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Amplification and over-expression of *erb*B-2 oncogene in primary human breast tumors

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Abstract

***erb*B-2 is a growth factor receptor gene, and it's product, p185, has tyrosine kinase activity and can transform NIH3T3 cell when overexpressed. *erb*B-2 oncogene has been reported to be amplified frequently in various human tumors of epithelial origin, such as breast, ovary and stomach. In breast cancer, the amplification and over-expression of *erb*B-2 protein, p185, have been implicated as parameters for prognosis. In this study, we analyzed 72 primary breast tumors obtained from Korean patients for gene amplification and mRNA over-expression, and 114 tumor tissue sections from paraffin blocks were analyzed for p185 expression using anti-p185 antibody. Ten (13.9%) of 72 DNA samples from primary breast tumors contained multiple copies of erbB-2 gene, ranging from 2 to 27. RNAs were prepared from 23 tumors among 72 tumors, of which 4 (17.4%) had increased amount of *erb*B-2 mRNA. Thirty-eight percent (43 of 114) of paraffin-embedded tissue sections were strongly reactive to the anti-p185 polyclonal antibody. No p185 was detectable in 37% (42 of 114) sections and the rest were weakly stained. Seventy percent (7 of 10) of tumors containing amplified *erb*B-2 gene were positive for p185 expression. But, 37% (20 of 54) of tumors with single gene copy were strongly reactive to anti-p185 antibody. These results could explain the higher rate of protein expression than gene amplification of *erb*B-2 in primary breast tumors. We also observed mRNA over-expression in 2 of 15 tumors with single copy *erb*B-2 gene, and furthermore, strong expression of p185 protein in 4 of 15 tumors without increased mRNA expresseion. These suggest that other mechanisms than gene amplification and/or mRNA over-expression for the over-production of p185 in primary breast tumors may exist.**

Keywords: *erb*B-2, amplification, over-expression, p185, breast tumor

Introduction

Breast cancer is one of the leading cause of cancer death among women, and the number has been increasing in Korean women. Therefore, there have been a lot of continuous efforts to elucidate the etiology of breast cancer. Several oncogenes have been implicated in the carcinogenesis of breast cancer, such as *ras*, c-*myc* (Guerin *et al.*, 1988), c-*int* (Machotaka *et al.*, 1989; Spandidos *et al.*, 1989) and *erb*B-2 (Slamon *et al.*, 1987). Among them, *erb*B-2 oncogene has been studied most extensively.

*erb*B-2 is a growth factor receptor oncogene which encodes a 185 kDa glycoprotein, with tyrosien kinase acitivtiy (Schechter *et al.*, 1984; King *et al.*, 1985; Akiyama *et al.*, 1986; Bargaman *et al.*, 1986; Yamamoto *et al.*, 1986). p185 has some functional relationships as well as extensive amino acid sequence homology with human epidermal growth factor (EGF)receptor (Coussens *et al.*, 1986). However, neither EGF nor TGF-α bind to p185. A group of specific ligands for p185, known as heregulin, neu differentiaton factor (NDF) or glial cell growth factor, have been reported. These ligands are a family of proteins produced by alternative splicing from a single gene (Lupu *et al.*, 1990).

*erb*B-2 oncogene can transform NIH3T3 cells when overexpressed, but the level of expression is critical for transformation (Di Fiore *et al.*, 1987). The transforming potential of *erb*B-2 oncogene is totally dependent on the tyrosine kinase activity as in the other tyrosine kinase family oncogene (Wildenhain *et al.*, 1990). The increase of tyrosine kinase acitivity of *erb*B-2 in a cell can be caused by three different mechanism; N-

terminal truncation, point mutation in transmembrane domain and mRNA over-expression. The first two mechanisms, N-terminal trucncation shown in v-*erb*B, a viral counter part of EGF receptor, and the point mutation shown in *neu* oncogene, a rat homologue of *erb*B-2 (Bargaman *et al.*, 1986), have not yet been demonstrated in human tumors (Lemoine *et al.*, 1990). The only mechanism of the elevated *erb*B-2 tyrosine kinase activity observed in human tumor is the increase of total amount of tyrosine kinase by the increase of p185 amount, which is caused by the over-expression of *erb*B-2 mRNA with or without gene amplification.

