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Correlation between c-*erb*B-2 Amplification and Risk of Recurrent Disease in Node-negative Breast Cancer[1]

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ABSTRACT

Drawing upon the comprehensive population-based Northern Alberta Breast Cancer Registry containing 704 patients with histologically negative axillary lymph nodes who have been followed for 5–16 years, we have undertaken a retrospective case-control study to evaluate the utility of genomic amplification of specific protooncogenes [c-*erb*B-2 (*nee* HER-2/*neu*), c-*erb*A, c-*myc*, *int*-2, and *hst*-1] as predictive indicators of clinical outcome in node-negative disease. To this end, 115 women with node-negative breast cancer who had recurred at any time up to 16 years posttreatment (cases) were matched pairwise for appropriate clinicopathological variables (size of primary tumor, menopausal state, estrogen receptor status, anniversary year of treatment, and patient age) with a second group of 115 women (controls) selected from a cohort of 502 node-negative patients who had not relapsed during long-term follow-up. Tumor DNA extracted from archival formalin-fixed, paraffin-embedded tissue blocks were analyzed for protooncogene copy number by slot-blot hybridization. Taking a gene copy number of 3 as the cutoff, 27 of the 230 tumor samples examined contained from 3- to 22-fold elevation in c-*erb*B-2 genomic equivalents. Twenty-one of the 27 tumors amplified for c-*erb*B-2 were derived from cases and 6 from controls, signifying that 18% of the node-negative patients who had relapsed harbored excessive copies of the protooncogene in their malignant tissue compared to only 5% for the patients who had remained in remission. Accordingly, the occurrence of amplification of c-*erb*B-2 proved to be a statistically significant predictor of poor prognosis, especially disease-free interval ($P = 0.006$). Moreover, this genetic alteration appeared to be independent of and to have greater predictive power than most commonly used prognostic factors. Our findings also indicated that as a clinical test, measurement of c-*erb*B-2 amplification suffers from low sensitivity; however, when >6 gene copies are present, the test has a positive predictive value for recurrence of 70%. Concurrent analysis of tumor DNA blots with probes for the other four protooncogenes examined revealed that their amplification, which others have reported to arise often, especially in node-positive disease, was seldom found even in our high-risk case group (2–3%). In short, our data strongly suggest that amplification of c-*erb*B-2 may contribute to the pathogenesis of some forms of node-negative breast cancer and thus may serve as a useful genetic marker to identify a subset of high-risk patients.

INTRODUCTION

Structural and functional modifications in protooncogenes, a group of phylogenetically conserved genes of diverse function (1–3), have been consistently observed in a diverse array of human malignancies (4–7). This striking relationship has prompted numerous investigators to explore the possibility that oncogenes may be of sufficient pathogenic significance in tumorigenesis to serve as genetic markers for assessing prognosis. For instance, N-*myc* amplification and elevated expression correlate with disease stage and overall survival in children with neuroblastoma; furthermore, the more copies of N-*myc* present in the tumor cells, the worse is the prognosis, irrespective of disease stage (8, 9). Similarly, patients with myelodysplasia who harbor a mutated c-Ki-*ras* or c-Ha-*ras* allele in the bone marrow are prone to develop leukemia; and c-Ha-*ras* and N-*ras* mutations may play a role in the progression of chronic myelogenous leukemia from the chronic to the acute phase (6, 10, 11).

One extensive inquiry recently undertaken to incriminate oncogenes in the cause and course of human cancer concerns the testing of an association between alterations in specific protooncogenes, namely, c-*erb*B-2 (synonymous with HER-2 or *neu*), c-*erb*A, c-*myc*, *int*-2, and *hst*-1, and poor prognosis for breast cancer (12, 13). In one of the initial studies (14), c-*erb*B-2, which encodes a M_r 185,000 epidermal growth factor receptor-like glycoprotein with tyrosine kinase activity (15, 16), was found to be amplified from 2- to greater than 20-fold in 28% of the primary breast cancer patients surveyed. Moreover, it was demonstrated that the greater the copy number of c-*erb*B-2 in the breast cancer cells, the shorter were both the disease-free interval and overall survival. This genetic change proved to be an independent prognostic factor which appeared to have greater predictive power than most currently used prognosticators including size of primary tumor and steroid receptor status. In later studies (17–39), however, the reported incidence of increased c-*erb*B-2 copy numbers has ranged from 8 to 40%, with a mean of 19% (17–34). In addition, genomic amplification of the proto-oncogene or overexpression of its cognate glycoprotein has been statistically correlated with axillary lymph node involvement (17, 21–23, 30, 34), absence of hormone (estrogen/progesterone) receptors (23, 28, 30, 34, 39), high nuclear grade (22, 30, 36), large tumor size (35), advanced clinical stage (21), and poor prognosis as evidenced by early tumor recurrence and/or shortened patient survival (19, 21, 29, 30, 38, 39), but at the same time the presence of elevated c-*erb*B-2 copy numbers in tumor DNA has been judged by others to have limited, if any, prognostic significance (18, 24–26, 32, 33, 37).

Two groups (18, 30) have observed that c-*erb*A, a member of the thyroid/steroid hormone receptor gene family (40, 41), is often coamplified with c-*erb*B-2 in breast carcinoma, a finding which is not unexpected given that both genes are located on chromosome 17q21–22 (15, 42, 43) and thus may sometimes be included within the same amplification unit. Unlike the

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situation for c-*erb*B-2 (15, 29), the occurrence of multiple genomic equivalents of c-*erb*A is not accompanied by a comparable increase in the levels of the corresponding RNA (18); this implies that amplification of c-*erb*A is a fortuitous event arising from its physical proximity to c-*erb*B-2, the presumed target gene in the amplicon.

As noted for c-*erb*B-2 above, discrepancies in both the incidence of genomic amplification and its association with accepted clinicopathological parameters have also surfaced for c-*myc* and for *int*-2 and *hst*-1, two angiogenesis-inducing, fibroblast growth factor-like genes localized in close proximity on chromosome 11q13 (44–46). The observed frequency of elevated copy numbers of c-*myc* has varied from 4 to 56% (mean, 17%) (17, 19, 23, 26, 30–33, 47, 48) and those for *int*-2 and *hst*-1 have ranged from 4 to 18% (mean, 12%) (30–33, 49–52) and 12 to 18% (mean, 15%) (30, 31, 51, 52), respectively. Where assayed (30, 31, 51, 52), *int*-2 and *hst*-1 have almost always been found coamplified with identical genomic equivalents, indicating their persistent coexistence in the same amplicon. While amplification (and/or overexpression) of either c-*myc* or *int*-2/*hst*-1 has been statistically linked to lymph node positivity (23, 31, 33) and poor prognosis (19, 30), perhaps the most notable relationships disclosed thus far have associated increased c-*myc* copy number with hormone receptor negativity (31), high tumor grade (31), and older patient age (47) and, in contrast, *int*-2/*hst*-1 coamplification with hormone receptor positivity (31), low tumor grade (31), and younger patient age (30, 33), suggesting that alterations in structural integrity and expression at certain loci may serve to delineate different subsets of breast carcinomas. Here again, however, these relationships have not been observed in similar investigations on other tumor panels (17, 32, 48, 51).

The clinical implications of protooncogene alterations as potential laboratory indicators of high-risk breast cancer are far-reaching, particularly in women whose malignancy has not spread to axillary lymph nodes. The prognosis for these node-negative patients is generally favorable, and yet, as shown elsewhere (53–55) and confirmed here, approximately one in five will relapse within 5 years following surgical removal of the primary tumor. In view of the limitations of conventional prognostic factors (12, 13, 53, 54, 56–58), a marker which would contribute to the identification of node-negative patients at high risk of recurrence (despite otherwise favorable predictive factors) would be valuable for selection of patients who would most likely benefit from adjuvant therapy. Accordingly, reports (14, 17–22, 25, 29–33) indicating, *e.g.*, an incidence of c-*erb*B-2 amplification (*i.e.*, mean, 13%; range, 0–27%) in node-negative patients approaching that seen in the aforementioned studies of breast cancer patients as a whole, has attracted considerable interest. The predictive significance of overexpression of this protooncogene in node-negative patients is controversial, however, with one investigative group finding a statistically significant correlation with overall patient survival (38) and three others failing to observe any association whatsoever with breast tumor aggressiveness (29, 30, 39). Clearly, there remains a paucity of data on disease outcome for node-negative patients with amplified c-*erb*B-2 copy number, highlighting the need for studies involving large numbers of patients with long-term clinical follow-up. To this end, we used the well-maintained tumor tissue repositories and extensive demographic and clinicopathological data on patients in northern Alberta in order to test retrospectively the hypothesis that amplification of c-*erb*B-2 (and possibly c-*myc*, *int*-2, and *hst*-1) may be a reliable predictor of poor prognosis for node-negative breast cancer. Women with node-negative disease who had recurred at any time up to 16 years posttreatment (cases) were matched for appropriate prognostic factors with node-negative patients who had been followed for similar periods and had not relapsed (controls), and tumor DNA extracted from their preserved tissue blocks was subjected to slot-blot hybridization analyses to measure protooncogene copy numbers. The significance of the resultant protooncogene profiles as predictors of disease progression and their correlation with traditional prognostic variables constitute the subject of this communication. Interim accounts of our findings have appeared elsewhere (59, 60).

