is a normal cellular gene which located on chromosome 17 (Fukushige *et al*, 1986). It belongs to the tyrosine kinase oncogene family and encodes a 185 KDa transmembrane glycoprotein ($p185^{HER-2}$) growth factor receptor that is highly homologous with, but distinct from, the epidermal growth factor receptor (EGFR) (Coussens *et al*, 1985; Schechter *et al*, 1984; Yamamoto *et al*, 1986). The two proteins (HER-2 and EGFR) are 40% identical in amino acid sequence forming the extracellular domain which may be responsible for ability to bind ligands, while the intracellular domains of the two proteins are 80% identical in amino acid sequence and possess tyrosine kinase activity. Overexpression of $p185^{HER-2}$ leads to receptor dimerization, tyrosine kinase activation, receptor autophosphorylation, and the subsequent activation of kinase substrates involved in the cellular signal-tansduction mechanisms, which eventually affects the nuclear transcription of genes regulating cell-cycle progression (Valeron *et al*, 1996). The first study which indicated that amplification of HER-2/neu/c-erbB-2 oncogene may be related to poor prognosis was published by Slamon *et al* (1987). Several studies have shown the correlation between HER-2/neu/c-erbB-2 oncogene amplification and overexpression of the oncoprotein, $p185^{HER-2}$ (Borg *et al*, 1990; Chen *et al*, 1995; Kerns *et al*, 1993; Liu *et al*, 1992; Scorilas *et al*, 1995; Slamon *et al*, 1989; Tandon *et al*, 1989; Valeron *et al*, 1996). In human breast cancer, amplification of HER-2/neu/c-erbB-2 has been found in 10% to 35% of patients (Table 3). Amplification and/or overexpression have been demonstrated to correlate with poor prognosis in breast cancer patients (Allred *et al*, 1992; Borresen *et al*, 1990; Descotes *et al*, 1993; Paik *et al*, 1990; Paterson *et al*, 1991; Seshadri *et al*, 1993; Slamon *et al*, 1987, 1989; Tandon *et al*, 1989; Tetu and Brisson, 1994; Tsuda *et al*, 1989; Varley *et al*, 1987; Walker *et al*, 1989; Wright *et al*, 1989) but its significant relationship still remains controversial (Ali *et al*, 1988; Berns *et al*, 1995; Clark and McGuire, 1991; Cline *et al*, 1987; Gusterson *et al*, 1988; van de Vijver *et al*, 1988; Zhou *et al*, 1989). It is believed that genetic alteration (*ie* loss of heterozygosity (LOH) and amplification of oncogene) has an importance in the origin and progression of human breast cancer (Berns *et al*, 1995). Since HER-2/neu/c-erbB-2 overexpression correlates with HER-2/neu/c-erbB-2 oncogene amplification not only in invasive tumors and breast cancer cell lines but also in *in situ* breast carcinoma (Liu *et*

al, 1992). During the process of tumor development, gene alterations such as HER-2/neu/c-erbB-2 oncogene amplification or others might occur in early or late events of the disease. However, little is known about the genetic sequence involved in the disease. HER-2/neu/c-erbB-2 oncogene amplification might also be detected in benign breast disease. In this study the differential polymerase chain reaction (differential PCR), which is a simple, rapid, and sensitive method (Frye *et al*, 1989; Neubauer *et al*, 1992) was used to examine the significance of HER-2/neu/c-erbB-2 oncogene amplification in Thai breast cancer and in benign breast disease (BBD). Its relationship to other clinicopathological and biological factors was also determined.

MATERIALS AND METHODS

Tissue specimens

Paraffin-embedded tissue specimens of 66 primary breast cancers were collected from patients attended at National Cancer Institute, Bangkok, Thailand, between January 1989-April 1995. All of them were diagnosed as having breast cancer. Their ages varied from 30-77 years. Ninety paraffin-embedded tissue specimens of benign breast disease (BBD) obtained from patients with benign breast disease who visited National Cancer Institute (NCI), Bangkok, Thailand during January-December in 1994. Their ages varied from 17-57 years. The histological types of samples enrolled in this study were 30 fibrocytic diseases; 30 benign mammary dysplasias; and 30 fibroadenomas. A microtome was used to cut several 10-μm sections of the paraffin-embedded tissue specimens. To visualize the tumor boundaries in each sample, the adjacent 4-μm sections were cut and stained with hematoxylin and eosin (H and E). The contiguous 10-μm sections of the paraffin-embedded tumor tissues were used for DNA extraction. The DNAs obtained were subjected for differential polymerase chain reaction (differential PCR) to determine gene amplification.

