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Determination of c-myc amplification and overexpression in breast cancer patients: evaluation of its prognostic value against c-erbB-2, cathepsin-D and clinicopathological characteristics using univariate and multivariate analysis

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Summary C-myc and c-erbB-2 amplification and/or overexpression as well as total cathepsin-D (CD) concentration have been reported to be associated with poor prognosis in breast cancer. The prognostic significance, however, remains somewhat controversial, partly because of discrepancies among the different methodologies used. We determined the amplification and overexpression of c-myc oncogene in 152 breast cancer patients and examined its prognostic value in relation to c-erbB-2 amplification and overexpression, high concentration of CD ($\geq$ 60 pmol mg^{-1} protein) and standard clinicopathological prognostic factors of the disease. High CD concentration, as well as c-myc amplification and overexpression, proved to be the best of the new variables examined for prediction of early relapse (ER; before 3 years). After multivariate analysis only CD remained significant, which suggests that the prognostic power of these variables is similar. Using univariate analysis we proved that c-myc amplification and overexpression were highly significant for disease-free survival (DFS) ($P = 0.0016$ and $P = 0.0001$ respectively) and overall survival (OS) ($P < 0.0001$ and $P = 0.0095$ respectively), although by multivariate analysis c-myc overexpression was statistically significant only for DFS ($P = 0.0001$) and c-myc amplification only for OS ($P = 0.0006$). With regard to c-erbB-2, only its overexpression appeared to be significant for DFS and OS, although after multivariate analysis its prognostic power was weaker ($P = 0.030$ and $P = 0.024$ respectively). c-myc amplification and overexpression exhibited a tendency for locoregional recurrence (LRR) ($P = 0.0024$ and $P = 0.0075$ respectively), however, their prognostic value was lower after multivariate analysis and only CD remained significant. © 1999 Cancer Research Campaign

Keywords: c-myc; c-erbB-2; cathepsin-D; early relapse; relapse-free survival; overall survival; locoregional recurrence; breast cancer

Despite major advances in therapy, survival of patients with breast cancer has not substantially improved. The search for reliable and sensitive prognostic tests is critical as they may help to identify patients for whom intensive adjuvant therapy is worthwhile. Histology alone is subjective and often not predictive of clinical behaviour. Many different tumour characteristics and cell components have been evaluated for prognostic significance in breast cancer. Regulatory and structural alterations of oncogenes appear to be one of the key events in the formation of most human cancers. Proto-oncogenes are present in all mammalian cells and are involved in normal growth and differentiation. Deregulated activation of the same genes can contribute to cancer development (Alitalo and Schwab, 1986). In particular, the presence of amplified oncogenes has been widely reported in human tumours, and in many cases a correlation of amplification degree with clinical indicators was detected.

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Expression of the c-myc proto-oncogene is involved in the regulation of cellular proliferation and terminal differentiation of human cells. Amplification of *c-myc* seems to associate with poor prognosis in breast cancer (Varley et al, 1987; Berns et al, 1992, 1996; Borg et al, 1992; Rous-Dosseto et al, 1992; Kreipe et al, 1993; Pertschuk et al, 1993; Pietilainen et al, 1995; Nass and Dickson, 1997) and to be a prognostic marker in node-negative patients (Berns et al, 1992; Borg et al, 1992). The *c-myc* oncogene produces a nuclear DNA-binding protein whose expression is regulated by oestrogen and down-regulated by tamoxifen in hormone-responsive human breast cancer in vitro (Santos et al, 1988; Van der Burg et al, 1989). Oestrogens are potent mitogens in a number of target tissues, including mammary glands where they play a pivotal role in the development and progression to mammary carcinoma. Oestrogens regulate the expression and function of c-myc and cyclin D1 and activate cyclin E–CdK2 complexes, all of which are rate-limiting for progression from G1 to S phase (Prall et al, 1998).

Reverse effects have been shown with respect to *c-erbB-2* expression (Dati et al, 1990; Read et al, 1990), i.e. oestrogen

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down-regulated the expression of c-erbB-2 and this effect was reversed by anti-oestrogens. It has been hypothesized that the activation of the two genes might share the same metabolic pathway; in fact, the c-erbB-2 gene has been identified as a cellular target for negative regulation by *c-myc* (Suen and Hung, 1991), whereas c-erbB-2 tyrosine kinase-mediated signals seem to down-regulate the immediate-early genes (Sistonen et al, 1990).