MATERIALS AND METHODS

Patients. The Breast Unit of the Cross Cancer Institute maintains a computerized population-based registry of all women who have been diagnosed with breast cancer in northern Alberta since 1971 (currently totaling ~7800 patients). Detailed demographic, clinicopathological, staging, treatment, and follow-up information can be readily accessed to address specific questions relating to the pathogenesis and natural history of breast cancer. A total of 704 female subjects diagnosed as having invasive carcinoma of the breast with negative axillary lymph nodes were registered during the 12-year period from 1971 to 1982 inclusive. This cohort formed the population base of our study group and, inasmuch as the end point for assessing clinical outcome was December 31, 1987, there was at least 5 years follow-up on all patients. In keeping with the observations of others (53–55), we observed actuarial relapse rates for the 704 node-negative patients of 18 and 27% and deceased rates (from all causes) of 14 and 28% for 5 and 10 years follow-up, respectively. Because of this low failure rate, it was decided that the most practical approach to achieving the primary aim of the present study, *i.e.*, evaluation of the utility of protooncogene amplification as a molecular indicator of poor prognosis for node-negative breast cancer, would be to match pairwise those patients (cases) who had relapsed (*i.e.*, developed locoregional recurrence and/or distant metastasis after initial surgery) with those (controls) who had not as yet recurred during long term follow-up. Absence of axillary node disease was determined by histological examination of lymph nodes resected at the time of primary treatment. Locoregional recurrence was defined as arising in the chest wall or ipsilateral regional nodes and distant metastasis as recurrence elsewhere.

Case-Control Selection. To control for the potential confounding effects of various prognostic factors and other biologic variables, pairwise case-control matches was made by the following criteria, considered hierarchically: size of primary tumor at clinical examination (T category, T_1–T_3; Ref. 61); menopausal status (pre-, peri-, post-, or unknown); ER[5] status (positive, negative, or unknown); anniversary year of treatment (within 3 years; 1971 through 1982, thus giving approximately the same potential follow-up time); and age at treatment (within 5 years). ER concentrations were measured in breast tumor cytosols with the use of specific [^{3}H]estradiol binding in a dextran-coated charcoal assay as described elsewhere (62). ER positivity was defined as ≥8 fmol of specific binding sites per mg cytosol protein.

Of the 704 entries for node-negative patients with an anniversary year from 1971 to 1982, the registry identified 179 cases (*i.e.*, patients who had relapsed), 475 patients who had not had disease recurrence by 1987, and 27 who had died of causes other than breast cancer, the latter 2 groups serving as a pool (502 in total) for selection of controls to pair with cases. The remaining 23 patients were lost to follow-up. Consequently, 97% of the entire node-negative patient population was potentially eligible for inclusion in the study. Initially then, 179 cases were matched with 179 controls. Of the 358 paraffin-embedded tumor blocks sought, only 75 (48 case and 27 control specimens) were unavailable. The 283 blocks received were first coded (*i.e.*, "blinded" as to whether

[5] The abbreviations used are: ER, estrogen receptor; cDNA, complementary DNA.

derived from cases or controls). Upon histopathological review, 33 (16 cases and 17 controls) of the blocks were rejected due to insufficient tumor material and a further 4 (all controls) were excluded because only intraductal carcinoma *in situ* was found. In the remaining 246 samples, adequate amounts of sufficiently intact DNA were extracted from all except 1 control, thereby permitting protooncogene profiling on 245 specimens. After completion of the protooncogene copy number measurements, the tumor code was broken, revealing that 115 cases and 130 controls had been analyzed. After reassigning the most appropriate of the 28 unpaired controls to the 13 unmatched cases, the remaining 15 controls were removed, leaving a final tally of 230 patients in our study. As is evident in Table 1, cases and controls were generally distributed in the same proportion in the different categories within the 5 clinicopathological criteria used for matching. Moreover, 73 (63%) of the 115 pairs were matched perfectly, and in 24 of the remaining 42 pairs, all except 1 (usually patient age largely because of the paucity of patients under 40 years of age available for matching) of the 5 criteria were concordant. These analyses indicated that there was no inherent bias in our case-control pairing. The mean number of nodes examined in both cases and controls was 9, ranging from 4 to 25 and 4 to 24, respectively. For cases and controls whose tumor DNA was found amplified for c-*erb*B-2, the sampling mean was 10 (range, 5–19) and 12 (range, 7–18) lymph nodes, respectively. Likewise, treatment regimens were essentially identical for the 2 groups. No patient received any form of treatment prior to surgery. The vast majority of the patients were treated for their primary tumor by either modified radical mastectomy (80 cases, 76 controls) or total mastectomy and sampling of the lower axillary lymph nodes (33 cases, 36 controls). Postoperative radiation therapy to the axilla, supraclavicular regions, and the chest wall was often given to those who underwent the latter surgical procedure (13 cases, 15 controls) but seldom to those who received the former treatment. Adjuvant hormone therapy (3 cases) or chemotherapy (2 cases, 1 control) was too infrequent to influence disease outcome. Therefore, there was no reason to suspect that either inadequate lymph node sampling (leading to contamination of the study group with node-positive patients) or differing treatment modalities may have resulted in selection bias.

Table 1 *Comparison of conventional disease prognosticators in the northern Alberta population-based cohort of 704 patients with the 115 cases and 115 controls selected for study*

Prognosticator	No. of patients			Survival (%)[a]	
	Cohort	Cases	Controls	5 yr	10 yr
T category					
T_1	306	39 (7)[b]	42 (0)[b]	92	86
T_2	358	68 (13)	68 (6)	89	82
T_3	40	8 (1)	5 (0)	84	68
Menopausal state					
Pre	210	35 (7)	39 (2)	89	80
Peri	36	5 (1)	4 (0)	87	83
Post	419	74 (13)	70 (4)	90	84
NR[c]	39	1 (0)	2 (0)	89	82
Estrogen receptor status					
Positive	151	29 (3)	27 (1)	91	NR
Negative	40	20 (4)	20 (1)	82	NR
NR	513	66 (14)	68 (4)	92	86
Anniversary year					
71	47	12 (3)	9 (1)		
72	42	7 (0)	11 (0)		
73	26	2 (0)	1 (0)		
74	60	11 (1)	13 (0)		
75	54	11 (1)	9 (1)		
76	68	11 (3)	14 (1)		
77	64	17 (5)	10 (2)		
78	63	11 (2)	9 (0)		
79	67	7 (1)	12 (0)		
80	70	11 (1)	12 (0)		
81	70	8 (1)	11 (0)		
82	73	7 (3)	4 (1)		
Age groups (yr)					
20–29	11	4 (0)	1 (0)	58	50
30–39	46	9 (1)	4 (0)	86	72
40–49	182	27 (8)	38 (2)	91	84
50–59	183	30 (4)	29 (4)	88	82
60–69	169	30 (4)	28 (0)	92	84
70–79	92	13 (4)	13 (0)	93	91
≥80	21	2 (0)	2 (0)	90	90

[a] For total cohort, corrected for cause of death.
[b] Number in which tumor DNA was amplified for c-*erb*B-2.
[c] NR, not recorded.

The 230 patients examined have been followed from 5 to 16 years. Median times to relapse and to death for the 115 patients comprising the case group were 36 (range, 2–151) and 56 (range, 10–166) months, respectively, and these times for the 179 patients originally assigned to the case group were 33 (range, 2–151) and 59 (range, 4–203) months, respectively. The similarity in both time to recurrence and time to death between these two groups suggests that no obvious selection bias was inadvertently introduced by our inability to obtain tumor DNA from the 64 members missing from the full cohort.

Pathological Examination of Tumor Blocks. All available formalin-fixed, paraffin-embedded tumor tissue blocks from cases and controls were collected and forwarded to the reference pathologist. Upon receipt, histological slides, uniformly stained with hematoxylin and eosin, were prepared, and the presence of invasive carcinoma was confirmed. The stained slides were reviewed, assigning each a nuclear grade according to the criteria established by Fisher *et al.* (63). The portion of each tumor exhibiting the greatest cellularity, least lymphocytic infiltration, and best preservation (*i.e.*, minimal tissue autolysis or necrosis) was located. This region was identified in the paraffin-embedded tissue, and 3-mm cores of material (usually three, yielding ~50 mg of tissue in total) were removed (with a dermal biopsy punch), coded, and forwarded for protooncogene analysis.