Cell line

The human breast cancer cell line SK-BR-3, which has 4-to 8-fold amplification of HER-2/neu/c-erbB-2 (Kraus *et al*, 1987) was used in this study. The cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD), maintained in McCoy's 5A medium (Gibco, BRL, USA) plus 10% fetal bovine serum and incubated at 37°C in a humidified 5% CO_2 incubator.

Preparation of specimens for differential polymerase chain reaction

Paraffin-embedded tissue specimens from 66 patients diagnosed with primary breast cancer, and 90 tissues from patients with benign breast disease were obtained and DNA were extracted. The ten micron thick specimens placed in 1.5 ml microcentrifuge tubes were deparaffinized by adding 0.5 ml of xylene, rotated for 30 minutes, and centrifuged at 10,000*g* for 10 minutes at room temperature. The tissue pellet and any residual paraffin was washed with 0.5 ml of absolute ethanol to remove the xylene. After centrifugation at 10,000*g* for 10 minutes at room temperature, the pellet was extracted by adding 5 ml of lysis buffer (0.32 M sucrose, 10 mM Tris-HCl pH 7.5, 1.5 mM $MgCl_2$, 1% triton x-100), vigorously mixed, and centrifuged at 3,000*g* for 10 minutes at room temperature. The pellet was then digested by adding 0.2 ml proteinase K solution (200 μg/ml proteinase K, 1% NP-40, 1% Tween 20, 1.5 mM $MgCl_2$, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 0.01% gelatin) and incubating at 37°C overnight. The digested sample was boiled for 10 minutes to inactivate the proteinase and then kept at -20°C for differential polymerase chain reaction (differential PCR).

The human breast cancer cell line, SK-BR-3 used as the positive control, was grown in McCoy's 5A medium supplemented with 10% fetal bovive serum in the 25 cm^2 growth area of tissue culture flask. The cell pellet was extracted by adding 5 ml of lysis buffer. The remainder of the procedure was performed as mentioned above. A 10 μl aliquot of the digested sample was then subjected for determination of HER-2/neu/c-erbB-2 gene amplification by differential PCR. Human genomic DNA from normal placenta which purchased from CLONTECH (USA) was used as the negative control.

Differential PCR

Differential PCR was carried out by coamplification of a target gene and a single-copy reference

gene in the same reaction tube. The differential PCR technique used in this study was performed according to the procedure described by Neubauer *et al* (1992). Briefly, 50 µl of the prepared specimens was made up to 100 µl with 10 mM Tris-HCl, pH 8.3 at room temperature, 50 mM KCl, 0.01% gelatin, 1.5 mM $MgCl_2$, 0.2 mM each of four dNTPs, 0.5 µM each of primers (except for primer γ-IFN 85, in which the primers were 0.1 µM) and 2.5 units of AmpliTaq DNA polymerase (Perkin Elmer, USA). The reaction mixture was overlaid with 100 µl of mineral oil and subjected to 1 amplification cycle, consisting of sample denaturation at 94°C for 5 minutes, primer annealing at 50°C for 1 minute and primer extension at 72°C for 1 minute. Followed by 33 cycles of amplification, each cycle consisting of 1 minute sample denaturation, primer annealing and primer extension at temperature of 94°C, 50°C, and 72°C respectively. The last cycle of PCR employed at 94°C for 1 minute, and at 60°C for 10 minutes.

Analysis of the amplified products

Ten µl of amplified products were analyzed by electrophoresis on 5% agarose gels. The Hae III-digested $\phi \times 174$ RF DNA (Gibco-BRL, USA) was used as the size standard marker. The gels were stained with ethidium bromide and photographed under UV illumination. The photographs were used to assess the HER-2/neu/c-erbB-2 gene amplification by comparing the relative intensity between the two resulting PCR product bands of the target and reference genes by densitometric scanning (BIO-RAD, USA). Analysis of the ratios obtained was done according to Neubauer *et al* (1992). In brief, four sets of primers were used. The first set of primers (γ-IFN 82/γ-IFN 150) was utilized to test adequate quality of target DNA, while the other sets of primers (neu 98/β-IFN 119, neu 98/γ-IFN 85, neu 98/N-ras110) were performed to determine the presence of HER-2/neu/c-erbB-2 gene amplification. The ratios obtained were then scored semiquantitatively into four amplification levels by comparison between the differential PCR results obtained from test samples and those obtained from the positive and the negative controls as the following criteria:

