MOLECULAR AND CELLULAR BIOLOGY, May 1987, p. 2019–2023 Vol. 7, No. 5
0270-7306/87/052019-05$02.00/0
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Amplification of the *neu* (c-*erbB*-2) Oncogene in Human Mammary Tumors Is Relatively Frequent and Is Often Accompanied by Amplification of the Linked c-*erbA* Oncogene

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Received 5 November 1986/Accepted 17 February 1987

We investigated alterations in the structure and expression of oncogenes in human mammary tumors and mammary tumor-derived cell lines. In 16 of 95 samples, we detected amplification of the human *neu* oncogene, also known as c-*erbB*-2, accompanied by overexpression in the tumors from which intact RNA could be isolated. In 10 of these DNAs, the linked oncogene c-*erbA* was also amplified, whereas another gene on human chromosome 17, p53, was present in normal copy numbers. Overexpression of c-*erbA* could not be detected in the tumors analyzed. The relatively high frequency of *neu* amplification points to a functional role in human breast cancer. Coamplification of the c-*erbA* oncogene could contribute to this disease as well but is most likely fortuitous.

The evidence that alterations in the structure and expression of known oncogenes contribute to the genesis of human tumors is often based on the observation that these genes are rearranged or amplified. For some human tumors, there is a high correlation between oncogene rearrangement and the cellular origin and even the prognosis of the particular type of tumor (24, 29, 39, 41–43). In other tumors, known oncogenes are less frequently implicated. Breast tumors, one of the most common types of human cancer, form a heterogeneous group, as different genes have been found to be amplified or rearranged (9, 10, 19, 20). In a recent study, 43 of 121 tumors contained an altered c-*myc* gene which was amplified 2- to 15-fold or otherwise rearranged (8).

We undertook a systematic study of oncogene alterations in a large group of human breast cancer samples and found occasional cases of c-*myc*, c-Ki-*ras*, and c-*erbB*-1 amplifications (data not shown). In this study, we report on the most frequent alteration we detected, i.e., coamplification of two linked oncogenes on chromosome 17.

A group of 95 histologically proven malignant mammary tumors from 95 patients and 10 established mammary tumor-derived cell lines were screened by extraction of DNA and restriction enzyme digestion, followed by blot hybridization with several probes. The cell lines used in this study were BT-20, MDA-157, SKBR-3, HST-578, MCF-7, PMC42, CAMA-1, HBL-100, and T47D (all described by others) and MPL-13 (isolated in the Netherlands Cancer Institute). Primary human breast tumors are often very heterogeneous and may consist of relatively few tumor cells and a large amount of connective tissue. Therefore, we took tumor material from the areas expected to be most rich in tumor cells and removed nontumorous tissue from the surgical specimen as much as possible. After being frozen in liquid nitrogen, the tumors were kept at −70°C; DNA was isolated by standard procedures after homogenization of the tumor (21). DNA was digested with *Eco*RI or *Bam*HI, and DNA content was measured again. Samples, all containing equal amounts of DNA, were run on an 0.8% agarose gel and transferred to nitrocellulose filters. Filters were hybridized to several ^{32}P-labeled oncogene probes.

In 15 of the tumors and the SKBR-3 cell line, we detected relatively intense hybridization of a probe derived from the rat *neu* gene (26) to two *Eco*RI fragments of approximately 9.5 and 2.0 kilobases (kb). The human homolog of *neu* (40) has been independently cloned from tumors in which the gene is amplified and is also known as c-*erbB*-2, MAC117, or HER2 (5, 18, 30). It is homologous but not identical to c-*erbB*-1, the gene encoding the epidermal growth factor receptor (4, 26, 40). The signal detected with the *neu* probe was not due to cross hybridization with c-*erbB*-1, since a probe specific for the latter reacted with fragments of a different size, which were not amplified in the samples studied. Figure 1 shows the data from 11 of the tumors, one of which was negative for amplification (lane 12), and from the SKBR-3 cells; the control lane contains DNA from normal lymphocytes of a healthy subject. Hybridization was performed with a 420-base-pair *Bam*HI fragment of the *neu* cDNA (3). One sample contained an additional fragment (6.6 kb) hybridizing with the *neu* probe, which could have been due to a polymorphism. Normal DNA from this patient was not available for analysis.

