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PROGNOSTIC FACTORS IN HUMAN PRIMARY BREAST CANCER: COMPARISON OF c-*myc* AND HER2/*neu* AMPLIFICATION

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Summary—Amplification of oncogenes in primary tumours may have prognostic and/or therapeutic significance for patients with breast cancer. We have studied HER2/*neu* and c-*myc* amplification together with steroid receptors in human primary breast tumours and related the outcome with (relapse-free) survival. A strong inverse correlation was found between HER2/*neu* amplification and the presence of oestrogen and progesterone receptors. Actuarial 5-years survival showed that breast cancer patients with c-*myc* amplification in their primary tumours experience a shorter relapse-free survival, especially in node-negative and in receptor-positive tumours, whereas HER2/*neu* amplification may be of prognostic value for overall survival in receptor-negative tumours. Overall, in our hands, c-*myc* amplification appeared to be a more potent prognosticator than HER2/*neu* amplification in human primary breast cancer.

INTRODUCTION

Cancer results from alterations that disrupt the appropriate controls and balances that direct normal cellular growth and development. These changes (resulting in altered gene products or altered gene expression) can occur in two classes of genes that interact with each other: genes that inhibit (tumour suppressor genes) and genes that facilitate cell growth and development [for reviews see 1, 2]. The loss of heterozygosity or heterozygous deletion as detected by restriction fragment length polymorphism of DNA from matched blood and tumour tissue, assists in the identification of these tumour suppressor genes. The same suppressor genes may be inactive in different tumour types. Furthermore, two or more different suppressor genes may be inactivated in the same tumour. Multiple allele losses have so far been noticed exclusively in adult solid tumours (i.e. tumours from patients generally older than 25 years of age). Two suppressor genes cloned to date are localized to chromosome regions commonly altered in human breast cancers (the retinoblastoma gene localized on chromosome 13q14 and the p53 gene on chromosome 17p) [3, 4]. In addition, in breast cancer, chromosomes 1p (ductal breast cancer); 1q; 3p; 11p; 13q (ductal breast cancer, and in premenopausal patients); 17p; 18q and 22q (in lobular breast cancer) are shown to be affected [5–11, for review see 12].

Cellular proto-oncogenes can be activated by many mechanisms including amplification, point mutation or association with an active promoter as a result of gene rearrangement. It is a general observation that amplification of cellular oncogenes is found more frequently in solid tumours and predominantly in cells of more aggressively growing tumours [for a review see 13]. In breast cancer, amplification of three oncogenes (HER2/*neu*, c-*myc* and int-2) has been observed using molecular hybridization techniques. The *neu* gene was identified upon transfection of DNA isolated from rat neuroglioblastoma (induced by exposure *in utero* to ethylnitrourea) into NIH 3T3 cells [14]. The human homologue called HER2 or a c-*erb*B-2 maps to chromosome 17q21 and shares extensive homology with the epidermal growth factor receptor (EGF-R, c-*erb*B-1) [15, 16]. The protein product of the HER2/*neu*/c-*erb*B-2 gene, $p185^{neu}$, encodes for a transmembrane receptor with tyrosine kinase activity [17]. Amplification of the c-*myc* oncogene has also been described for human breast cancer. The c-*myc* oncogene which is homologous to the transforming gene (v-*myc*) of avian

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myelocytomatosis, was originally shown to be involved in Burkitt lymphoma and in undifferentiated B-cell lymphoma. Specific chromosomal translocation results in transcriptional activation [18]. The c-*myc* oncogene belongs to a family of at least three members (c-*myc*, L-*myc* and N-*myc*) and has been implicated in cell proliferation, differentiation and neoplasia. The protein (a nuclear phosphoprotein) is expressed in most cell types at low concentrations and is thought to play a role in the competence phase of the cell cycle (G_0 to G_1 transition). The recently discovered nuclear protein called "Max" specifically associates with c-*myc*, L-*myc* and N-*myc* proteins [19]. The proto-oncogenes int-1 and int-2 were discovered as cellular genes which were activated through insertional mutagenesis by a MMTV provirus in mouse mammary tumours [20]. The int-2 gene shows homology to the fibroblast growth factor gene family.

