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Overexpression of the c-erbB-2 Protein in Human Breast Tumor Cell Lines

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The c-erbB-2 proto-oncogene is amplified in a high percentage of primary human breast tumors, suggesting that the overexpression of this gene may be involved in the development of human breast cancer. We have investigated five human breast tumor cell lines and have detected amplified c-erbB-2 gene copies in two of them. This amplification leads to overexpression of the c-erbB-2 protein. In addition, two other cell lines have elevated protein levels without gene amplification, suggesting that other mechanisms can lead to overexpression of the c-erbB-2 protein. These results are similar to those that we obtained during a study of primary breast tumors (Berger et al.: Cancer Res 48:1238–1243, 1988). These breast tumor cell lines should be useful for an analysis of c-erbB-2 expression and of the mechanisms that in some cases lead to overexpression.

Key words: gene amplification, mammary cancer, tyrosine kinase, proto-oncogene

INTRODUCTION

Altered proto-oncogene expression has been implicated in the development of certain human and animal tumors [1]. Proto-oncogenes can be activated by point mutations or genomic DNA rearrangements, leading to the production of altered protein products. Another mechanism of activation is the overproduction of an apparently normal protein product that in some cases is due to DNA amplification. Various proto-oncogenes have been shown to be amplified in different tumor cells [2], and studies of the N-myc gene were the first to show that there is a relationship between N-myc amplification and survival of neuroblastoma patients [3].

The c-erbB-2 gene was first isolated because of its sequence similarity with v-erbB and human epidermal growth factor (EGF) receptor probes [4–6]. Based upon the deduced amino acid sequence, it has been postulated that c-erbB-2 encodes a growth factor receptor similar to the EGF receptor. There are two mechanisms that

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lead to the oncogenic activation of c-erbB-2. First, a point mutation in the *trans*-membrane domain of the *neu* gene, the rat homologue of c-erbB-2, has been found in rat neuroblastoma DNA [7]. Second, overexpression of the normal human c-erbB-2 protein leads to transformation of NIH/3T3 fibroblasts [8,9]. Recent studies have shown that the c-erbB-2 proto-oncogene is amplified in up to 33% of primary human breast tumors [10–13] and breast tumor cell lines [14]. The detection of this consistent alteration suggests that overexpression of the c-erbB-2 proto-oncogene is involved in the development of human breast cancer. We have shown that there is a good correlation between c-erbB-2 gene amplification and high c-erbB-2 protein expression [13]. In addition, we have observed that some primary breast tumors with single-copy c-erbB-2 DNA sequences stained strongly with the c-erbB-2 specific antiserum [13], suggesting that mechanisms other than gene amplification may lead to elevated protein levels. This phenomenon was more closely studied using human breast tumor cell lines. In this paper we present the results of an analysis of c-erbB-2 DNA, RNA, and protein levels. As seen for the primary tumors, some of the tumor cell lines overexpress the c-erbB-2 protein in the absence of gene amplification.

MATERIALS AND METHODS

Nucleic Acid Isolation and Hybridization Analyses

High molecular weight DNA was isolated from the different cell lines and analyzed for the c-erbB-2 gene copy number as described [13]. Total RNA was isolated by the guanidinium-cesium chloride method [15]. The RNA dot-blot analysis was done by applying twofold dilutions of glyoxal denatured total RNA in 20 × SSC to replicate nitrocellulose filters mounted in a dot-blot apparatus. Filters were hybridized with the c-erbB-2 probe [16] or a control human cDNA probe as described [17]. Hybridization signals were quantitated using an LKB laser densitometer. Northern blot analysis on total SK-BR-3 cell line RNA was performed as described [18]

Immunoblotting

Cells were lysed in 50 mM Tris, pH 7.4, 5 mM EGTA, 1% Triton X-100, 150 mM NaCl, and 3 mM paramethylsulfonyl fluoride, and the supernatant was tested for protein concentration using the method of Bradford. Different amounts of extract were electrophoresed through a 6% polyacrylamide gel and electroblotted onto a nitrocellulose filter. The filter was exposed to the c-erbB-2-specific antiserum 21N [19], and the immunoglobulin reactive band was visualized by ^{125}I-labeled protein A.

Immunohistochemistry

Cells were fixed overnight in 4% formalin, pH 7.0, washed in PBS, suspended in 0.9% agar, and then dehydrated and embedded in paraffin. The cells were stained for c-erbB-2 protein by an immunohistochemical technique on 7-μ paraffin sections using the avidin-biotin complex immunoperoxidase assay [20]. The c-erbB-2-specific antipeptide antisera, 20N and 21N, were used in the assay [13,19]. The dilutions of the antisera and the control staining were as described previously [13]. The scoring is described in the legend to Table I.