Measurement of Protooncogene Copy Number. DNA was isolated from the tumor core sections following a modification of our protocol for rapid extraction and sensitive alkaline blotting of DNA from freshly frozen human tissue (64). After extraneous paraffin was discarded, the sections were sliced manually into 1–2-mm fragments using a scalpel and forceps. The fragments were suspended in 5 ml of extraction buffer [0.1 M Tris (pH 8.0)-0.2 M NaCl-0.4 M urea-10 mM cyclohexanediamine tetraacetate-1% (w/v) *N*-laurylsarcosine], after which 2.5 mg of proteinase K [Boehringer Mannheim Canada, Dorval, Quebec, Canada] was added, and the reaction mixture was incubated, with continuous gentle shaking, at 55°C for 24 h. A second equivalent concentration of proteinase K was added, and the incubation was continued for a further 24 h. (Note: In those few instances in which sample dissolution was inadequate to allow DNA extraction, a third cycle of proteinase K supplementation and 24 h incubation was performed.) Contaminating RNA was then digested in each dissolved sample by addition of RNase A (final concentration, ~100 μg/ml; rendered DNase-free by boiling for 15 min; Pharmacia, Dorval, Quebec, Canada) followed by incubation at 55°C for 4–8 h. Proteinase K (final concentration, ~500 μg/ml) was once again added to the sample and incubation was continued for 2–4 h. The digested samples were then organically extracted four times at ambient temperature in the following sequence: once with Tris (pH 8.0)-saturated phenol; twice with Tris-buffered phenol:chloroform: isoamyl alcohol (25:24:1); and once with chloroform:isoamyl alcohol (24:1). DNA was: (*a*) precipitated from the aqueous solution by addition of 0.1 volume of 2.5 M sodium acetate (pH 5.3) and 2 volumes of ice-cold ethanol and incubated at −20°C for 12–24 h; (*b*) recovered by centrifugation at 11,000 × *g* for 15 min at 4°C; (*c*) rinsed briefly in 70% ethanol; (*d*) dried under vacuum to eliminate excess ethanol; and finally (*e*) resuspended in 10 mM Tris-HCl (pH 7.6)–1 mM EDTA. Upon estimating the concentration of DNA spectrophotometrically (50 μg/ml = 1.0 A_{260}), samples were stored at 4°C until further use.

Two nonestablished dermal fibroblast strains served as standards for the determination of tumor DNA samples of normal diploid constitution (*i.e.*, those said by convention to contain a single copy of a particular gene): GM 38 (9-year-old healthy female), and GM 43 (32-year-old healthy female). (Both strains were obtained from the NIGMS Human Genetic Cell Repository, Camden, NJ.) The cultivation of these

strains and the extraction of their genomic DNA have both been detailed earlier (64).

A small portion (2–3 μg) of each DNA sample was routinely examined by agarose gel electrophoresis followed by ethidium bromide staining to verify the absence of contaminating RNA and to determine the extent of genomic DNA degradation. To this end, unrestricted DNA was electrophoresed on 1% agarose gels with an appropriate range of molecular weight markers, stained in buffered ethidium bromide, and photographed, essentially as detailed elsewhere (65). In no instance was any difficulty encountered with RNA contamination, indicating in retrospect that the RNase treatment was not noticeably inhibited by the detergent (*N*-laurylsarcosine) present in the buffer.

To determine gene copy number accurately and thus to detect amplification of a particular protooncogene, DNA samples were subjected to conventional slot-blot hybridization analysis. Five μg of each DNA sample were denatured by boiling for 2–3 min, then cooled to ambient temperature, and raised to alkaline pH by addition of an equal volume of 0.8 N NaOH. Three 2-fold serial dilutions were made in 0.4 N NaOH and applied directly to a charge-modified nylon membrane (Biotrace RP membrane; Gelman Sciences, Ann Arbor, MI) using a slot-blot apparatus (Minifold II Slot-Blotter; Scheicher and Schuell, Keene, NH), after which the samples were irreversibly fixed to the membrane by incubation for 24 h in the presence of 0.4 N NaOH and then air-dried. Following prehybridization, each blotted membrane was sequentially hybridized with eight probes: (*a*) 1.4-kilobase *Hind*III-*Pvu*II human c-*erb*B-2 cDNA sequence (14, 15); (*b*) 1.55-kilobase *Pst*I arginase gene cDNA fragment of plasmid pKT218 (66); (*c*) 2.0-kilobase *Bam*HI human p53 antigen gene cDNA segment of plasmid php53B (ATCC 57254); (*d*) 3.9-kilobase *Eco*RI human c-*erb*A DNA segment of plasmid pHE-A1 (ATCC 57334); (*e*) 1.2-kilobase *Eco*RI-*Cla*I human exon 3-specific c-*myc* DNA segment of plasmid pHSR-1 (ATCC 41010); (*f*) 2.4-kilobase *Cla*I human c-*erb*B-1 cDNA fragment of plasmid pE7 (ATCC 57346); (*g*) 1.0-kilobase *Sac*I human *int*-2 genomic sequence (encompassing putative exon 2) of plasmid pSS6 (44, 67); and (*h*) 0.6-kilobase *Sac*I-*Eco*RI human *hst*-1 cDNA fragment (*i.e.*, nucleotides 535–1152 of KS3 cDNA) of plasmid pKS(Sac) (68). The first and second probes were kindly made available by Dr. A. Ullrich (Genentech, Inc., San Francisco, CA) and Dr. S. D. Cederbaum (UCLA), respectively; the next four were obtained from the American Type Culture Collection (Rockville, MD); and the last two were generous gifts of Dr. C. Casey (University of California at Irvine) and Dr. G. Paterno (Memorial University of Newfoundland) through Dr. D. R. Edwards (University of Calgary), respectively. The second and third probes served as internal controls, the former to normalize for loading of an equivalent amount of DNA in each slot and the latter, because of its location on the same chromosome (No. 17) as c-*erb*B-2 (15, 41–43, 69), to discriminate between true gene amplification due to allelic duplication as distinct from chromosomal duplication. Probes were labeled to high specific activity (10^8–10^9 cpm/μg) with [^{32}P]dCTP (~3000 Ci/mmol) using the random hexanucleotide sequence-primed synthesis procedure of Feinberg and Vogelstein (70). Membranes were washed under high stringency (twice for 30 min each at 55°C in 0.1 × standard saline-citrate (3.0M NaCl-0.3M sodium citrate, pH 7.0) containing 0.1% sodium dodecyl sulfate) and autoradiographed using XAR-5 film exposed at −70°C for 12–48 h with Dupont Cronex Lightning Plus screens. The intensity of the hybridization signals was quantitated by soft laser densitometry (model Ultrascan XL; LKB Instruments, Montreal, Quebec, Canada).

Using the arginase gene probe to normalize for amount of slot-blotted DNA, the number of copies of a given protooncogene was estimated for individual tumor specimens by comparing the signal intensity of serially diluted DNA blots of a given specimen with the average of that found (taken as the single copy value) for the two normal fibroblast strains GM 38 and GM 43. In this analysis, a tumor specimen was categorized as amplified for a specific protooncogene if the extracted DNA was found to contain at least three genomic equivalents of that protooncogene. As reported by others (71, 72) and verified here (see "Results"), considerable variation has been regularly observed in the quality and in turn the size of the DNA recovered from archival formalin-fixed tissue. This fluctuation in DNA size, among other variables, can influence the signal intensity of a given probe (71) and, consequently, gene copy values of <3 were regarded as indistinguishable from single copy, a cutoff value also selected by other investigative groups (see, *e.g.*, Refs. 23 and 30).

Statistical Methods. The relative risk for disease progression associated with protooncogene amplification for the matched case-control study was estimated by the odds ratio using the Cox proportional hazards regression model (73). The odds ratio was then adjusted for the effects of nuclear grade. The Cox regression model was also utilized to compare the fraction of tumor DNA samples containing an amplified protooncogene in the case and control groups. The effect on prognosis of protooncogene amplification and other disease parameters was examined by a standard univariate χ^2 test. To determine the effect of magnitude of protooncogene amplification on survival in the case group, survival curves were calculated using the Kaplan-Meier estimate (74), and the resulting curves were compared for significant differences by the log rank test as described by Peto *et al.* (75). The factors used for the initial matching of cases with controls were examined as prognostic factors for the whole group ($n = 704$) by the Cox regression model using univariate and multivariate analyses. These analyses enabled indirect comparison of the predictive power of protooncogene amplification with that of standard disease parameters. All P values reported are two-sided, and values less than 0.05 are considered statistically significant.