Data on the incidence of amplification in breast tumours, described in the literature on the oncogene HER2/*neu* range from 8–46%; on c-*myc* from 1–41%; and on int-2 from 9–23% [for reviews see 21–24]. Increased protein levels have been reported in the absence of gene amplification (e.g. for HER2/*neu*/c-*erb* B-2), but in general gene amplification results in overexpression of the gene-product. An exception is the int-2 gene on chromosome 11q13, which is often amplified in an amplicon with hst-1 and bcl-1 [25], while the int-2 protein is not expressed [26]. Amplification and overexpression of c-*myc* and HER2/*neu*, detected in tumour biopsies of patients with breast cancer, have been related to more aggressive tumours and to poor prognosis, the latter issue being, however, under debate. We have therefore studied amplification of HER2/*neu* and c-*myc* genes in human primary breast tumours and in this paper we will discuss amplification data on the HER2/*neu* and c-*myc* genes and their relation with tumour and patient characteristics and prognosis of human breast cancer.

MATERIALS AND METHODS

Tumour tissues

Tumour specimens were drawn from a pool of frozen (liquid nitrogen) specimens, which were originally submitted to our laboratory for steroid receptor analysis. Only tissues from breast cancer cases collected between 1978 and 1988 were used for analysis of relapse-free and overall survival. In addition these tissues and those collected from 1990 onwards were used for incidence of amplification and correlation with receptors.

Receptor assays

Tumour tissue (0.4–0.8 g) was pulverized and homogenized as recommended by the EORTC for processing of breast tumour tissue for cytosolic oestrogen (ER) and progesterone receptor (PgR) determinations [27]. A part of the homogenate was used for DNA analysis. The remainder of the homogenate was centrifuged for 30 min at 100,000 *g* at 4°C, and the supernatant fraction (cytosolic extract) was used for ER and PgR determinations, either with enzyme immunoassays (ER-EIA and PgR-EIA kits, Abbott Laboratories, Abbott Park, IL) or with radioligand binding assays as described previously [28] and as recommended by the EORTC [27].

DNA analysis

DNA was isolated from an aliquot of the total tissue homogenate. Southern blotting of EcoR1 digested DNA was performed by standard techniques [29]. In brief, digested DNA was size fractionated on a 0.6% agarose gel and transferred to a nylon membrane (Hybond N^+, Amersham, Bucks., England) and hybridized overnight at 65°C with randomly primed [30], [^{32}P]dATP labelled probes (sp. act. $1–2 \times 10^9$ cpm/μg DNA), obtained from American Type Culture Collection (Rockville, MD). After washing at high stringency (0.3 × SSC), autoradiography with intensifying screens was performed for 1–2 days at −70°C using Kodak XAR-5 films, and autoradiograms were scanned with a Bio-Rad Video densitometer 620.

RESULTS AND DISCUSSION

HER2/neu Amplification

Amplification or overexpression of the HER2/*neu* gene or product has been described in 8 to 46% (mean 20%, in 7549 tumour tissues, as reviewed by us [24]) of breast tumours samples [for reviews see 24, 31–34].

Tumour characteristics

As shown in Table 1, we and others observed that HER2/*neu* amplification is found more

Table 1. HER2/*neu*, the 5-year actuarial (relapse-free) survival data

	Patients		Relapse-free survival (%)		Overall survival (%)	
Status	No.[a]	%[b]	Normal	Amplified	Normal	Amplified
Nodal						
Negative	95	19	62	67	82	67
Positive	182	26	29	31	50	38
ER/PgR						
Positive[c]	172	38	44	59	65	65
Negative[d]	47	16	34	17	43	19

[a]Number of patients per subgroup, [b]percentage amplified; [c]positive for both the ER and PgR (> 10 fmol/mg protein), and [d]negative for both the ER and PgR

frequently in steroid (oestrogen and progesterone)-receptor-negative tumours [35–40]. In contrast, other authors [41–43] did not observe a negative association between HER2/*neu* amplification and steroid-hormone-receptors. The reported data on HER2/*neu* amplification and tumour size and differentiation grade indicate a lack of association between these parameters, although Borg [38] reported on an association of HER2/*neu* amplification with tumour size and Zhou *et al.* [41] reported on an association of HER2/*neu* amplification with tumour grade. In invasive breast carcinoma, $p185^{neu}$ staining is mainly seen in a subgroup of ductal tumours [44]. Lovekin *et al.* [45] reported a strong association between $p185^{neu}$ status and grade. There appeared to be no relation between EGF-R (the other member of the c-*erb* B family) levels and either HER2/*neu* amplification [35] or overexpression [46]. With respect to overexpression of the HER2/*neu* protein, data suggest that there is an inverse association with steroid-hormone-receptor levels and a tentative relation with tumour size and grade [for review see 32].