The over-expression of p185 has been observed in various human carcinomas of epithelial origin. In primary human breast cancer, the frequency of *erb*B-2 amplification has been reported to be in between 10 to 40% (Slamon *et al.*, 1987, 1989; van der Vijver *et al.*, 1987; Ali *et al.*, 1988; Bacus *et al.*, 1990; Paterson *et al.*, 1991). Several studies suggested that amplification of *erb*B-2 gene has significant relationship with the clinical parameters of breast cancers such as axillary lymph node metastasis, tumor size, stage of the disease and estrogen receptor status (Zhou *et al.*, 1987; Tauchi *et al.*, 1989). Particularly, in node positive cases, the increase of p185 has been reported to be related to the recurrence and poor prognosis (Slamon *et al.*, 1987, 1989; Borg *et al.*, 1990). But other studies showed no apparent relationship between the amplification and clinical parameters in breast cancers (Ali *et al.*, 1988; Uehara *et al.*, 1990; Clark *et al.*, 1991; Ciocca *et al.*, 1992). Therefore, it needs more studies to clarify the clinical importance of amplification and over-expression of *erb*B-2 in breast cancer.

Since the frequency of *erb*B-2 gene amplification and over-expression in breast cancer are varied, we examined the amplification, mRNA and p185 over-expression of *erb*B-2 in human primary tumor tissues obtained from Korean patients.

Materials and Methods

Tumor samples

Primary breast cancer tissues from a 72 patients were obtained after surgery and kept in liquid nitrogen until use. Both DNA and RNA were prepared from 23 tumor tissues, and DNA were prepared from the rest 49 tumor tissues. For analysis of p185 expression, 123 paraffin-embedded tissue sections were obtained from the Department of Pathology, Seoul National University Hospital. These 123 cases include 115 invasive ductal, 4 medullary, 1 infiltrative papillary, 1 intraductal, 1 mucinous and 1 metaplastic carcinomas.

Preparation of DNA and RNA

For DNA preparation, tumor tissues were ground to powder in liquid nitrogen and digested with 100 μg/ml proteinase K in detergent containing lysis buffer. DNA was purified by subsequent phenol, phenol/chloroform (1:1), and chloroform extraction and precipitated with ethanol. Total RNA was isolated by acid guanidium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987).

Southern, Northern and dot blot analysis

The genomic DNA (25 μg) digested with *Eco*RI was fractionated on 0.8% agarose gel, and transferred to nitrocellulose membrane as described (Maniatis, 1989). The total RNA (20 μg) was denatured and electrophoresed on 1% denaturing agarose gel containing 20% formamide, and transferred to nitrocellulose membrane. The blots were hybridized with ^{32}P-labeled 1.6 kb internal *Eco*RI fragment of *erb*B-2 c-DNA (kindly provided by M. Kraus) at 42°C for 16 h under stringent condition. The filters were washed and exposed to X-ray film with intensifying screen overnight. For dot blot analysis, the genomic DNA was digested with *Eco*RI, denatured with 0.1 M NaOH and neutralized in 1 M ammonium acetate. The neutralized DNA was diluted serialy by two-fold and the diluted DNA was applied in duplicate to nitrocellulose
filters by using a dot blot manifold.

Immunohistochemistry with anti-p185 antibody

The avidin-biotin complex immunoperoxidase assay was performed on 5-μm sections from paraffin embedded tissues. The sections were treated with a polyclonal anti-p185 antibody (1:100 dilution) (Bae *et al.* 1993) and then with second biotinylated anti-rabbit antibody, followed by avidin-biotin complex. The section was counterstained with hematoxylin. The amount of p185 protein was grouped in three stages of expression; strong positive (++), positive(+) and negative. Only strong posivtive tumors were classified as overexpressed.