To express its full potential in systemic disease, however, the gene may have to act in concert with other events which also render the cell capable of metastasizing. Tumours with activated c-erbB-2 show several characteristics of the aggressive phenotype and are implicated in early relapse and shortened overall survival (Allred et al, 1998; Sjogren et al, 1998). One of the molecular mechanisms involved in the process of metastasis may be over-production of proteases that degrade the basement membrane and the extracellular matrix (Rochefort, 1992). The most extensively studied protease in human breast cancer is cathepsin-D (CD). Several reports on the prognostic value of CD in breast cancer have revealed poor survival for patients with high CD levels (Spyratos et al, 1989; Scorilas et al, 1993, 1995, 1999; Ferradina et al, 1997; Losch et al, 1998).

The present analysis was designed to extend and complete our previous work (Scorilas et al, 1993, 1995, 1999) and to assess the prognostic significance of the overexpression and amplification of *c-myc* in relation to various established prognostic factors as well as c-erbB-2 oncogene amplification and overexpression and CD concentration in Greek breast cancer patients, in an effort to better characterize this marker. The interrelationship was tested by univariate and multivariate analysis in a series of 152 breast cancer patients with a median follow-up of 5 years in our hospital.

MATERIALS AND METHODS

Patients

Tumour specimens from 152 patients (age: mean, 60 years; range 24–92 years) with no signs of distant metastasis and who underwent surgery for primary breast cancer from 1990 to 1995 (modified mastectomy 50 patients (32.9%); breast-conserving lumpectomy 102 patients (67.1%)) at the Oncologic Hospital of Athens 'St Savas', were evaluated in this study. Tumour specimens were drawn from a pool of frozen specimens originally submitted to the Laboratory of Hormone Receptors for steroid receptor analysis. Most of the women with positive lymph nodes generally received adjuvant chemotherapy (cyclophosphamide–methotrexate–5-fluorouracil for 6 cycles every 28 days; 70 patients); 102 patients received adjuvant (Tamoxifen) therapy (20 mg daily for 5 years), whereas 115 were irradiated. Twenty-one patients (13.8%) developed locoregional recurrence. Median follow-up for patients was 5 years (range 4–8 years). A computerized database containing updated information concerning each patient, together with receptor status, nodal status, size of the primary tumour, number of positive nodes, age and menopausal status of the patients, and/or differentiation grade of the tumour, was available for statistical analysis.

Tumour sample processing

Tumour tissue was stored in liquid nitrogen. Samples were processed as we described previously (Scorilas et al, 1993, 1995). Tissue was pulverized in the frozen state and homogenized in 5 ml cytosol buffer (10 mM Tris, 1.5 mM EDTA, 5 mM NaMolybdate pH = 7.4, 5 mM dithiothreiol DTT). The homogenates were subjected to centrifugation at 40 000 rpm for 1 h at 4°C and the cytosols were kept at –80°C for later processing. The same cytosols were used for hormone receptors and for CD assays. DNA was isolated from 100 mg of tumour tissue, which was minced finely using a pair of scalpels, dispersed in 1 ml of 2 × TNE (20 mM Tris pH = 8.0, 300 mM NaCI, 20 mM EDTA) containing 0.5% sodium dodecyl sulphate (SDS) and digested with proteinase K (100 μg ml^{-1}) at 37°C. After repeated phenol, phenol–chloroform and chloroform–isoamyl alcohol extractions, intact genomic DNA was pooled following precipitation with 2 volumes of ethanol. RNA was isolated from frozen samples, ground to a fine powder in liquid nitrogen and subsequently homogenized in an acid guanidine thiocyanate–phenol–chloroform solution according to Chomczynski et al (1987). Southern blotting of *Eco*R1-digested DNA was performed by standard techniques (Feinberg and Vogelstein, 1983; Thomas, 1980). The integrity of the RNA was confirmed by formaldehyde-agarose gel electrophoresis. Northern blotting was performed according to Thomas (1980). Equal amounts of DNA (20 μg) were slot blotted on nylon membranes (Hybond N^+, Amersham). RNA (20 μg) was slot blotted according to Maniatis et al (1982).