1. No amplification:

All genes (neu 98/β-IFN 119, neu 98/γ-IFN 85, neu 98/N-ras 110) in the test samples gave identical results to the negative control or showing no gene amplification by neu 98/γ-IFN 85.

2. Two-to fourfold amplification:

All genes (neu 98/β-IFN 119, neu 98/γ-IFN 85, neu 98/N-ras 110) in the test samples gave greater results than the negative control but less than the midpoint between the negative and the positive controls.

3. Four-to eightfold amplification:

All genes (neu 98/β-IFN 119, neu 98/γ-IFN 85, neu 98/N-ras 110) in the test samples gave lesser results than the positive control but greater than the midpoint between the negative and the positive controls or at least neu 98/γ-IFN 85 result matching to the positive control.

4. Greater than eightfold amplification:

All genes (neu 98/β-IFN 119, neu 98/γ-IFN 85, neu 98/N-ras 110) in the test samples gave greater results than the positive control.

Table 1

Primers used in this study.

Gene and size (bp)	forward primer 5' → 3'	reverse primer 5' → 3'	Nucleotide position
HER-2/neu98	CCTCTGACGTCCATCATCTC	ATCTTCTGCTGCCGTCGCTT	2122-2219 (Yamamoto *et al*, 1986)
γ-IFN 150	TCTTTTCTTTCCCGATAGGT	CTGGGATGCTCTTCGACCTC	4582-4731 (Gray and Goeddel, 1982)
γ-IFN 85	AGTGATGGCTGAACTGTCGC	CTGGGATGCTCTTCGACCTC	4647-4731 (Gray and Goeddel, 1982)
γ-IFN 82	GCAGAGCCAAATTGTCTCCT	GGTCTCCACACTCTTTTGGA	2012-2093 (Gray and Goeddel, 1982)
β-IFN 119	GTGTCTCCTCCAAATTGCTC	GCCACAGGAGCTTCTGACAC	12-130 (Ohno and Taniguchi, 1982)
N-ras 110	ATGACTGAGTACAAACTGGT	CTCTATGGTGGGATCATATT	1-111 (Taparowsky *et al*, 1983)

Statistics

To study the association between the HER-2/neu/c-erbB-2 gene amplification status and clinicopathological or biological factors, the chi-squared test was used. Fisher's exact test was assessed when the chi-squared test was not valid. Statistical significance was reported if $p < 0.05$ was achieved.

RESULTS

Clinicopathological and biological data

Clinicopathological and biological data of 90 samples with histological analysis confirmed benign breast disease (BBD) and 66 samples with primary breast cancer are demonstrated in Table 2.

HER-2/neu/c-erbB-2 gene amplification analysis

In this study, DNA in the paraffin-embedded tissue specimens from BBD and breast cancer patients were examined for HER-2/neu/c-erbB-2 gene amplification by differential PCR, using the four sets of primers. The amplified products were electrophoresed through a 5% agarose gel. The photographs of ethidium bromide stained gels were quantitated by means of scanning densitometer and then classified into 4 levels of amplification (no amplification, two-to fourfold, four-to eightfold and greater than eightfold amplification). Fig 1 shows that the two DNA bands of the target (98 bp of HER-2/neu/c-erbB-2) and reference (119 bp, 85 bp, 110 bp of β-IFN, γ-IFN, N-ras, respectively) genes could be detected and quantitated for gene amplification. The integrity of DNA was determined with γ-IFN 82:γ-IFN 150 amplimers. If this ratio less than or equal to 3 indicates that the sample contains target DNA of adequate quality. HER-2/neu/c-erbB-2 gene amplification was noted in lane 6 (greater than eightfold amplification), in lane 5