Results

Amplification of *erb*B-2 oncogene in primary breast tumors

Seventy-two primary human breast tumor tissues were exmained for the amplification of *erb*B-2 oncogene. Hybridization with 1.6 kb middle *Eco*RI frgment of *erb*B-2 cDNA under high stringent condition revealed 6.4 and 4.1 kb bands in all tumor DNAs as expected (Figure 1). Amplification of *erb*B-2 oncogene was observed in 10 of 72 DNA from tumors (13.9%). To estimate the gene copy numbers in these amplified cases, the genomic DNA with higher band densities were analyzed by dot blot hybridization. duplicate dot blots containing tumor and placental DNA were hybridized with *erb*B-2 cDNA

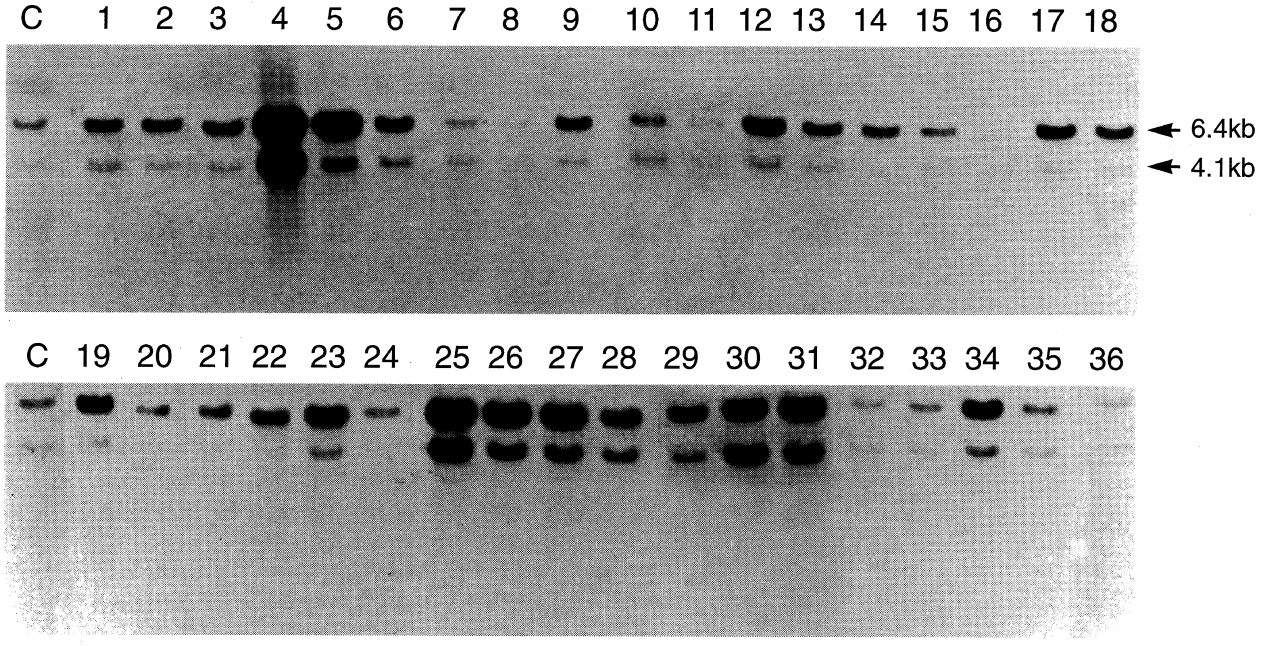

Figure 1. Amplification of *erb*B-2 gene in primary human breast tumors . DNA from primary human breast tumors (29 of 72 are shown) were digested with *Eco*RI and separated in 0.8% agarose gel. The DNA transferred blot were hybridized with 1.6 kb *Eco*RI fragment of *erb*B-2 c-DNA. C (control), human placental DNA.