As a control on the amounts of DNA loaded on the gel, the blot was hybridized to two probes derived from the human thyroglobulin gene, situated on chromosome 8 (2). These probes, 8- and 2.7-kb *Eco*RI fragments of the human thyroglobulin gene, detected fragments of approximately the same size as the *neu* probe did, which are present in normal copy number. To discriminate between amplification of the *neu* gene and the possible presence of multiple copies of chromosome 17, we annealed the filter to a probe for c-*erbA* (17) which has been mapped to region q21 of chromosome 17 (6, 32), the same band where *neu* is situated (5, 12, 26). The probe to detect c-*erbA* used in this study was an 8.5-kb *Eco*RI fragment of the cloned genomic c-*erbA*-1 gene designated HAT2 (22). Cell line SKBR-3 and 6 of the 10 tumors with an amplification of *neu* had amplified c-*erbA* fragments as well, whereas others were normal or had a slightly less intense hybridization. We then hybridized the samples to a

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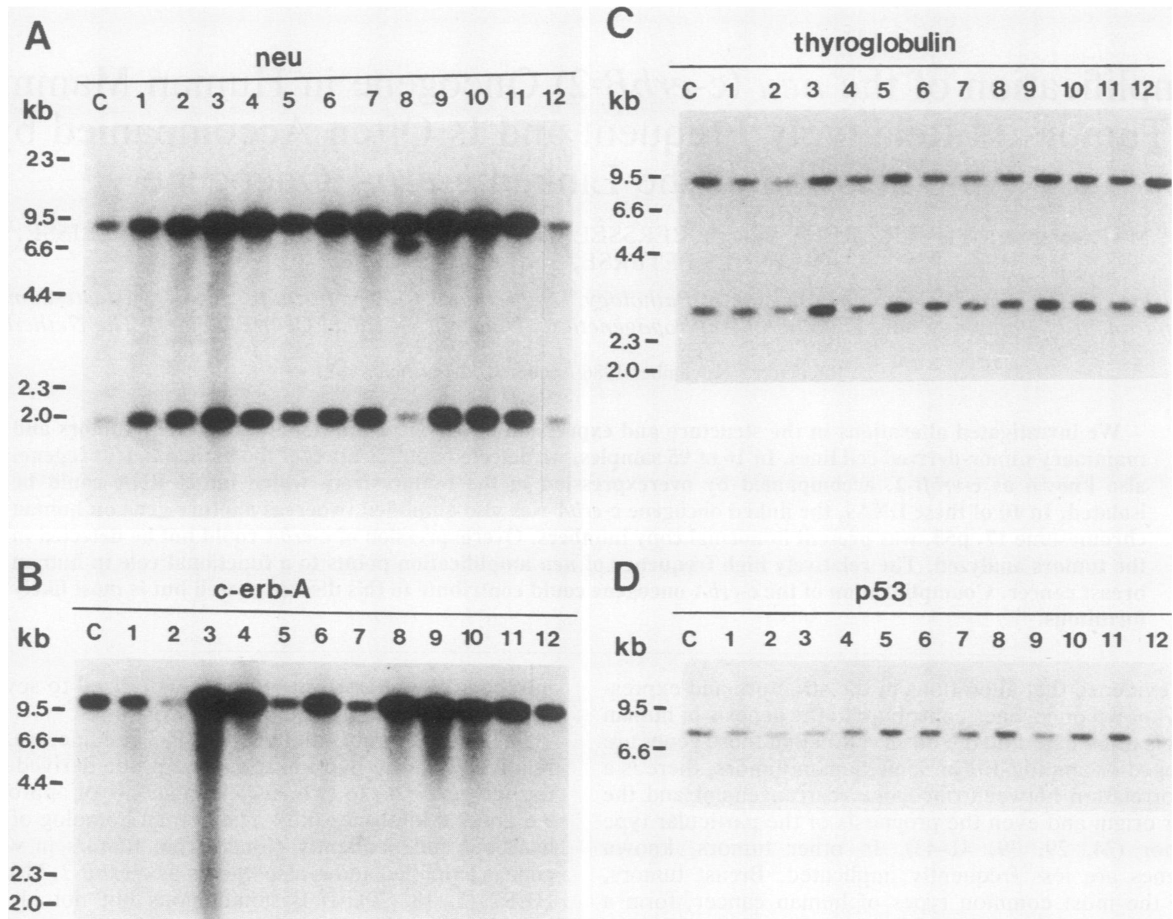

FIG. 1. Southern blot analysis of DNA from 11 mammary tumors and mammary tumor cell line SKBR-3. (A, B, and C) The same filter, containing *Eco*RI-digested DNA, was hybridized with probes for *neu*, c-*erbA*, and thyroglobulin, respectively. (D) The DNA was digested with *Bam*HI and hybridized with p53. Lanes: C, control DNA from lymphocytes of a healthy individual; 1 to 10 and 12, mammary tumors; 11, cell line SKBR-3. Molecular weight markers are derived from electrophoresis of lambda DNA digested by *Hin*dIII.