Detection of oncogenes

To determine c-myc overexpression or amplification, blots were hybridized overnight at 42°C to randomly primed [α-^{32}P]dCTP-labelled c-myc probe (1.3 kb fragment, *Cla*I-*Eco*RI, from pHSR-1 plasmid-PBR 322-*Hin*dIII *Eco*RI-human genomic c-myc exon 3 from Colo 320-ATCC). To determine c-erbB-2 overexpression or amplification, blots were hybridized to c-erbB-2 (Oncogene Science) by a 5′ end labelling procedure (Promega), using [γ^{32}P]ATP. The hybridization was performed according to the instructions of the manufacturer and others (Miyada and Wallace, 1987). Briefly, after washing the blots at high stringency (2 × CCS, 0.1% SDS), autoradiography with intensifying screens was performed for 2–4 days at –70°C using Kodak X-OMAT-100 films and autoradiograms were scanned with a BioRad video densitometer 620. DNA and RNA extracted from paired normal breast tissue (obtained from radical mastectomies from areas distant to the cancer) was used as normal control. The values obtained for c-myc and c-erbB-2 by densitometer scanning were normalized to values obtained for *β-actin*. The ratios obtained were compared to average values obtained from 25 normal samples processed in order to determine amplification or overexpression.

Hormone receptors

Oestrogen and progesterone receptors were assayed by ligand binding assay procedure using the dextran-coated charcoal technique as previously described (EORTC Breast Cancer Group, 1980; Kute et al, 1980). Results were expressed as specific binding sites per mg of cytosolic protein (fmol mg^{-1} protein). The cut-off value for both oestrogen receptors and progesterone receptors was 10 fmol mg^{-1} protein as established in our laboratory.

Cathepsin-D assay

Total CD concentrations were measured using a standard assay (IRMA, ELISA Cath-D kit; CIS Bio International, Gif-sur-Yvette,

Table 1 Distribution of 152 patients on the basis of factors examined

Factor	No. of patients	%
Age		
50	38	25.0
50–56	58	38.2
>60	56	36.8
Menopausal status		
Pre/peri	43	28.3
Post	109	71.7
Tumour size		
T1	44	29.0
T2	81	53.3
T3	27	27.7
Lymph node status		
Positive	88	57.9
Negative	64	42.1
Grade		
I	22	14.5
II	97	63.8
III	33	21.7
Oestrogen receptor		
Positive	125	82.5
Negative	27	17.8
Progesterone receptor		
Positive	130	85.5
Negative	22	14.5
c-erbB-2		
Amplification	31	20.4
Overexpression	45	29.6
Cathepsin-D		
Positive	72	47.4
Negative	80	52.6
C-myc		
Overexpression	43	28.3
Amplification	41	26.9
3 years relapse-free survival	106	69.7
Relapse-free survival[a]	85	55.9
Overall survival[a]	104	68.4
Locoregional recurrence	21	13.8

[a]Median follow up 5 years

Table 2 Univariate and multivariate analysis[a] for early recurrence of 152 primary breast cancer patients

Factor	Univariate *P*-value	Multivariate *P*-value	Relative risk	95% confidence intervals
C-myc amplification	0.012	NS	–	–
c-myc overexpression	0.0088	NS	–	–
Cathepsin-D	<0.0001	0.0001	3.12	2.32–4.19
c-erbB-2 amplification	NS	NS	–	–
c-erbB-2 overexpression	NS	NS	–	–
Oestrogen receptor	0.018	NS	–	–
Progesterone receptor	NS	NS	–	–
Differentiation grade	0.0084	0.035	–	1.07–3.20
Lymph node status	0.0018	0.0012	2.15	1.32–3.51
Tumour size	0.0027	NS	–	–
Menopausal status	NS	NS	–	–
Age	0.046	NS	–	–

[a]Cox regression analysis; NS, not significant ($P < 0.05$); median follow-up 60 months.