RESULTS

In our previous work [13], 51 primary human breast tumor DNAs were analyzed for c-erbB-2 gene copy number. Thirteen (25%) of the tumor DNAs contained

TABLE I. Relative Level of c-erbB-2

			Protein	
Cell line	DNA	RNA	Western	Immunohistochemical
BT20	1	1	4	++/+
HBL-100	1	1	1	+/−
MDA-MB231	1	1	1	+/−
MDA-MB361	6–8	23	20	+++
SKBR3	10	23	20	+++
T47D	1	1	4	++

Quantitation of the DNA, RNA, and Western blots was done using a laser densitometer. The immunohistochemical staining for c-erbB-2 on formalin-fixed, paraffin-embedded cells was scored as follows: strong cytoplasmic with a predominant plasma membrane staining (+++), strong cytoplasmic staining (++), weak cytoplasmic staining (+), and little or no staining (+/−).

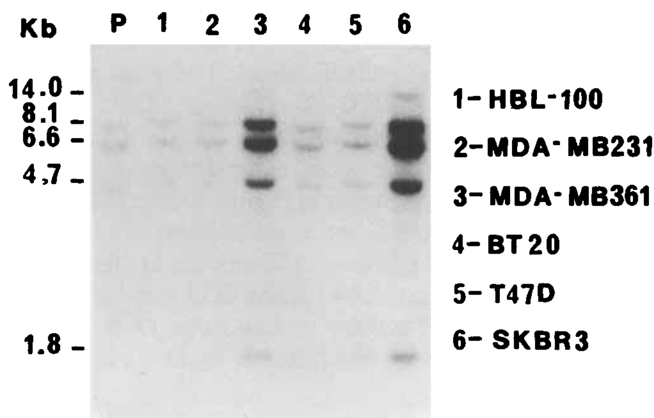

Fig. 1. Analysis of the c-erbB-2 gene in genomic DNA. Five micrograms of DNA isolated from placenta (**P**), a human breast epithelial cell line, HBL-100 (lane **1**), and five different human breast tumor cell lines (lanes **2–6**) were digested with the restriction enzyme EcoR1and analyzed by the Southern blotting technique [21]. The filter was hybridized with a 3.0-kb HindIII-KpnI fragment containing c-erbB-2 cDNA sequences [16]. The sizes of the hybridizing fragments are indicated on the left.

multiple gene copies. Using an immunohistochemical technique, we determined that the majority of these overexpressed the protein, but additional tumors with no apparent gene amplification also stained strongly with the c-erbB-2-specific antiserum [13]. These results suggested that mechanisms other than gene amplification may lead to elevated levels of c-erbB-2 protein. To study this result in more detail, we analyzed five human breast tumor cell lines quantitatively for their c-erbB-2-specific DNA, RNA, and protein content. The cell lines were also stained for c-erbB-2 protein using the same immunohistochemical technique that we have previously employed [13].

The c-erbB-2 gene copy number in six different cell lines was determined by the blotting technique of Southern [21]. The results of the DNA analysis are shown in Figure 1. The SK-BR-3 and MDA-MB361 breast tumor cells have, respectively, ten- and seven-fold c-erbB-2 gene amplification compared with placenta (P), a human breast epithelial cell line, HBL-100, and three other breast tumor cell lines, MDA-MB231, BT-20, and T47D.

To test for c-erbB-2 overexpression at the RNA level, serial two-fold dilutions of total cellular RNA were subjected to a dot- blot analysis. The filter was hybridized with a c-erbB-2 cDNA probe. The specificity of this cDNA probe is shown in the Northern analysis of Figure 2C. RNA isolated from SK-BR-3 cells contains the approximately 5-kb c-erbB-2 transcript that has previously been described [11, 14]. A duplicate control filter was hybridized with a control human cDNA probe that we have recently cloned. The cDNA is specific for a relatively abundant 1.2-kb mRNA that is transcribed to the same extent in all human cell lines that have been tested [17]. The results of the RNA analyses are shown in Figure 2A,B. As expected, the SK-BR-3 and MDA-MB361 cells that have amplified c-erbB-2 gene copies have the highest amounts of c-erbB-2 mRNA. These cells express approximately 20 times more c-erbB-2 mRNA than do the other four cell lines.