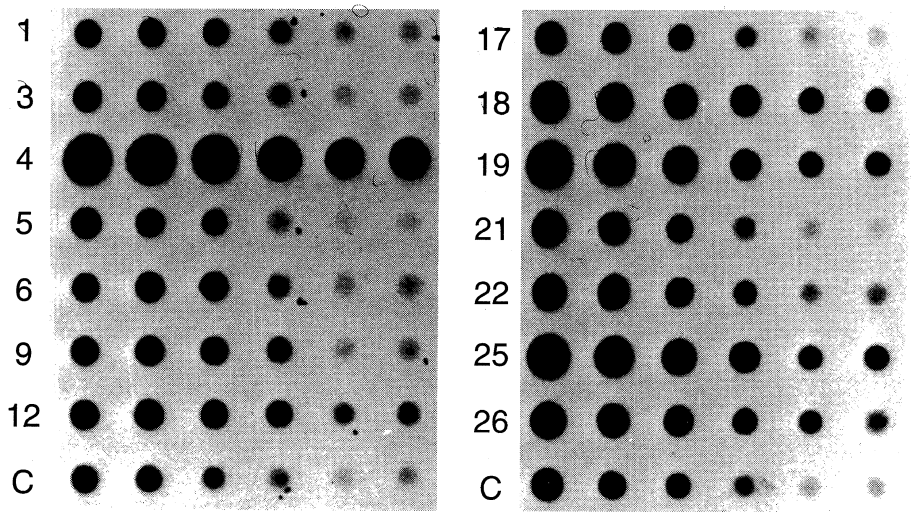

Figure 2. Estimation of number of *erb*B-2 gene copies in primary human breast tumors. To confirm gene amplification, erbB-2 gene copies were subsequently quantitiated by dot blot analysis in comparison to normal diploid human DNA. Replication dot blots containing serial twofold dilution of tumor DNA were hybridized with the *erb*B-2 cDNA. C (control), human placental DNA.

(Figure 2), and β-actin probe was used to estimate mRNA amount (data not shown). Comparison of the hybridization signal of the two probes revealed that the copy number of *erb*B-2 gene in these 10 amplified cases ranged from 2 to 27.

Overexpression of *erb*B-2 mRNA in primary breast tumors

To study whether *erb*B-2 mRNA over-expression in breast tumor is associated with gene amplification, total RNAs prepared from 23 primary breast tumors were subjected to Northern blot analysis with middle *Eco*RI fragment of *erb*B-2 c-DNA as a probe. The filter was subsequently rehybridized with human β-actin c-DNA to control the mRNA amount. Among 23 RNAs, only 4 showed detectable amount of 4.5 kb *erb*B-2 mRNA as shown in Figure 3 (17.4%). In the rest of the tumors, the *erb*B-2 mRNA was hardly detectable.

Expression of p185 protein in primary breast tumor tissues

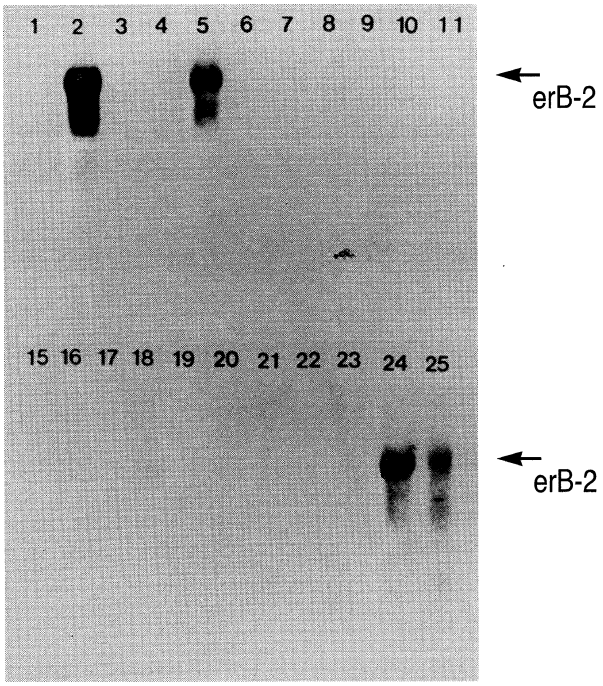

Figure 3. Expression of *erb*B-2 in primary human breast tumors. The expressed mRNA from primary human breast tumors were analyzed by Northern blot analysis. The blots were hybridized with *erb*B-2 c-DNA. All RNA sample were checked for integrity of the 28S and 18S ribosomal RNA.