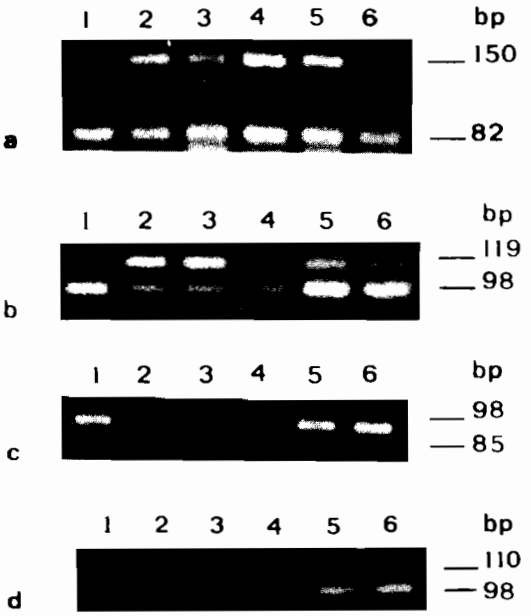

Fig 1–Differential PCR analysis of breast tumor DNA
(a) PCR product using γ-IFN 82/γ-IFN 150 amplimers
(b) PCR product using neu 98/β-IFN 119 amplimers
(c) PCR product using neu 98/γ-IFN85 amplimers
(d) PCR product using neu 98/N-ras 110 amplimers
lane 1, SK-BR-3
lane 2, normal human placenta
lane 3, no amplification of HER-2/neu
lane 4, two-to fourfold amplification of HER-2/neu
lane 5, four-to eightfold amplification of HER-2/neu
lane 6, greater than eightfold amplification of HER-2/neu

Table 2

Clinicopathological and biological data of benign breast disease and primary breast cancer patients.

Disease	Symptom	No./total	Range age (years)	Median age (years)
Benign breast disease	fibrocytic disease	30/90	19-57	38
	fibroadenoma	30/90	17-52	29.5
	benign mammary dysplasia	30/90	23-53	31
Primary breast cancer	premenopausal	36/66	30-57	43
	postmenopausal	30/66	31-77	57

Table 3

Summary on incidence of HER-2/neu/c-erbB-2 gene amplification in breast cancer patients.

Authors	Amplified/total (%)	Country
Cline *et al* (1987)	8/53 (15)	USA
Slamon *et al* (1987)	53/189 (28)	USA
Varley *et al* (1987)	7/37 (19)	England
Venter *et al* (1987)	12/36 (33)	England
Ali *et al* (1988)	12/122 (10)	USA
Seshadri *et al* (1989)	17/73 (23)	Australia
Zeillinger *et al* (1989)	52/291 (18)	Austria
Borg *et al* (1990)	52/310 (17)	Sweden
Borresen *et al* (1990)	20/89 (22.5)	Norway
Clark and McGuire (1991)	120/362 (33)	USA
Liu *et al* (1992)	26/122 (21)	USA
Tiwari *et al* (1992)	17/61 (28)	USA
Tommasi *et al* (1992)	9/26 (35)	Italy
Descotes *et al* (1993)	28/149 (18.8)	France
Imyanitov *et al* (1993)	36/142 (25)	Russia
Knyazev *et al* (1993)	15/60 (25)	Russia
Seshadri *et al* (1993)	222/1,056 (21)	Australia
Berns *et al* (1995)	63/259 (24)	The Netherlands
Chen *et al* (1995)	35/101 (34.7)	China
Valeron *et al* (1996)	62/415 (15)	Spain

Table 4

HER-2/neu/c-erbB-2 gene amplification in benign and malignant breast disease.

	Fold HER-2/neu/c-erbB-2 amplification				
Sample	1	2-4 (%)	4-8 (%)	> 8 (%)	Total
Fibrocytic disease	30	0	0	0	30
Fibroadenoma	30	0	0	0	30
Mammary dysplasia	30	0	0	0	30
Breast carcinoma	47	6 (31.6)	11 (57.9)	2 (10.5)	66

(four-to eightfold amplification), in land 4 (two-to fourfold amplification), and in lane 3 (no amplification). Human breast cancer cell line, SK-BR-3 DNA was used as a positive control, and the ratio of HER-2/neu/c-erbB-2:β-IFN; HER-2/neu/c-erbB-2:γ-IFN; HER-2/neu/c-erbB-2:N-ras was found to be 1.40; 1.70; 1.87 respectively (lane 1). Normal human placental DNA was used as a negative control, and the ratio of those was found to be approximately 1 (lane 2).