Patient characteristics

HER2/*neu* amplification is independent of age. We observed a higher frequency of amplification in pre- and perimenopausal women [47]. HER2/*neu* amplification is more prevalent in tumours from node-positive patients (Table 1). A weak association between HER2/*neu* amplification and lymph node status has been observed in other studies [38, 48–51], but no consensus exists [33, 36, 39, 41, 42, 52–55]. With respect to $p185^{neu}$ expression, a tentative relation with nodal involvement was observed.

Survival

In the initial study by Slamon *et al.* [48], a strong association of HER2/*neu* amplification (high copy numbers) and a shorter relapse-free survival was suggested, especially in node-positive patients. Numerous reports followed on the subject, but there is no agreement regarding HER2/*neu* amplification and length of relapse-free and overall survival. We (Table 1) observed no difference in 5-year relapse-free survival in node-negative and -positive patients with HER2/*neu* amplified tumours when compared to tumours with normal gene copy numbers. There was, however, a difference in 5-year overall survival, although marginal. Patients with tumours negative for ER and PgR and with an amplified HER2/*neu* gene, tend to relapse earlier (Table 1) and also have a higher death rate. Although the mechanism of action of HER2/*neu* in breast cancer is not clear it is interesting that the expression is inhibited by oestrogens *in vitro* [56]. In analogy to the data showed by Slamon [48], studies showed a significant correlation between HER2/*neu* amplification and worse prognosis, almost exclusively in node-positive patients [38, 52–54, 58]. Others failed to find a significant association between HER2/*neu* amplification and clinical outcome [33, 39, 42, 59, 60] or the discriminative power of the HER2/*neu* amplification disappeared in multivariate analysis [38]. With respect to $p185^{neu}$ expression, Van de Vijver *et al.* [61] found an effect of borderline statistical significance in analysis for overall survival but not in analysis for recurrence-free survival. This effect on overall survival disappeared in multivariate analysis and it was concluded that $p185^{neu}$ status was of limited prognostic value. Several studies showed that the prognostic effect of $p185^{neu}$ expression is stronger for survival than for recurrence [for review see 62]. However, others have shown an overall prognostic effect of $p185^{neu}$ status in recurrence-free and overall survival analysis [62 and references therein], which was retained in multivariate analysis. In addition the prognostic effect was equal in node-negative and -positive patients. One of the prerequisites seems to be that the group studied must be large. On the other hand recent reports on amplification showed that HER2/*neu* gene amplification will only be of

Table 2 Amplification of c-*myc* in human breast cancer tumour tissues. a literature survey

Author *et al.*	Ref No	Year	Tumours (No.)	Amplification (%)	Notes
Escot	64	1986	121	32	
Yokota	72	1986	10	20	
Varley	57	1987	41	22	
Cline	49	1987	53	16	
Bonilla	65	1988	48	41	
Guerin	73	1988	50	6	
Lidereau	69	1988	121	32	32% (Primary breast cancer) 74% (Metastasis)
Seshadri	74	1989	73	15	
Tsuda	53	1989	169	4	
Adnane	37	1989	219	9 and 21	Different geographical areas
Garcia	36	1989	125	18	
Tavasoli	51	1989	52	21	
Brouillet	68	1990	140	23	
Meyers	75	1990	100	1	
Saint-Ruf	76	1990	6	33	
Roux-Dousetto	77	1990	173	27	
Roy	71	1991	17	24	
Total[a]			1518		

[a]In this survey, 324 out of 1518 tumours are amplified (21%)

marginal utility as a prognostic factor for prediction of clinical outcome [33]. Our data indicate a shorter survival for patients having a tumour with a HER2/*neu* amplified gene in the ER/PgR-negative subgroup. We conclude that future studies on a large series of tumours, in which both expression and amplification of HER2/*neu* and receptor data are included are needed to reveal the prognostic value of this gene.

c-myc Amplification

Early studies on c-*myc* amplification in breast cancer, showed that amplification of the c-*myc* gene occurred only in one out of five human breast cancer cell lines studied, and the authors concluded that c-*myc* amplification may be found only in a minority of human breast carcinomas [63]. Few reports are available on c-*myc* alteration in human primary breast tumour biopsies. Deregulation, including rearrangement [64, 65] and amplification, of the c-*myc* gene has been observed in 1 to 56% of the breast cancer tissues studied. A literature survey on c-*myc* amplification in 1518 human breast tumours is depicted in Table 2. The mean incidence of amplification is 21%. Amplification of other members of the *myc* family, N-*myc* and L-*myc*, has not been frequently observed in breast cancer DNAs.