One hundred fourteen primary breast tumor tissues were analyzed for the expression of p185 *erb*B-2 protein expression. The tissue sections from paraffin blocks were deparaffinized and then immunostained with a polyclonal anti-p185 antibody (Bae *et al.*, 1993). High expression of p185 was observed in the 43 primary breast cancer tissues (37.7%, Figure 4A). Fourty-two (36.8%) primary breast cancer tissues showed no or very little p185 protein and 39 were stained weakly (Figure 4, B and C).

Figure 4. Immunohistochemical staining of erbyB-2 protein in paraffin-embedded sections of primary human breast tumors. Tissue sections of infiltrating ductal carcinoma of breast were first treated with anti-p185 polyclonal antibody and then with mouse anti-rabbit antibody conjugated with avidin. The color was develpoed by by peroxidase reaction with diaminobenzidine, and counterstained with hematoxylin. A, strong positive (400X). B, weak positive (200X). C, negative (200X).

Relavance of p185 protein expression to the amplification and mRNA over-expression of erbB-2

A correlation between *erb*B-2 gene amplification, mRNA expression and protein expression is shown in Table 1. Among the 72 primary breast tumors, we could analyze both DNA and RNA in 19 tumors. *erb*B-2 genes was amplified in 4 cases, and it was overexpressed in 4 of 19 primary tumors. In the rest of the tumors, the mRNA were hardly detectable. Most of the primary tumors with a single copy *erb*B-2 gene had very little amount of gene transcripts, but two of them showed increased expression of mRNA. Among the 4 primary breast tumor tissues with amplified *erb*B-2 genes, two had increased amounts of mRNA, while the other two had negligeble amount of mRNA (Table 1A).

Seven out of 10 tumor tissues (70%) with amplified *erb*B-2 gene were stained strongly by the polyclonal antibody against kinase and C-terminal domain of p185. Therefore the amplification of *erb*B-2 gene is highly correlated with the protein expression. But, twenty out of 54 tumors (37%) with single copy *erb*B-2 gene also showed high expression of p185 (Table1B), which indicated that the amount of p185 in tumor tissuess is not strictly realated with number of the gene copies.

We also examined the amount of p185 protein on the basis of *erb*B-2 mRNA expression. Table 1C shows that 3 of 4 tumors with high mRNA expression contain increased amount of p185 protein, but p185 was not detective in one tumor with increased mRNA. Surprisingly, 4 out of 15 tumors (26%) without overexpressed mRNA were reactive to the antibody. This results suggest that the amount of p185 is not totally dependent on the amount of *erb*B-2 mRNA.

Discussions

The detection of consistent genetic alterations in the DNA of breast tumors might provide insight into the mechanism of carcinogenesis, and possibly suggest the early diagnostic tools or rational strategies for the treatment. These genetic alterations may be the activation of dominant acting oncogenes and/or defect in tumor suppressor genes. Both of the genetic alterations have been suggested to play a role in the development of primary breast cancers.

In this study, we investigated the possible involvement of *erb*B-2 oncogene in breast cancer development by analysis of DNA and mRNA in primary breast tumor tissues, and immunohistochemical ananlysis of p185 proteins in tissue sections from paraffin blocks. We have observed *erb*B-2 gene amplification in 13.9% (10 in 72) of primary breast tumors. This value is lower than other reports ranging from 15 to 30% (Slamon *et al.*, 1987), but is almost same as that reported by a Japanese study (15%) (Uehara *et al.*, 1990). In our preivious study, we also observed that less than 10% of the primary breast tumors had increased number of *erb*B-2 gene (Kim *et al.*, 1991). These results indicated that *erb*B-2 gene amplification in Korean primary breast tumors is not as frequent as in western countries. The difference could reflect the racial differences and different factors might be involved in the carcinogenesis of breast cancer. Four (17.4%) of 23 primary breast tumors contained increased amount of *erb*B-2 mRNA. Although the number of tumors for the analysis of mRNA was not sufficient to get confirming result, it is compatible to get the frequency of gene amplification. Eventhough, the frequency of gene amplification in Korean breast tumors was relatively lower than that of other countries, the positive rate of immunologically stained p185 (37.7%) showed no big differences (Berger *et al.*, 1988)