RESULTS

Quality of Tumor DNA. Genomic DNA extracted from surgical pathology specimens stored as formalin-fixed, paraffin-embedded tissue typically exhibits varying degrees of degradation and structural modification (*e.g.*, formation of protein-DNA cross-links) depending upon such factors as: (*a*) time between surgical removal of the tissue and its fixation in formaldehyde; (*b*) use of insufficiently fixed tissue; (*c*) extent of tissue necrosis; and (*d*) duration of fixation and storage in the paraffin block (71, 72). Because the molecular weight of DNA, if too small, can affect both background noise and signal intensity in slot-blot hybridization experiments (71), we first monitored each breast tumor DNA sample by gel electrophoresis analysis to verify that extensive degradation had not taken place. As illustrated in Fig. 1, DNA extracted from formalin-preserved breast tissue (*Lanes 1–5*) was invariably of lower molecular weight than intact, chemically unmodified DNA obtained from freshly cultured skin fibroblasts (*Lanes 6* and *7*). Whereas the fibroblast DNA was always well above the largest size marker (23.1 kilobases), the size of the tumor DNA ranged all the way from 0.6 to ~25 kilobases, with the bulk between 2 and 10 kilobases. Appreciable variation in the extent of DNA degradation was observed from one tumor specimen to another (*cf. Lanes 1* and *2*). No concerted effort was made, however, to identify the factors causing this variation, although no obvious relationship was noted between the size of a given DNA sample and either the anniversary year of the donor (Fig. 1) or the pathology department from which the tissue block was obtained.[6] In general, the DNA used here was of considerably higher molecular weight than that reported by others for archival colon tumors (71). We attribute this difference at least in part to our careful selection of well-fixed viable tissue (free of necrosis or autolysis) as the source of DNA, a practice advocated recently by Dubeau *et al.* (72). In short, DNA extracted from the breast tumor specimens, although damaged to varying extents, was nonetheless of sufficient quality for reliable determination of protooncogene copy number.

[6] Unpublished data.

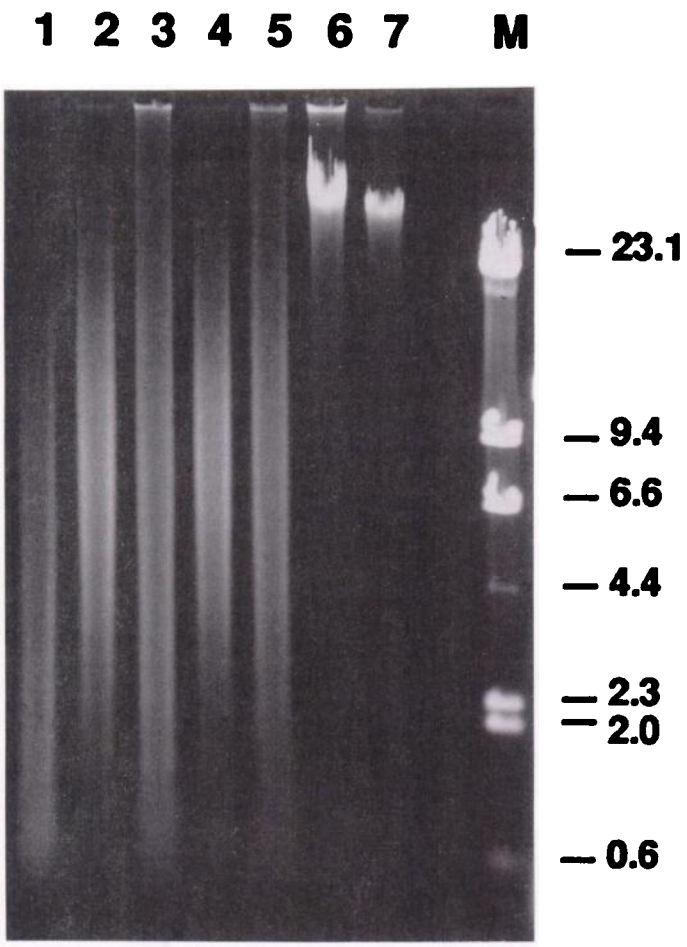

Fig. 1. Ethidium bromide-stained 1% agarose gels containing unrestricted human DNA extracted from archival formalin-fixed, paraffin-embedded breast carcinoma tissue and freshly cultured skin fibroblasts. DNA was isolated, electrophoresed on agarose gels, and stained with ethidium bromide according to an earlier protocol (65). *Lanes 1–5*, representative DNA samples (5 μg each) recovered from breast tumor tissue resected and preserved in 1979, 1972, 1982, 1982, and 1976, respectively. *Lanes 6* and *7*, control DNA samples (2 μg each) extracted from GM 38 and GM 43 fibroblast cultures, respectively. *Lane M* contains 3 μg of standard DNA size markers (7 λ DNA/*Hind*III fragments spanning 23.1 to 0.6 kilobases) (Catalogue No. 5612SA; Bethesda Research Laboratories, Bethesda, MD). *Right ordinate*, size of each fragment marker.

c-*erb*B-2 Gene Amplification. DNA extracted from core sections of 230 tumors was subjected to slot-blot hybridization to appropriate probes followed by autoradiography. Fig. 2 depicts representative autoradiographic results for 14 samples, including two derived from the nontransformed human fibroblast strains GM 38 and GM 43, each containing a full complement of genes present in normal (single) copy number. Based on the relative intensities of the probe for the human arginase gene, it is apparent that comparable amounts of DNA were slot-blotted for each sample. In addition, it can be seen that the signal for the p53 gene is of normal intensity in each tumor DNA sample, indicating no reduplication of chromosome 17, where c-*erb*B-2 is located (15, 42, 43). In contrast, markedly enhanced hybridization signals are evident with c-*erb*B-2 for four tumor specimens (coded as Nos. 161, 164, 166, and 144 in Fig. 2) and, to a lesser extent, for two others (Nos. 156 and 159). It should also be noted that the probe for c-*erb*A, which has been colocalized with c-*erb*B-2 on chromosome 17 (40, 41), gives a normal signal for all samples. Hence the increased signal for c-*erb*B-2 in 6 of the 12 tumor DNA samples shown in Fig. 2 is a manifestation of true gene amplification and is not due to isochromosomy, a type of genetic alteration sporadically found in certain hematopoietic malignancies (76) and colon cancer (77) in which only a single arm of a chromosome is duplicated. Laser densitometric scanning of representative slot blots presented in Fig. 2 revealed that the degree of c-*erb*B-2 amplification in the six tumor specimens ranged from 6 to 22 times, with individual values as follows: No. 156, 6 copies; No. 159, 10 copies; Nos. 161, 164, and 166, 20 copies; and No. 144, 22 copies. The first 5 tumor specimens were obtained from patients in the case group and the 6th one was derived from a control patient. Similarly, of the 230 tumor DNA samples assayed, 27 showed evidence of c-*erb*B-2 gene amplification at levels of 3-fold or greater (Table 2). In contrast, hybridization analysis with the p53 probe detected a multiple genomic equivalent in only one tumor specimen (see below) and that one was distinct from those amplified for c-*erb*B-2, thereby excluding aneuploidy for chromosome 17 as a possible explanation for the observed increase in c-*erb*B-2 copy number in certain tumors. In a similar vein, only 2 of the 27 tumors amplified for c-*erb*B-2 also contained elevated copies of c-*erb*A (see below). Almost certainly, coamplification of these two protooncogenes reflected the presence of a common amplicon (15, 42, 43) and hence isochromosomy for chromosome 17 is not a viable mechanism for c-*erb*B-2 amplification. Twenty-one of the 27 tumors amplified for c-*erb*B-2 were obtained from the case group and the remaining 6 came from the control group, indicating that 18% of the node-negative patients who relapsed had multiple copies of c-*erb*B-2 in their malignant tissue compared to only 5% for the patients who have remained disease-free for protracted periods. From our data, the odds ratio of disease progression associated with c-*erb*B-2 amplification is 4.0 (95% confidence limits, 1.5–10.9; $P = 0.0073$), suggesting that this genetic alteration may be a reliable predictor of poor prognosis.