1.8-kb mouse-derived cDNA probe for p53 (pM8 [38]), a gene located on the short arm of human chromosome 17 (16, 23). The *Eco*RI digest of the tumor DNAs did not reveal high copy numbers of p53, but since the probe recognized an *Eco*RI fragment of a different size than the *neu* probe did, we repeated this analysis on a *Bam*HI digest, which produced a 7-kb p53 fragment, present in equal amounts in all samples. This filter was also hybridized to the thyroglobulin probe, showing that equal amounts of DNA were loaded on the gels (data not shown). Thus, in cell line SKBR-3 and in nine tumors, including three tumors not shown in Fig. 1, *neu* amplification was accompanied by higher copy numbers of the c-*erbA* gene, and this was not owing to the presence of multiple copies of the whole chromosome. A total of six tumors contained amplification of the *neu* gene only, whereas none of the tumors contained elevated copy numbers of c-*erbA* in the absence of amplified *neu*. We estimated the degree of amplification by serial dilutions of the DNA samples and by comparing the intensity of the signal on blots exposed for different times. The degree of amplification thus measured ranged from 2 to 5.

We analyzed for the presence of oncogene transcripts in the cell lines (Fig. 2A) and in the tumors (Fig. 2B), but only a few tumors yielded enough material for analysis. RNA was isolated by homogenizing cells or tumors in 6 M urea–3 M $LiCl_2$ as previously described (1). The cell line RNAs were enriched for poly(A)$^+$ RNA by oligo(dT) chromatography. Samples were run on 1% formaldehyde denaturing gels and transferred to nitrocellulose filters. These filters were hybridized to the ^{32}P-labeled *neu* and c-*erbA* probes used for hybridization in the Southern blots shown in Fig. 1. The *neu* probe detected high levels of a 4.8-kb mRNA in the SKBR-3 cell line and in three tumors in which *neu* amplification was present; the transcript was present in lower quantities in nine cell lines and in eight tumors in which no *neu* amplification was found, providing a good control for the level of *neu* transcription in cells with a normal copy number of the gene. On the same RNA blots, the c-*erbA* probe did not detect an mRNA in any of the cell line or tumor RNAs. Hybridization with a probe for actin showed that the RNAs in all samples were of similar quality. Apparently, amplification of *neu* correlated with overexpression, but c-*erbA* was not detectably expressed, regardless of amplification of the gene.

Correlations between *neu* amplification and a number of clinicopathological parameters were determined (Table 1). *neu* amplification was independent of histological classification (most tumors were invasive intraductal carcinomas not otherwise specified) and grade of malignancy. As can be seen in Table 1, two of five tumors derived from patients with recurrent disease contained amplification of the *neu*

locus; these two tumors also contained a c-*erbA* amplification. *neu* amplification was independent of estrogen receptor content, age, or clinical stage of disease. Clinical staging was done after pathology according to International Union Against Cancer rules. Estrogen receptor content was assayed by standard procedures used in the hospitals participating in this study. Data listed as unknown in the table could not be retrieved or are not applicable to some of the patients. As most of the patients in this study were treated during the past 2 years, correlation with prognosis will have to be examined later.

The implications of these data are twofold. First, a relatively high percentage of human breast cancers contain an amplified *neu* gene. This gene has been shown to be amplified in other human tumors of glandular origin (12, 18, 30, 42), including three mammary tumors. In addition, we have shown here that the gene was expressed at high levels in tumors and in a cell line containing amplification of the *neu* gene. In quite another type of neoplasia, rat neuroblastomas induced by chemical carcinogens, *neu* is activated by virtue of a point mutation, leading to a gene product that is able to transform NIH/3T3 cells (3). Overexpression of the wild-type rat *neu* gene does not lead to morphological transformation of fibroblastic cells (15). It is not clear whether the amplified *neu* gene in the mammary tumors studied has undergone mutations, but the repeated finding of amplifications of this gene in glandular tumors strongly suggests that some step in tumorigenesis or progression is caused by overexpression of this gene. The finding that a mammary tumor-derived cell line has amplified *neu* may allow us to test this notion, perhaps by generating antisera specific for the extracellular domain of this growth-factor-receptor-like molecule and by examining the effects on growth in vitro.