The level of c-erbB-2 protein was analyzed in each of the cell lines using the Western protein blotting technique and the 21N antipeptide antiserum. This serum has been well characterized by ourselves and others [12,13,19]. In Western blotting analyses, the 21N antiserum detects a protein of approximately 185-kd in all the human and rodent cell extracts that we have tested. The antiserum rarely reacts with other proteins on the filter (unpublished observations). Different amounts of total cellular protein were separated by electrophoresis, transferred to nitrocellulose, and incubated with the 21N antiserum. The bound antibodies were revealed by incubation with [^{125}I]Protein A. The results are shown in Figure 3. The MDA-MB231 and HBL-100 cells have low levels of the p185, c-erbB-2 protein. A similar low level of c-erbB-2 protein is also present in the human epidermoid carcinoma cell line, A431, a cell line that overexpresses the structurally related EGF receptor protein [22]. The c-erbB-2 protein in 75 and 120 μg of cellular protein from, respectively, the T47D and BT-20 cell lines appears as a strong band that is approximately equivalent to the

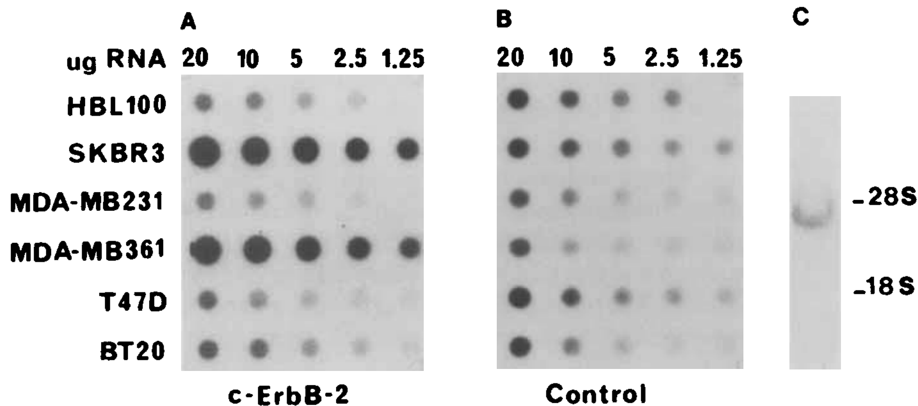

Fig. 2. Quantitation of the c-erbB-2 RNA levels in different cell lines. Twofold dilutions of total cellular RNA from the indicated cell lines were applied to duplicate nitrocellulose filters. One filter (A) was hybridized with a c-erbB-2 cDNA probe [16], while the other (B) was hybridized with a human cDNA that is equally expressed in all human cells that have been tested [17]. This serves to control for the amounts of RNA applied to the filters. Quantitation of the results is shown in Table I. (C) Fifteen micrograms of total RNA isolated from the SK-BR-3 cell line was electrophoresed, blotted, and hybridized with a c-erbB-2 cDNA probe [16].

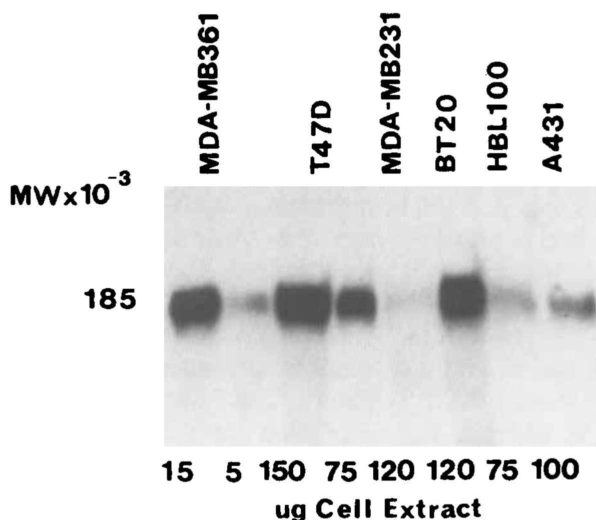

Fig. 3. Detection of c-erbB-2 protein by Western blotting. The indicated amounts of cellular protein from each of the human cell lines were separated on a 6% polyacrylamide gel, transferred to nitrocellulose exposed to the 21N c-erbB-2 specific antiserum [19], and visualized by ^{125}I-labeled protein A. The molecular weight of the c-erbB-2 protein is indicated on the left.

amount in 15 μg of MDA-MB361 cell extract. The latter cells and SK-BR-3 cells have equivalent amounts of c-erbB-2 protein (data not shown).