Amplification of Other Protooncogenes. Typical results arising from a second round of analysis of the 230 tumor DNA blots with probes for c-*erb*B-1 and c-*myc*, *e.g.*, are presented in Fig. 3. Only 1 of the 12 samples appears to be amplified, *i.e.*, No. 192, when probed with c-*erb*B-1. The presence of multiple copies of this gene was observed in only four tumor specimens; two, including No. 192, were derived from cases and the other two were from control patients (see Table 3). These incidences [~2% (2 of 115) for both cases and controls], which were too low to attempt any meaningful statistical correlations, imply that the occurrence of gene amplification in node-negative breast cancer may be peculiar to c-*erb*B-2 and is not a hallmark of protooncogenes coding for transmembrane tyrosine kinases such as c-*erb*B-1. Likewise, the presence of multiple copies of each of the remaining four protooncogenes examined was also rare, ranging from total absence for c-*erb*A and *hst*-1 in the control group to ~3% for c-*myc* in the case group (Table 3). Although difficult to assess due to the small number, the level of amplification of these protooncogenes tended to be relatively low (*i.e.*, 3–11 copies) with the possible exception of c-*erb*B-1 for which the copy number in the 4 amplified tumors extended from 9 to 23. Together, these data demonstrate that c-*erb*B-2 was the only protooncogene of the 6 examined that was amplified to any significant degree in the node-negative breast cancer population of northern Alberta, an observation which is at variance with those reported for some other groups of patients (see Introduction). In addition, our patient cohort appeared to differ from others with respect to the incidence of coamplification of c-*erb*B-2/c-*erb*A and *int*-2/*hst*-1 loci, with ours being much lower for both presumed coamplicon units, *i.e.*, ~7% (2

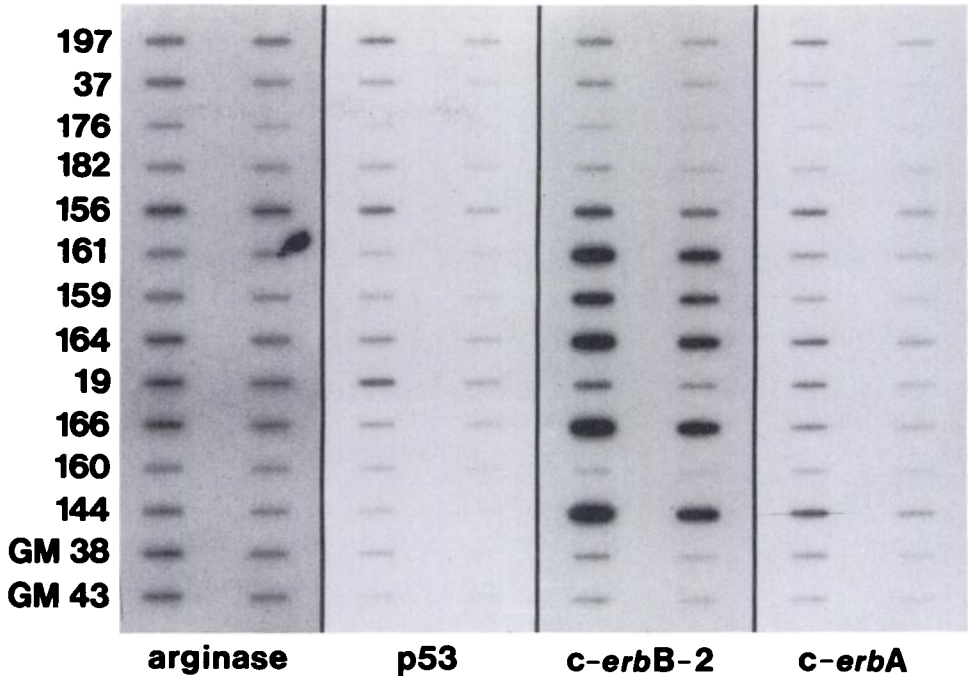

Fig. 2. Slot-blot hybridization autoradiograms of genomic DNA extracted from preserved breast carcinoma specimens and probed to detect amplification of c-*erb*B-2 and c-*erb*A protooncogenes. Copy numbers of the arginase and p53 genes were also measured to normalize for differences in amount of DNA slot-blotted and to monitor for aberrant chromosomal duplication, respectively. *Numbers* to the *left* of the top 12 rows of slot blots correspond to those assigned blindly to paraffin-embedded tumor blocks from node-negative patients under survey, whereas the bottom two, GM 38 and GM 43, denote two normal fibroblast strains derived from healthy volunteers and hence standards for singly gene-copy determinations. DNA samples were applied to the nylon membrane at two concentrations, 1.25 μg (*left columns*) and 0.625 μg (*right columns*), and each autoradiogram was exposed for 36 h. Conditions for DNA extraction and nylon membrane fixation, as well as probe hybridization, autoradiography, and laser densitometric scanning, were as described in "Materials and Methods."

Table 2 *Amplification of* c-erb*B-2 in resected tumors from case and control patients*

No. of gene copies in tumor	No. of patients				Confidence limits			
	Cases	Controls	Total	Odds ratio	Low	High	P^a	P^b
1	94	109	203					
≥3	21	6	27	4.00	1.48	10.9	0.0056	0.0073
>6	12	2	14	5.99	1.30	27.7	0.019	0.021
>12	9	1	10	9.03	1.11	73.7	0.037	0.036

[a] Cox proportional hazards general linear model comparing the number of case and control patients whose tumor DNA contained c-*erb*B-2 amplified at the levels indicated.

[b] Same test as above, adjusted for nuclear grade.

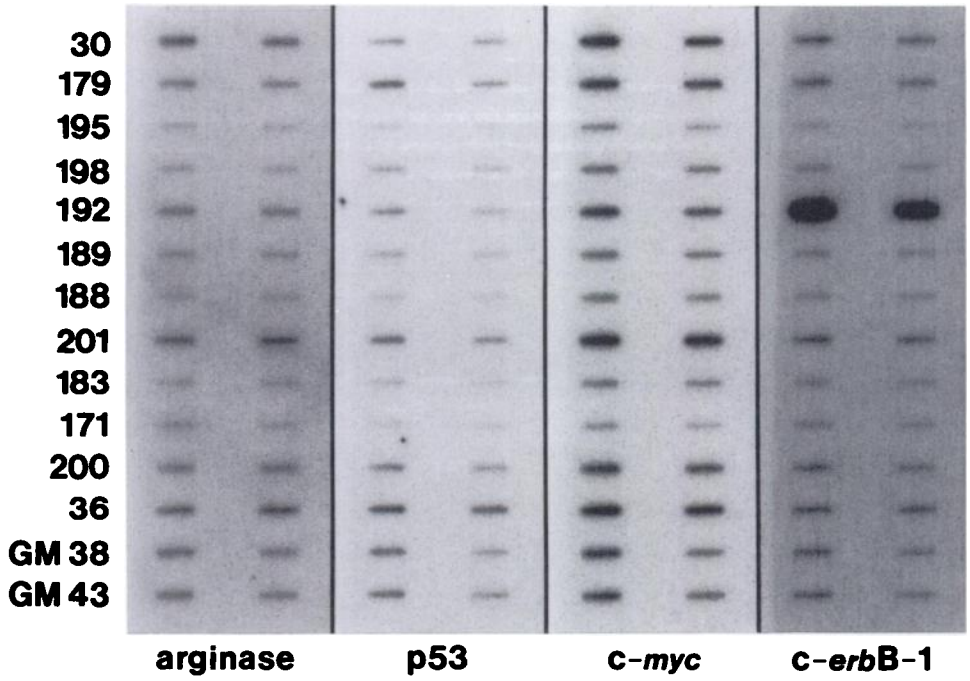

Fig. 3. Slot-blot hybridization autoradiograms of genomic DNA extracted from preserved breast carcinoma specimens and probed to detect amplification of c-*myc* and c-*erb*B-1 protooncogenes. Experimental details were as given in the legend to Fig. 2, except that autoradiograms were exposed for 24 h.

of 27; Tables 2 and 3) *versus* ~60% (40, 41) and 50% (2 of 4; Table 3) *versus* 97% (116 of 118; Refs. 30, 31, 51, and 52), respectively.

Because the p53 gene product is thought to have a tumor suppressor function (78) the inactivation of which may be a frequent event in the genesis of several common malignancies including breast carcinoma (79), it was of interest to observe closely the status of this gene in our tumor panel. In none of the 230 tumor DNA samples examined was there any indication of allelic loss of p53 (*i.e.*, deletion of 1 of the 2 constitutive

Table 3 *Incidence of amplification of the other protooncogenes examined in case and control patients*

	No. of patients							
	Cases				Controls			
	No. of copies in tumor			Total	No. of copies in tumor			Total
Protooncogene	1	3–6	>6	amplified	1	3–6	>6	amplified
c-*erb*A	113	2	0	2 (2)[a]	115	0	0	0 (0)
c-*erb*B1	113	0	2	2 (2)	113	0	2	2 (2)
int-2	113	1	1	2 (2)	113	1	1	2 (2)
hst-1	113	1	1	2 (2)	115	0	0	0 (0)
c-*myc*	111	3	1	4 (3)	114	1	0	1 (1)

[a] Percentage of tumors with multiple genomic equivalents.

copies of the gene), although such genomic alterations could have conceivably arisen and gone undetected due to significant contamination of excised malignant tissue with normal stromal elements and inflammatory cells. As mentioned earlier, one tumor specimen (No. 253 from a case) was found to be highly amplified for p53, containing 22 genomic equivalents. To our knowledge this observation is unprecedented, and efforts are under way to assay the DNA sample for evidence of structural alterations in the p53 gene itself or adjoining sequences.