Nineteen of 66 samples (28.8%) from patients with primary breast cancer showed amplification of HER-2/neu/c-erbB-2 gene. The results were categorized into 4 levels of gene amplification: 47 had no amplification and 19 had amplification. Among these, 6/19 (31.6%) had two-fourfold amplification, 11/19 (57.9%) had four to eightfold amplification, and 2/19 (10.5%) had greater than eightfold amplification as shown in Table 4.

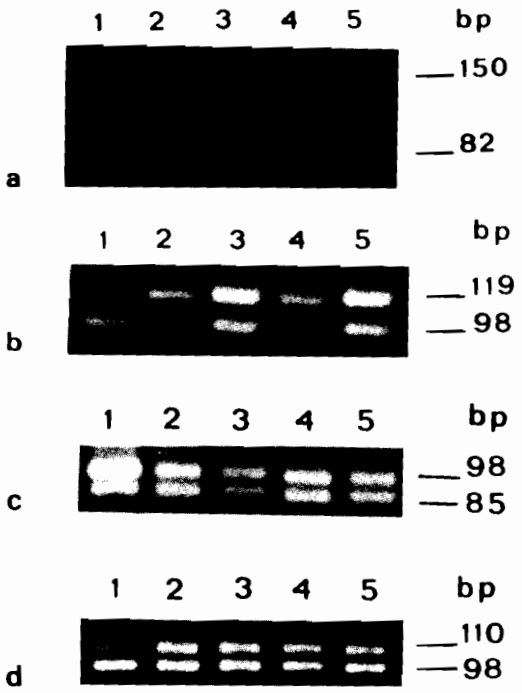

Fig 2–Differential PCR analysis of BBD DNA
(a) PCR product using γ-IFN 82/γ-IFN 150 amplimers
(b) PCR product using neu 98/β-IFN 119 amplimers
(c) PCR product using neu 98/γ-IFN85 amplimers
(d) PCR product using neu 98/N-ras 110 amplimers
lane 1, SK-BR-3
lane 2, normal human placenta
lane 3, no amplification of HER-2/neu in fibroadenoma
lane 4, no amplification of HER-2/neu in fibrocytic disease
lane 5, no amplification of HER-2/neu in mammary dysplasia

No amplification of HER-2/neu/c-erbB-2 gene was detected in 90 BBD samples (Fig 2, Table 4).

HER-2/neu/c-erbB-2 gene amplification association with various factors

Table 5 shows the association between HER-2/neu/c-erbB-2 gene amplification and clinicopathological or biological factors from patients with breast cancer. There was no correlation between amplification of HER-2/neu/c-erbB-2 gene and age, menopausal status, the number of positive nodes, tumor size, estrogen receptor (p = 0.3089, 0.3089, 0.0813, 0.3684, 1.0000, respectively). In contrast, there was a statistically significant correlation between the gene amplification and nodal status (p = 0.0049).

DISCUSSION

Southern blot or dot blot hybridization has been used as the conventional standard method to study HER-2/neu/c-erbB-2 oncogene amplification. This method requires a large quantity and high quality of DNA, time consuming, and radioactive reagents. In this study, the differential PCR technique was used to determine HER-2/neu/c-erbB-2 oncogene amplification in the DNA of human breast cancer and BBD samples. This procedure is a simple, rapid, and sensitive method. No radioactive reagent is required and only a small amount of DNA is needed for quantitative detection of the amplified gene in small samples of cells or paraffin-embedded specimens (Frye *et al*, 1989; Neubauer *et al*, 1992). Therefore, it is a suitable method to determine the significance of HER-2/neu/c-erbB-2 oncogene amplification in benign breast disease and breast cancer. A relationship between HER-2/neu/c-erbB-2 oncogene amplification and overexpression of the oncoprotein in human breast cancer have been reported by Borg *et al* (1990), Chen *et al* (1995), Kerns *et al* (1993), Liu *et al* (1992), Scorilas *et al* (1995), Slamon *et al* (1989), Tandon *et al* (1989), Valeron *et al* (1996). In this study, the amplification of HER-2/neu/c-erbB-2 gene in patients with breast cancer, node negative and node positive, was found 28.8%, 7%, and 43% respectively. Statistical analysis between gene amplification and nodal status in breast cancer patients was significant at p = 0.0049. This result is similar to the observation reported by Tiwari *et al* (1992) but is different from the observation of Borresen *et al* (1990), Clark and McGuire (1991), Imyanitov *et al* (1993), Scorilas *et al* (1995), Seshadri *et al* (1993). The age, menopausal status, the number of positive nodes, tumor size, and estrogen receptor are not associated with HER-2/neu/c-erbB-2 gene amplification in breast cancer patients (p = 0.3089, 0.3089, 0.0813, 0.3684, 1.0000 respectively). This finding is similar to the previous results reported by Borresen *et al* (1990), Imyanitov *et al* (1993), Scorilas *et al* (1995), Seshadri *et al* (1993), Tiwari *et al* (1992), but is different from the reports by Berns *et al* (1992); Borg *et al* (1990), Chen *et al* (1995). The