It has been shown that amplification of *erb*B-2 gene is highly related to the expression of p185. Our present result also supports the previous studies, in which seventy percent (7 of 10) of the tumors with amplified *erb*B-2 gene reacted strongly to the anti-p185 antibody. But our results is lower than the values from other studies. Berger *et al.*(1988) and Ventor *et al.* (1987) reported that over 90% of tumors with gene amplification contained detectable p185 protein, of which 70% reacted strongly to the antibody. Since we classifed weakly reactive cases as negative, if we include the weak positive cases, our result is almost same as those of others.

We have also observed that a considerable amount of p185 protein was detectable in 37% (20 of 54) of primary breast tumors with single copy of *erb*B-2 gene (Table 1B). Berger *et al.* (1988) reported a similar result. Fourty-eight percent of tumors with single copy *erb*B-2 gene contained detectable *erb*B-2 protein. But, Ventor *et al.* (1987) reported even higher positive rate, 79%, of p185 expression in tumors without gene amplification. Our results suggest that the primary breast tumors of Korean women with a single copy of *erb*B-2 gene may have low frequency of p185 expression. But the difference could be due to the differences in the immunohistochemical technique and/or the differences in the antibody used in the studies. However, the results of our and other studies suggest that primary breast tumors without any apparent amplification of *erb*B-2 gene can over-express p185 protein. Similar result was reported from cell line studies. Kraus *et al.* (1987) reported the expression of protein in cell lines without gene amplification, but these cell lines expressed mRNA of *erb*B-2 higher than other cell lines. Therefore, increased expression of mRNA may be one of the mechanism in which p185 is overexpressed without increase of the gene copy number.

Table 1. Corelation of gene amplification, mRNA over-expression and protein expression of *erb*B-2 oncogene in primary human breast tumors.

A. Gene Amplification and mRNA expression[b].

erbB-2 gene copy No.	mRNA over-expression	
	+	-
single	2	13
> 2	2	2

B. Gene amplification and protein expression[c].

erbB-2 gene copy No.	Protein staining	
	+[a]	-
single	20	34
> 2	7	3

C. mRNA expression and protein expression[d].

m-RNA expression	Protein staining	
	+[a]	-
increased	3	1
-	4	11

[a] strong positive.
[b] and [d] For the study of corelation between gene amplification and mRNA expression, and mRNA protein expression, 19 tumor smaples, from which we prepared RNA, were selected.
[c] For the study of corelation between gene amplification and protein expression, 64 tumor samples, from which we prepared DNA, were selected.

But, as shown in Table 1C, p185 protein was detected in 4 of 15 (27%) primary breast tumors without increased expression of *erb*B-2 mRNA. This suggests that the *erb*B-2 protein could be increased without increase of mRNA and this is a rather common mechanism of p185 increase in primary breast cancers. If this is true, there may be three different mechanisms of p185 increase in priamry breast cancers; gene amplification with mRNA over-expression, mRNA over-expression without gene amplification and p185 over-expression without mRNA over-expression. The third mechanism, the increase of p185 without increase of its mRNA, could be explained by either the increase of the translational efficiency and/or the prolongation of the half-life of the p185 protein. These three mechanism could explain the discrepances in the rate of gene amplification (13.9%), mRNA over-expression (17.4%) and p185 expression (37.7%) in primary breast tumors. Kornilova *et al.* (1992) have reported that the changes in protein contents were not due to variations in the *erb*B-2 mRNA level but by post-transcriptional regulation by epithelial growth factor and by the culture density. They suggested that c-*erb*B-2 expression was controlled at various level, both transcriptional and post-transcriptional. But it will need more studies on the gene amplification, mRNA and protein over-expression of *erb*B-2 gene in primary breast tumors to determine the mechanism(s) which can lead to the over-expression of *erb*B-2 protein.

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