Coamplification of Cancer Genes Found on Different Chromosomes. Three tumor DNA samples, all derived from cases, simultaneously contained elevated copies of cancer genes located on different chromosomes: No. 161 amplified for c-*erb*B-2 (20-fold) and *int*-2/*hst*-1 (4-fold each); No. 253 for c-*myc* (11-fold) and p53 (22-fold); and No. 321 for c-*erb*B-2 (15-fold) and c-*myc* (3-fold). This incidence of coamplification was too infrequent to make any prediction of disease outcome in the cognate patients.

Association between c-*erb*B-2 Amplification and Disease Outcome. As shown in Table 2, a statistically significant correlation was observed between c-*erb*B-2 amplification and disease recurrence; *i.e.*, ≥3 copies were found in 21 of 115 cases *versus* 6 of 115 controls ($P = 0.0056$). This significant difference between cases and controls was maintained at higher levels of gene amplification (>6 copies, $P = 0.019$; >12 copies, $P = 0.037$). The relationship between c-*erb*B-2 copy number and disease outcome in the case group is depicted graphically in the survival curves presented in Figs. 4 and 5. While little difference was observed in either disease-free interval ($P = 0.21$) or overall survival ($P = 0.46$) when patients without gene amplification ($n = 94$) were compared with those having any amplification ($n = 21$) (Fig. 4), a significant association between magnitude of c-*erb*B-2 amplification and decreased survival interval was revealed when the survival data were stratified for gene copy number (Fig. 5). This tendency for patients with elevated copy numbers of c-*erb*B-2 to have a worse prognosis was more striking for disease recurrence (χ^2 for trend = 4.37; $P = 0.04$) than for death (χ^2 for trend = 2.01; $P = 0.16$). Pairwise comparisons of cases assigned to three subgroups containing, respectively, 1, 3–6, and >6 genomic equivalents of c-*erb*B-2 provided statistical confirmation that the last subgroup of patients had significantly shorter disease-free ($P = 0.007$) and overall ($P = 0.04$) survival than did the first subgroup. On the other hand, neither the progression-free ($P = 0.71$) nor the overall survival ($P = 0.46$) interval for patients with low gene amplification was statistically different than that experienced by those with a normal gene copy. Moreover, within the case group no association was observed between level of c-*erb*B-2 amplification and tumor size, ER activity, age at initial surgery, or menopausal status (Table 4), indicating that the prognostic value of c-*erb*B-2 copy number was independent of various

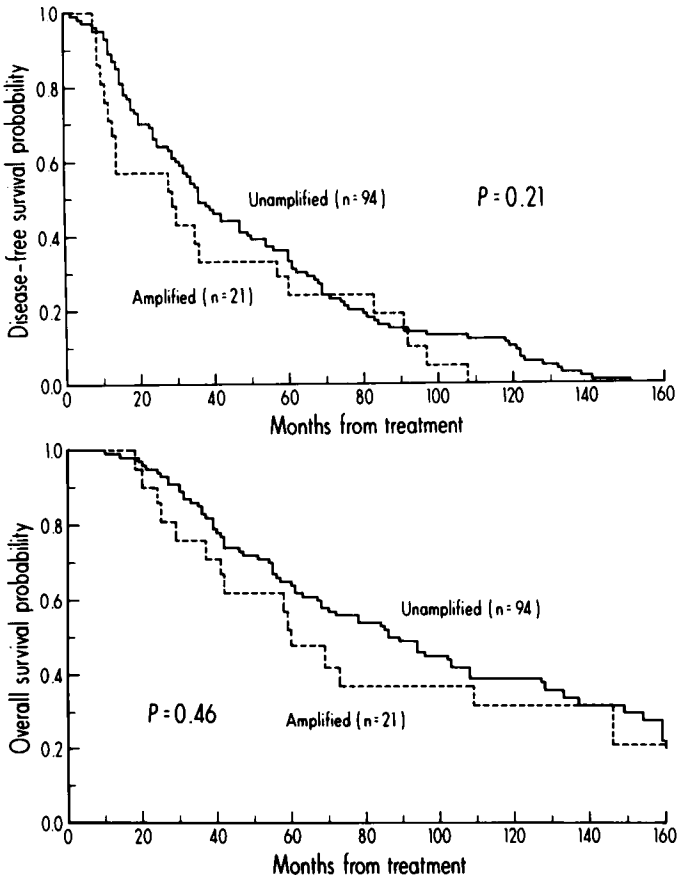

Fig. 4. Kaplan-Meier computed curves for disease-free survival (*top*) and overall survival (*bottom*) in cases (*i.e.*, patients who had relapsed) with no amplification of c-*erb*B-2 in their resected DNA versus cases with any amplification (*i.e.*, ≥3 copies) of the protooncogene.

classical disease markers. Together, these data imply that the presence of c-*erb*B-2 at high copy number in primary tumors is a powerful independent predictor of both shortened remission and reduced life expectancy.

We next studied the relationship between nuclear grade, a recognized histopathological predictor of disease behaviour (53, 57), and risk of relapse in our case-control cohort of 230 patients. The tendency of tumors from the case group to be of higher nuclear grade (*i.e.*, grade 2 *versus* grade 1) than those from control patients is evident in Table 5. However, this difference in nuclear grade between cases and controls proved to be of only borderline significance ($P = 0.03$). Furthermore, when univariate survival analysis was performed to examine nuclear grade in relation to disease-free interval, no significant association was found (regression coefficient, 0.3124 ± 0.2317 (SE); $P = 0.18$). In contrast, a similar analysis for c-*erb*B-2 revealed a highly significant correlation between increased gene

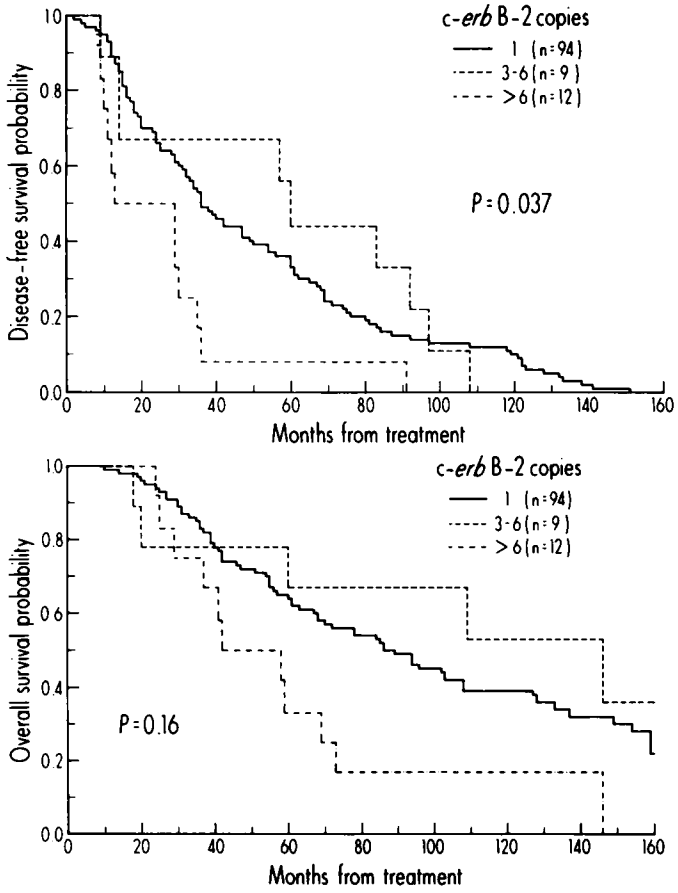

Fig. 5. Relationship between number of c-*erb*B-2 gene copies in tumor DNA and disease-free survival (*top*) and overall survival (*bottom*) for the 115 high-risk patients in the case group.