Table 3 Univariate and multivariate analysis[a] for relapse-free survival of 152 primary breast cancer patients

Factor	Univariate *P*-value	Multivariate *P*-value	Relative risk	95% confidence intervals
c-myc amplification	0.0016	NS	–	–
c-myc overexpression	<0.0001	0.0001	2.25	1.55–3.05
Cathepsin-D	0.0022	NS	–	–
c-erbB-2 amplification	NS	NS	–	–
c-erbB-2 overexpression	0.031	0.0302	1.88	1.15–3.07
Oestrogen recepter	0.019	0.0030	0.25	0.11–0.63
Progesterone recepter	0.028	NS	–	–
Differentiation grade	0.0074	NS	–	–
Lymph node status	0.0055	0.0047	2.54	1.65–3.91
Tumour size	0.0010	NS	–	–
Menopausal status	NS	NS	–	–
Age NS		NS	–	–

[a]Cox regression analysis; NS, not significant ($P < 0.05$); median follow-up 60 months.

France) according to the procedure described by the manufacturer in 1/40 and 1/80 dilutions of the reconstituted cytosols, both in duplicate.

Statistics

Survival analyses were performed by constructing Kaplan–Meier DFS, OS and LRR curves (Kaplan and Meier, 1957), where differences between curves were evaluated by the log-rank test. Cox and logistic regression analysis were used to estimate the relative risks for relapse, locoregional recurrent and death (Tormod and Egil, 1985). Selection of prognostic variables with the highest significant effect in DFS, LLR and OS was performed in the Cox's model using the step-wise regression method in multivariate analysis. Only variables for which $P < 0.05$ were retained in the final model. Relative risks and 95% confidence intervals are presented only for retained variables, significant in the multivariate analysis. Tumour size and differentiation grade are continuous variables with scores 1–3.

RESULTS

The frequency of amplification measured in 152 primary breast tumours was 26.9% for c-myc and 20.4% for c-erbB-2, whereas 47.4% of tumours produced high concentrations of CD (≥ 60 pmol mg^{-1} protein). Moreover, overexpression of c-myc was found in 28.3% and of c-erbB-2 in 29.6% of tumours (Table 1). The magnitude of c-myc amplification ranged between 3 and 7 gene copies, whereas c-erbB-2 amplification ranged between 3 and 10 copies. The overexpression was stronger for c-erbB-2 (3–12 times) than for c-myc (3–6 times) (data not shown). The median CD concentration was 59 pmol mg^{-1} protein (range 23.2–132 pmol mg^{-1} protein). The patients examined were divided in subgroups: (1) according to survival: early relapse (ER; before 3 years); disease-free survival (DFS; median 5 years); overall survival (OS; median 5 years) and (2) according to locoregional recurrence (LRR).

Statistic analysis for the ER

By univariate analysis, the variables found to negatively affect ER were CD, c-myc amplification, lymph node status, tumour size, differentiation grade, c-myc overexpression and age. ER was