A quantitation of the results shown in Figures 1–3 are presented in Table I. In addition, the results of an immunohistochemical analysis for c-erbB-2 protein in each of the cell lines is also presented. The cells were formalin-fixed and paraffin-embedded before staining. This approximately mimics the state of the primary breast tumor material that has been stained for c-erbB-2 protein [13]. The results show that the two cell lines with amplified c-erbB-2 gene copies, SK-BR-3 and MDA-MB361, have the highest amounts of c-erbB-2 RNA and protein. They also display the strongest staining with the c-erbB-2 specific antisera. The BT-20 and T47D cell lines also contain elevated levels of c-erbB-2 protein when compared with the HBL-100 and MDA-MB231 cell lines. The former two cell lines do not contain amplified gene copies, and we have not seen an obvious increase in c-erbB-2 RNA when compared to the level in the HBL-100 or MDA-MB231 cells. Slightly elevated levels, for example, two-fold, of c-erbB-2 RNA would be difficult to quantitate. In the immunohistochemical analysis, these cells are also clearly positive for c-erbB-2 staining. The HBL-100 and MDA-MB231 cells contain low levels of c-erbB-2 RNA and protein; they were barely, if at all, stained in the immunohistochemical analysis. In conclusion, the results show that the c-erbB-2 protein level in human breast tumor cell lines can be elevated either in the presence or absence of gene amplification. In addition, the results from the immunohistochemical and the Western analyses for c-erbB-2 protein are in good agreement. Thus, the ABC immunoperoxidase staining technique can be used for the detection of c-erbB-2 protein in primary breast tumors.

DISCUSSION

It has been shown that c-erbB-2 gene amplification [10–14] and protein over-expression [12,13] occur in a high percentage of breast tumors. Our own work has

shown that there is a statistically significant correlation between c-erbB-2 protein expression and parameters, such as lymph node involvement, which are used in breast cancer prognosis [13]. This suggests that the detection of c-erbB-2 protein in breast tumor sections may have value in predicting the course of the disease. The role that the c-erbB-2 protein plays in the development of breast cancer is unclear. Its normal function in the growth and differentiation of breast cells is also unknown. To understand more about this protein it will be important to study the mechanisms that control its expression. The results presented here show that c-erbB-2 RNA and protein are expressed in all the breast tumor cell lines examined. This agrees with other published work in which breast [14] as well as other tissues and cell lines were found to be positive for c-erbB-2 expression [6,19]. The promoter region of the c-erbB-2 gene has been sequenced, and it is different from the promoter of the closely related EGF receptor gene. It possesses a TATA box and a CAAT box as well as two putative SP1 binding sequences. In addition, its transcription appears to initiate at multiple sites [23,24]. Therefore, its expression may be controlled by multiple protein factors of which one or more may be present in most cell types.

The results also show that the SK-BR-3 and MDA-MB361 cells that contain amplified c-erbB-2 gene copies and elevated RNA levels also contain the most c-erbB-2 protein. The other four cell lines examined have similar amounts of c-erbB-2 RNA, yet the T47D and BT-20 cells contain approximately fourfold more c-erbB-2 protein than MDA-MB231 and HBL-100 cells. Some of these cell lines have been examined previously, and it was also found that the HBL-100 and BT-20 cells expressed the same low level of c-erbB-2 RNA [14]. In this previous study, the c-erbB-2 protein level was not determined. One mechanism that could explain the difference in c-erbB-2 protein levels in cells with similar c-erbB-2 mRNA levels is differences in protein stability. It has been observed for a number of peptide growth factor receptors that they are down-regulated by their ligand. Fibroblasts grown in the presence of EGF have only 20% of the EGF receptors present on the untreated control cells [25]. The ligand for c-erbB-2 (*neu*) has not yet been described, but addition of *neu* specific monoclonal antibodies to cells expressing the rat *neu* protein causes p185 *neu* down-regulation. This down-modulation is controlled by changes in protein stability [26]. Cells expressing low levels of c-erbB-2 protein could also be synthesizing its ligand. Such a situation would lead to continuous down-modulation of the c-erbB-2 protein. A comparison of the c-erbB-2 protein stability in the different breast tumor cell lines could help clarify this point.

This study shows that high levels of c-erbB-2 protein are present in four out of five of the breast tumor cell lines that have been studied. The elevated protein level is seen either in the presence or absence of gene amplification. The phenomenon of protein overexpression without gene amplification has also been reported for the EGF receptor [2]. One can speculate that those transformed cells that express high levels of growth factor receptors have a proliferative advantage in a tumor. The deregulation of c-erbB-2 expression at different levels of control of gene expression might indicate different mechanisms of transformation. Whereas gene amplification might be restricted to the c-erbB-2 locus, transcriptional deregulation or posttranscriptional effects are most likely mediated by *trans*-acting factors. Insights into the components involved in c-erbB-2 deregulation might provide targets for directed intervention with the growth of tumor cells.

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