Table 5

Relationships between clinicopathological or biological factors and HER-2/neu/c-erbB-2 gene amplification.

Factors	HER-2/neu/c-erbB-2 amplification			%HER-2/neu/c-erbB-2	
	positive	negative	total	amplified	p-value
Age (y)					
< 50	8	28	36	22	NS
≥ 50	11	19	30	37	
Menopausal status					
Pre	8	28	36	22	NS
Post	11	19	30	37	
Node status					
Negative	2	25	27	7	S
Positive	15	20	35	43	
NR	2	2	4		
No. of positive nodes					
1-3	3	11	14	21	NS
> 3	12	9	21	57	
Tumor size (cm)					
≤ 2	5	9	14	35.7	NS
> 2	14	38	52	26.9	
ER					
Negative	5	15	20	25	NS
Positive	6	14	20	30	
NR	8	18	26		

NS = not significant; ER = estrogen receptor; S = significant; NR = not report

finding in our study suggested that the relatively high incidence of HER-2/neu/c-erbB-2 oncogene amplification may have important role in progression of human breast cancer, particularly as a prognostic indicator in node positive patients. There are several studies that report the correlation between HER-2/neu/c-erbB-2 oncogene amplification and poor prognosis in node positive breast cancer patients (Borg *et al*, 1990; Borresen *et al*, 1990; Rilke *et al*, 1991; Seshadri *et al*, 1993; Slamon *et al*, 1987, 1989; Tandon *et al*, 1989; Tetu and Brisson, 1994; Tsuda *et al*, 1989) and in node negative patients (Allred *et al*, 1992; Borresen *et al*, 1990; Paterson *et al*, 1991; Seshadri *et al*, 1993). In contrast, there are some studies that report no such a relationship in node positive patients (Clark and McGuire, 1991) and in node negative patients (Berns *et al*, 1995; Borg *et al*, 1990; Clark and McGuire, 1991; Rilke *et al*, 1991; Tandon *et al*, 1989). However, our report is a retrospective study, we do not have clinical follow up data to determine if the association between HER-2/neu/c-erbB-2 oncogene amplification and prognosis exists. Furthermore, a large numbers of samples studied would be required to provide a definitive answer.

No gene amplification occurred in 90 paraffin-embedded samples of BBD. This finding is similar to previous results reported by Gusterson *et al* (1988), Lizard-Nacol *et al* (1995), Millikan *et al* (1995), Regidor *et al* (1995), Riviere *et al* (1991), Tsutsumi *et al* (1990) in that no detectable gene amplification in benign breast samples. Although only the small series were analyzed, our data suggest that HER 2/neu/c-erbB-2 oncogene amplification is a late molecular event involved in human breast cancer pathogenesis. Since the early detection and prevention are useful strategies among various tumors including breast cancer. It should be also noted that little is known about the early molecular alteration events associated with carcinogenesis of human breast cancer. The study for

investigation and elucidation the early molecular alteration events in other genes in this harmful disease by using the advance and sensitive technology such as PCR method should be further continue.

In conclusion, this study is the first report that describes the distribution of the HER-2/neu/c-erbB-2 gene in the human breast cancer in Thailand. A large prospective study with long term follow up using the PCR technique should be performed to investigate the advantage of knowledge on early molecular alteration events in several aspects, especially those that are more frequently involved in the breast cancer for the early detection and prevention of such disease as well as those of prognostic sig-nificance of HER-2/neu/c-erbB-2 gene amplification for the treatment of such patients.

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