TABLE 1. Correlations between *neu* amplification and relevant clinical data

Characteristic	No. of tumor DNAs (%) with *neu* amplification (n = 15)	No. in total population (%) (n = 95)
Stage of disease		
I	1 (7)	6 (6)
II	9 (60)	56 (59)
III	2 (13)	19 (20)
IV	0 (0)	3 (3)
Recurrent	2 (13)	5 (5)
Unknown	1 (7)	6 (6)
Age (yr)		
<50	5 (33)	32 (34)
>50	10 (67)	63 (66)
Estrogen receptor		
Positive	7 (47)	46 (48)
Negative	5 (33)	18 (19)
Unknown	3 (20)	31 (33)
Lymph node involvement		
Positive	6 (40)	41 (43)
Negative	6 (40)	44 (46)
Unknown	3 (20)	10 (11)

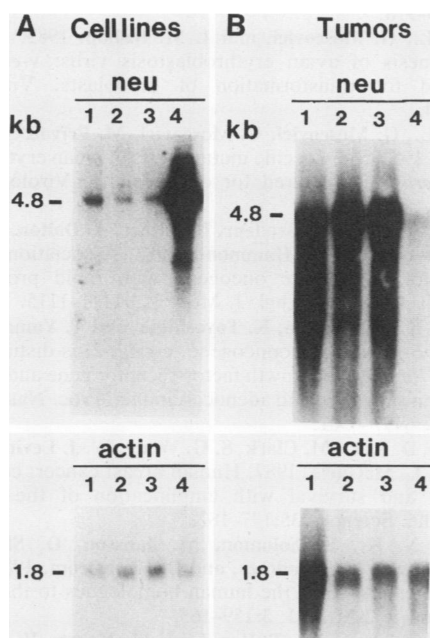

FIG. 2. RNA blot analysis. (A) Poly(A)$^+$ RNA from cells lines. Lanes: 1, T47D; 2, MDA-157; 3, HST-578; 4, SKBR-3. (B) Total RNA from tumors. Lanes: 1, 2, and 3, RNA from the same tumors as shown in lanes 1, 2, and 3 of Fig. 1; 4, RNA from a tumor without *neu* amplification. The upper panels show hybridization with the *neu* probe; the lower panels show control hybridizations of the same filters with a probe for actin. The size of the hybridizing RNA species is derived from the migration of the two large rRNAs as visualized by staining with ethidium bromide.

We also found the amplification of another proto-oncogene in many of the tumors with a *neu* amplification. A naive but not necessarily incorrect interpretation of this finding is that amplification of two oncogenes is indeed important for human breast cancer. The homology of the *erbA* oncogene with steroid hormone receptors (13, 14, 36) and the recent finding that c-*erbA* encodes the receptor for thyroid hormone T3 (25, 37) give this argument even more force; human breast tumors can evolve from a hormone-dependent stage to hormone independency, and overexpression of a hormone receptor gene could theoretically cause such a change. Moreover, a transforming retrovirus, avian erythroblastosis virus, carries modified forms of both c-*erbA* and c-*erbB* (34), the latter gene being highly homologous to *neu*. Mutations in either of these genes lead to loss of some oncogenic properties of this virus (11, 27, 28), showing that both genes cooperate in cell transformation.

We nevertheless consider the amplification of c-*erbA* a fortuitous consequence of the amplification of the closely situated *neu* gene, mainly because amplification of c-*erbA* does not result in detectable overexpression in the tumor and cell line studied. In addition, it is known that selection for amplification of a gene which confers drug resistance leads to coamplification of relatively large domains of surrounding sequences (7, 33, 35). Our observations reinforce the conclusion that amplification of oncogenes cannot directly be taken as evidence for involvement in tumorigenesis but that the actual selection may be for amplification of other nearby genes.

After submission of the manuscript, Slamon et al. reported on the correlation between amplification of the *neu* gene and relapse and survival in breast cancer (31).

We thank P. Hageman, M. van Doornewaard, J. Verrijk, M. Gorsira, and C. van den Berg for providing tumor material and N.

Dijkshoorn for technical assistance. We also thank the following investigators for providing probes: R. Weinberg (*neu*), D. Stehelin (c-*erbA*), V. Rotter (p53), and F. Baas (thyroglobulin). We thank P. Borst and S. Rodenhuis for critical comments on the manuscript.

M.v.d.V., R.v.d.B., and P.D. were supported by the Koningin Wilhelmina Fonds.

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