Table 4 *Relationship between c-*erb*B-2 amplification and several conventional disease parameters in the case group*

Disease parameter[a]	No. of tumors containing			P^b
	Single copy	3–6 copies	>6 copies	
Tumor size				0.39
T_1	32	1	6	
T_2	55	7	6	
T_3	7	1	0	
ER status				0.59
Positive	26	0	3	
Negative	16	0	4	
Unknown	52	9	5	
Age (yr)				0.17
≤50	32	2	7	
>50	62	7	5	
Menopausal status				0.69
Pre-	28	2	5	
Peri-	4	1	0	
Post-	61	6	7	
Unknown	1	0	0	

[a] See "Materials and Methods" for descriptions of the different disease parameters.
[b] Based on the χ^2 test excluding the unknown categories.

copy number and reduced time in remission (regression coefficient, 1.386 ± 0.500; $P = 0.006$). The association of c-*erb*B-2 amplification with a worse prognosis remained strong after adjusting for nuclear grade using the Cox proportional hazard regression model (regression coefficient, 1.569 ± 0.550; $P = 0.004$) (see also Table 2), indicating that this genetic marker is

Table 5 *Nuclear grade of resected tumors from case and control patients*

Grade[a]	No. of patients			P^b
	Cases	Controls	Total	
1	7 (0)[c]	19 (0)	26 (0)	
2	80 (13)	66 (4)	146 (17)	0.03
3	27 (8)	29 (1)	56 (9)	
Unknown	1 (0)	1 (1)	2 (1)	
Total	115 (21)	115 (6)	230 (27)	

[a] Assigned according to the criteria recommended by Fisher *et al.* (63).
[b] Based on the χ^2 test excluding the unknowns.
[c] Number in which tumor DNA was amplified for c-*erb*B-2.

Table 6 *Univariate and multivariate survival analyses comparing disease-free survival to various conventional disease prognosticators in the northern Alberta population-based cohort of 704 node-negative breast cancer patients*

Prognostic factor[a]	Disease-free survival	
	Univariate (P)[b]	Multivariate (P)[b]
Tumor size	0.002 (0.2325 ± 0.0755)	0.002 (0.2363 ± 0.0759)
ER status	0.06	0.04 (−0.1840 ± 0.0891)
Age	0.002 (−0.0190 ± 0.0060)	0.01 (−0.0201 ± 0.0081)
Menopausal status	0.01 (−0.1802 ± 0.0708)	0.77

[a] Consult "Materials and Methods" for descriptions of the various prognostic factors.
[b] Numbers in parentheses, regression coefficient ± SE.

both independent of and far superior to nuclear grade as a prognosticator of disease-free survival in our node-negative breast cancer patient population. This strong correlation between c-*erb*B-2 amplification and disease outcome was also independent of size of the primary tumor and ER activity since these two other well-known prognostic factors were used to match the case and control groups.

While our study design precluded direct determination of the interrelationships of c-*erb*B-2 amplification with conventional disease parameters, it was nonetheless informative to use the Cox regression model to assess the powers of various commonly used clinicopathological factors for predicting disease-free survival in the entire cohort of 704 node-negative patients presenting from 1971 to 1982. As documented in Table 6, tumor size ($P = 0.002$) proved to be the only other independent factor comparable to c-*erb*B-2 for predicting high risk for relapse. Patient age at initial surgery ($P = 0.002$) was also equal to the genetic marker in the univariate analysis. This association was undoubtedly due to exceptionally poor prognosis for patients below age 40 (see Table 1), a conclusion reached by us earlier (80). Interestingly, it would appear that amplification of c-*erb*B-2 is not a determining factor in the low survival rates experienced by these young node-negative patients as only 1 of 13 cases and none of the 4 controls had multiple copies of the protooncogene in tumor DNA (Table 1). The predictive significance of age was reduced considerably on multivariate analysis. In short, these data suggest that c-*erb*B-2 amplification is superior to several of the known disease prognosticators with the exception of tumor size.

Examination of the anatomical sites of disease recurrence in the 21 cases with amplified c-*erb*B-2 revealed that 17 of the patients had developed distant metastatic disease, predominantly in bone and/or liver. Seven of these patients had also experienced locoregional recurrence whereas only three patients relapsed solely at the site of the original tumor. The recurrence site was unknown for one patient. A similar distribution of relapse sites was observed in the remaining 94 cases without c-*erb*B-2 amplification, implying that the presence of multiple genomic equivalents of the protooncogene did not appear to influence the site of recurrence in the cohort studied.

DISCUSSION

To assist in resolving the controversy surrounding the incidence of amplification of c-*erb*B-2, c-*myc*, and *int*-2/*hst*-1 in node-negative breast cancer and the potential utility of these genetic alterations as predictors of clinical outcome, we systemically determined the copy number of each of these protooncogenes in tumor DNA from 115 case-control matched pairs selected from a cohort of ~700 node-negative breast cancer patients with 5–16 years follow-up. The most noteworthy observation emerging from this analysis is that amplification of c-*erb*B-2 is found in a much higher portion (18 *versus* 5%) of the tumors from the patients who had relapsed (cases) than from those who had not (controls) (Table 2). Accordingly, the occurrence of this protooncogene at increased copy number is a statistically significant predictor of both time to recurrence and overall survival in our patient population. Furthermore, amplification of c-*erb*B-2 appears to have greater predictive power than most currently used prognostic factors with the possible exception of tumor size which appears to serve as an independent disease marker of equivalent prognostic value. Last but not least, 12 of the 21 cases with amplified c-*erb*B-2 had 7 or more genomic equivalents and 11 of these 12 patients relapsed within the unusually short span of 36 months (Fig. 5, *top*), implying that the magnitude of gene amplification, and not merely its presence, may affect tumor behavior. Statistical analysis confirmed that the presence of this protooncogene at high copy number may, by itself, influence the natural history of the disease.

Since c-*erb*B-2 encodes a putative growth factor receptor (15, 42), a reasonable interpretation of our results is that amplification of the normal, unaltered gene itself, and the ensuing overexpression of the corresponding 4.6–4.8-kilobase mRNA transcript (15, 81) and the M_r 185,000 transmembrane glycoprotein (15, 16, 42), may be a predisposing determinant, via increased protein kinase activity, in the aggressive behavior of some forms of node-negative breast cancer. There is compelling corroborative evidence in experimental systems indicating that structural or functional changes in the c-*erb*B-2 gene can underlie *in vitro* cell transformation to anchorage independence (82, 83), tumorigenicity in nude mice (83), and accelerated induction of mammary adenocarcinoma in transgenic mice (84). Other investigators have observed, both in biopsied material from patients with various stages of breast cancer (20, 22, 29) and in cultured breast tumor cell lines (85), that tumors with increased genomic equivalents of c-*erb*B-2 invariably contain elevated amounts of the corresponding glycoprotein. This complete concordance between multiple genomic equivalents of the protooncogene and overproduction of the cognate protein is likewise consistent with a possible etiological role for elevated c-*erb*B-2 expression in mammary tumorigenesis. It has also been noted that some tumors with no detectable amplification of the protooncogene still overexpress the gene product (20, 22, 29) presumably due to anomalies in mechanisms controlling gene expression. It is thus possible that a small portion (perhaps 5–10%) of the primary tumors from the case group in particular may contain excessive quantities of the protein in the absence of genomic amplification of the protooncogene, inferring that as many as 25% or so of the node-negative breast cancer patients at high risk of relapse may be identified by assaying immunohistochemically for overproduction of c-*erb*B-2 protein in their excised tumors.

It is instructive to compare the amplification frequencies of the various protooncogenes examined here with those reported elsewhere for other populations of breast cancer patients with histologically negative axillary nodes. In the present investigation, amplification of c-*erb*B-2 was observed in 18% of the tumors resected from the cases and in 5% from the controls (Table 2). Since, in our registry, patients who remained disease free outnumbered those who relapsed by about 3:1, it can be estimated that approximately 8% of all node-negative patients in northern Alberta harbor multiple genomic equivalents of c-*erb*B-2 in their tumor DNA. This amplification frequency is roughly one-half the average value (13%) reported recently by numerous other research teams who have collectively surveyed a patient population ($n = 698$) that is comparable in size to the Albertan cohort used for our case-control matching (14, 17–22, 25, 29–33). Our incidence value of 8% is also one-third that found by the only other groups thus far who have had more than 100 specimens in their tumor panel (mean, 25%, $n = 181$, Ref. 29; mean, 23%, $n = 111$, Ref. 31), suggesting that amplification of c-*erb*B-2 may be associated with tumor development less frequently in our population than in most others. The discrepancy in amplification incidence between the present and published results is more pronounced for the remaining protooncogenes. Adjusting for the ratio of 3:1 control:case in our patient population, the frequencies of increased copy numbers of c-*myc* and *int*-2 are each 2% (Table 3). Mean values of published data for these two protooncogenes are 31% (range, 14–48%, $n = 133$; Refs. 31 and 47) and 12% (range, 11–13%, $n = 300$; Refs. 30, 31, and 51), respectively, indicating that amplification of c-*myc* or *int*-2 is at least 6-fold less common in our tumor panel than in those examined elsewhere. Moreover, our disclosure that c-*erb*A was present at an elevated copy number in only 2 of 27 tumors possessing multiple genomic equivalents of the closely linked c-*erb*B-2 gene is in stark contrast with the observations of 2 other groups. Tsuda *et al.* (30) detected multiple copies of c-*erb*B-2 in 10 of 72 tumors from node-negative patients and c-*erb*A was coamplified with the same copy number in all 10 tumor specimens. This latter finding is consistent with an earlier one of van de Vijver *et al.* (18) who demonstrated that in stage II and III breast carcinomas, amplification of c-*erb*B-2 is often accompanied by increased copies of c-*erb*A (*i.e.*, in 9 of 15 tumors). Similarly, although our numbers are small, the rate at which *int*-2 and *hst*-1 are coamplified may be considerably lower in the Albertan tumor panel than in others, *i.e.*, 50% (Table 3) *versus* 97% (30, 31, 51, 52).