Tumour characteristics

As shown in Table 3, we did not observe a relation between ER status and c-*myc* amplification. This is in agreement with other reports [36, 57, 64, 66] and additionally we did not observe a relation between PgR status and c-*myc* amplification (Table 3). This has also been observed by others [36, 64]. However in a large series ($n = 1052$ tumours) recently studied by us we found an inverse relation between PgR status and c-*myc* gene amplification [40] which is in agreement with the observation made by Adnane *et al.* [37] in 219 tumours.

Patient characteristics

One report [57] described a positive relation between c-*myc* amplification and age (>50 years), whereas others did not find such a relation. We observed a higher incidence of amplification in node-positive patients ($P = 0.01$) which was not found by others [36, 49, 51, 64, 66]. Interestingly, in a sub-

Table 3 c-*myc*, the 5-year actuarial (relapse-free) survival data

	Patients		Relapse-free survival (%)		Overall survival (%)	
Status	No[a]	%[b]	Normal	Amplified	Normal	Amplified
Nodal						
Negative	95	12	69	18	80	72
Positive	182	24	36	9	52	33
ER/PgR						
Positive[c]	172	21	56	10	71	42
Negative[d]	47	22	30	20	36	30

[a]Number of patients per subgroup; [b]percentage amplified; [c]positive for both the ER and PgR (>10 fmol/mg protein); and [d]negative for both the ER and PgR.

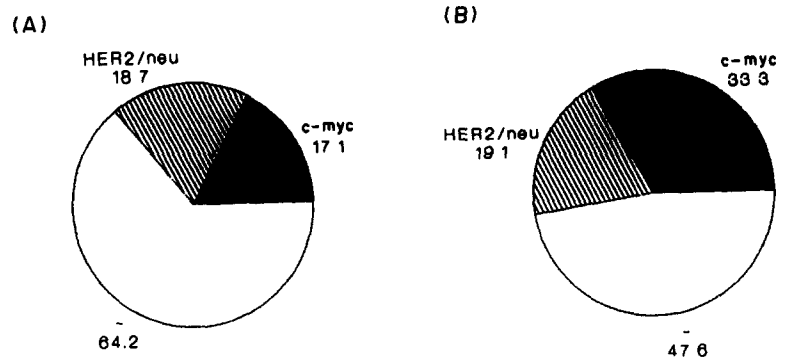

Fig. 1. Amplification of c-*myc* and HER2/*neu* in human primary breast cancer. (A) All tumour tissues and (B) tumour tissues from patients selected for advanced disease.

group of patients selected for development of advanced disease, the incidence of c-*myc* amplification in the primary tumours was increased twice (17 to 33%, see Fig. 1). As yet, the mechanism is unclear but may indicate a relation between c-*myc* amplification and aggressive behaviour of the tumour [67]. In a study by Brouillet *et al.* [68], c-*myc* has been related to cathepsin-D, a marker for metastasis. Tumours having high levels of cathepsin-D (>60 pmol/mg protein) showed a higher incidence of amplification (32 vs 15% amplification, in tumours with low levels of cathepsin-D, <60 pmol/mg protein). In addition Lidereau *et al.* [69] observed c-*myc* amplification more frequently in recurrences.

Survival

In Table 3, we show that c-*myc* amplification predicts an early relapse in patients with both node-positive (27% difference) and -negative (51% difference) tumours and ER/PgR -positive tumours (46% difference). *In vitro*, 17β-oestradiol has been shown to stimulate c-*myc* expression in human breast cancer cells [70]. Moreover, in breast cancer patients treated with the anti-oestrogen tamoxifen, this compound decreased expression of c-*myc* in the ER positive population [17] which may explain differences observed between relapse-free and overall survival (Table 3). The 5-year actuarial overall survival, shown in Table 3, indicates that c-*myc* amplification discriminates in node-positive (19% difference) and steroid-receptor-positive (29% difference) subgroups of patients. Data in the literature on c-*myc* oncogene amplification and prognosis in human primary breast cancer are scarce and include only few patients. c-*myc* amplification or overexpression has been found to be associated with poor short-term prognosis in univariate analysis [53, 57, 77] and by Guerin *et al.* [73] who looked at expression. Cline *et al.* [49] and Locker *et al.* [66], who studied expression of the c-*myc* protein, did not observe a significant association between (relapse-free) survival.

In conclusion, this report shows that: (1) c-*myc* amplification is an indicator of early recurrence and/or death, (a) recurrence especially in node-negative and -positive patients and in patients with steroid-receptor-positive tumours, (b) death in node-positive patients and patients with steroid-receptor-positive tumours; and (2) HER2/*neu* amplification may be of prognostic value for overall survival in patients with receptor-negative tumours.

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