There are a number of plausible explanations for the inconsistent results on protooncogene amplification presented in this and earlier reports. Plausible factors contributing to these discrepancies fall into two major categories, namely, (*a*) methodological pitfalls and (*b*) differences in study design and composition of the patient cohorts examined (12, 24, 25, 27, 29). At the technical level, resected malignant breast tissue typically contains variable amounts of normal stroma and infiltrating lymphocytes, resulting in varying degrees of underestimation of gene amplification in tumor DNA. As noted earlier, however, particular care was taken to minimize the amount of normal cellular elements in the core samples removed from the archival blocks and therefore excessive dilution of tumor DNA cannot account for the relatively low amplification incidences observed in our tumor panel. A second technical difficulty arises from the unavoidable fluctuation in the size distribution of DNA recovered from individual tumor specimens and its associated effect on signal intensity in slot-blot hybridization assays. Be-

cause of this confounding variable, it will be recalled, we chose a gene copy number of 3 as the lower limit for amplification; in contrast, in many of the earlier studies, including several of those recording the highest amplification frequencies (27, 29, 31), a sample was considered amplified if 2 or more gene copies were detected. While it is thus probable that elevated genomic equivalents of protooncogenes went undetected in some tumors analyzed here, the number would have almost certainly been too small to contribute significantly to our low amplification rates. In all likelihood then, the reasons for the discrepancies may be found primarily at the level of study design and cohort composition. In addition to wide variations in such factors as sample size and postsurgical treatment, we have conducted a case-control study in which patients were selected from a population-based registry representing a small geographic region whereas most of the previous workers examined groups of patients for which the referral bases were not as well circumscribed geographically. There is increasing awareness that the interaction of various ill-defined nutritional, environmental, and genetic background factors may contribute significantly to the evolution of breast cancer. The relative contributions of these disparate factors and their relationship with any given genetic alteration, such as c-*erb*B-2 amplification, in tumor progression will require analyses of large tumor panels from different geographical locations and genetic backgrounds.

To date, four reports have appeared specifically addressing the relationship between c-*erb*B-2 amplification or overexpression and clinical outcome in node-negative patients with breast cancer. In the first report in which 72 patients were followed for 10 years, neither disease-free nor overall survival was different in patients with increased gene copy numbers compared to those with a normal number (30). In the second study which examined 181 patients with a median follow-up of 59 months, no association was evident between gene amplification and disease behavior (29). However, in the third investigation involving 44 patients followed for the same period, overproduction of c-*erb*B-2 protein was shown to be predictive of overall survival (albeit of borderline significance with $P < 0.05$) but not of disease-free survival (38). However, this positive correlation between c-*erb*B-2 overexpression and poor prognosis was not observed in the fourth report containing 378 node-negative patients with 6 years of follow-up (39). Since node-negative breast cancer is widely recognized as a chronic disease characterized by an unusually extended remission period (*i.e.*, a progression rate after 5 and 10 years follow-up of only about 20 and 30%, respectively), there may not have been a sufficient number of relapses or deaths in the relatively small numbers of patients examined in the first three studies, at least, to disclose the full magnitude of the association between the genetic alteration and the ultimate outcome of the disease. Data presented here, using the equivalent of 700 unselected patients followed for 5–16 years, are consistent with this interpretation. Alternatively, c-*erb*B-2 overexpression may predict disease outcome in some, but not all tumor panels, depending upon the interplay of various ecogenetic factors. This may account for the different results presented here in comparison to the fourth report as the latter study was almost as comprehensive as the present one.

Approximately 100,000 women in North America alone develop axillary node-negative breast cancer each year, and it is therefore germane to ask how the present findings may contribute to improved management of their disease. The question as to whether node-negative patients as a whole would benefit from postoperative adjuvant therapy remains unresolved despite three decades of clinical trials including four completed recently (86–89). In each of these recent trials, systemic treatment (combination chemotherapy or endocrine therapy) was found to produce a significant improvement in disease-free survival but no detectable gain in overall survival after follow-up times of 3 to 4 years. While the observed effects of adjuvant therapy on the disease-free interval appear to be significant and almost certainly indicate that an increase in overall survival will be observed with further follow-up (90), it can be argued that the risk of chemotherapy does not warrant its routine administration (55). An alternative approach would be to identify a subset of node-negative patients most likely to profit from systemic treatment and to selectively enroll them in clinical trials (55). Current prognostic factors that might be used to select these high-risk patients include size of primary tumor (54, 56), steroid receptor status (91), nuclear grade (57), DNA content (92), and thymidine-labeling index (93, 94). To explore the desirability of adding c-*erb*B-2 to the list of potential prognostic factors for bedside application, it is necessary to extrapolate the c-*erb*B-2 amplification data in our case-control study to the original node-negative cohort, discounting the 23 patients who were lost to follow-up. In the resulting group of 681 women (179 cases and 502 controls), the breakdown for the number of patients whose tumor DNA contained 1, 3–6, and >6 copies of c-*erb*B-2 would be as follows: 94, 14, and 19 for the cases, and 476, 18, and 8 for the controls. If all 681 patients were subjected to the amplification analysis, 59 positive results would be recorded, of which 33 would be true positives (*i.e.*, cases), yielding a sensitivity value for the test of 18% (33 of 179) and a positive predictive value of 56% [33 of (33 + 26)]. Using a more rigid definition of positivity, namely, >6 gene copies, the positive predictive value would increase to 70% [19 of (19 + 8)] but the sensitivity would decrease to 11% (19 of 179), and only 4% [(19 + 8) of 681] of all patients tested would score positive. While it would be advantageous if the amplification test were to have higher positive predictivity, a value of 70% for the more rigid definition of amplification nonetheless constitutes a considerable improvement over the current predictive rate of ~25% in the absence of any test. Hence the major drawback of c-*erb*B-2 amplification measurement as a routine prognostic test is its low sensitivity, as approximately 5 of every 6 patients who are destined to relapse would go undetected by the test. Despite this limitation, evaluation of c-*erb*B-2 copy number (or from a practical viewpoint, production of the cognate protein measured immunohistochemically) may still prove useful as part of the aforementioned multivariate risk assessment of patients for entry into experimental regimens.

In summary, we have demonstrated that the presence of increased gene copies of c-*erb*B-2 in tumor DNA identifies a subset of node-negative patients with breast cancer who are at high risk of disease recurrence and poor overall survival. The subset singled out constitutes only a small portion (~18%) of those patients prone to relapse and amplification of other candidate protooncogenes (*i.e.*, *c-myc*, *int*-2, *hst*-1, c-*erb*A) was rarely associated with node-negative disease, leaving the vast majority of the high-risk patients without any known genetic marker. In view of the well-documented heterogeneous etiology of breast cancer presumably involving a complex interaction of multiple environmental and genetic determinants (12, 13), it seems likely that mammary tumors exploit multiple genetic pathways in their initiation and progression. Consequently, any one gene alone might not be expected to contribute greatly to the natural history of a substantial portion of the tumors, an

interpretation supported by the current data. There may exist, however, a limited number of genetic markers, each capable of detecting a different subset, which collectively might serve to pick out most of the patients with a poor prognosis. Large, well-controlled, and comprehensive tumor panels representing different geographic regions and genetic backgrounds, analogous to the northern Alberta panel used here, may be required to identify and establish clearly the prognostic value of these genetic markers. Studies of this magnitude are warranted as disclosure of such markers and their role in the pathogenesis of human mammary carcinoma promise to be of assistance for the realization of improved management of breast cancer.

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