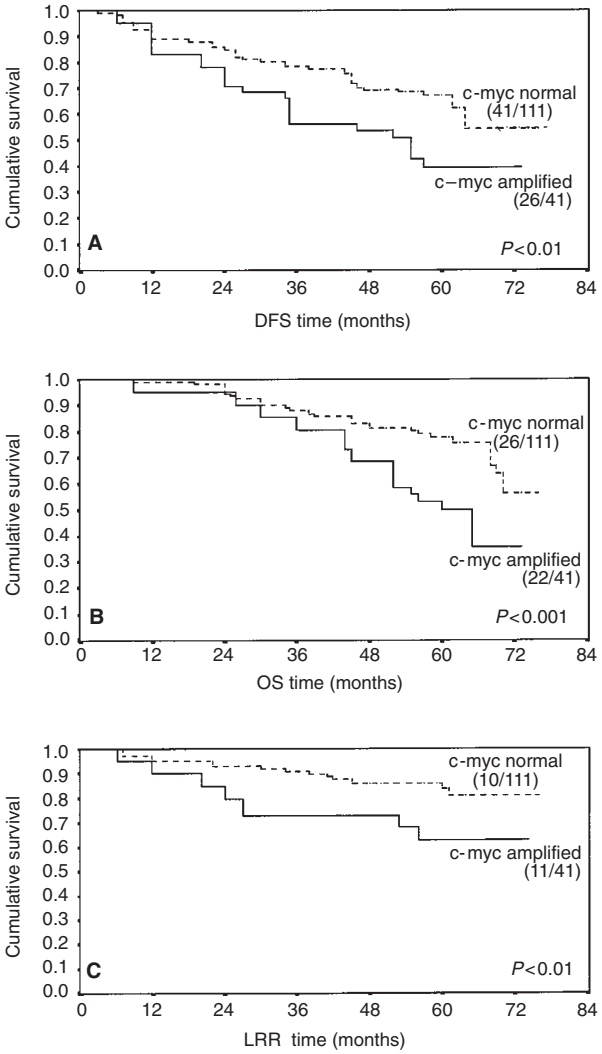

Figure 1 Kaplan–Meier survival plots of DFS (**A**), OS (**B**) and LRR (**C**) of 152 patients with c-myc normal and c-myc amplified. Differences among the two groups for DFS, OS and LRR were determined by log-rank test. Numbers in parentheses indicate the number of failures/total number of patients in each group

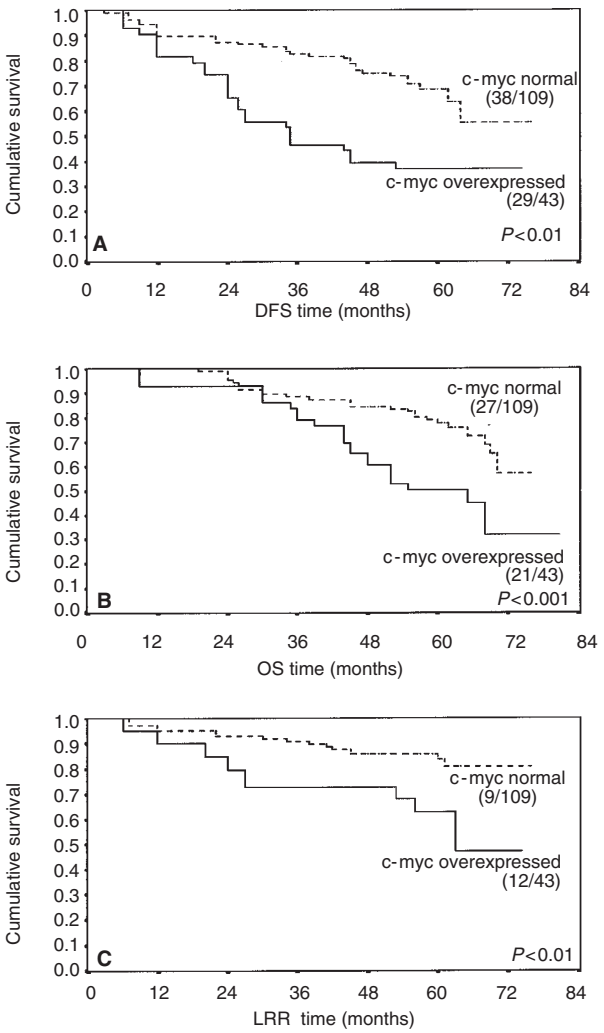

Figure 2 Kaplan–Meier survival plots of DFS (**A**), OS (**B**) and LRR (**C**) of 152 patients with c-myc normal and c-myc overexpressed. Differences among the two groups for DFS, OS and LRR were determined by log-rank test. Numbers in parentheses indicate the number of failures/total number of patients in each group

positively affected by oestrogen receptor but was unaffected by menopausal status, progesterone receptor, or amplification and overexpression of c-erbB-2. Multivariate analysis again reveals CD (P = 0.0001) as the most important variable influencing ER and the precision of the prediction is statistically improved when lymph node status and grade (P = 0.012 and P = 0.035 respectively) are considered (Table 2). In a previous study we showed that the concentration of CD was found to be positively correlated with c-myc amplification and overexpression (Scorilas et al, 1993). This finding, by multivariate analysis, reduces the significance of c-myc oncogene, due to technically easier determination of CD.

Univariate and multivariate analysis for DFS

Relapse free survival as shown in Table 3 was negatively affected by c-myc overexpression and amplification, tumour size, CD concentration, lymph node status, differentiation grade and c-erbB-2 overexpression; however, it was positively affected by the oestrogen and progesterone receptors, and remained unaffected by age and c-erbB-2 amplification. By multivariate analysis, c-myc overexpression and lymph node status emerged as the variables with the strongest influence on DFS. Prediction, however, is improved statistically when oestrogen receptor and C-erbB-2 overexpression are considered. In our previous study (Scorilas et al, 1993) we reported positive correlation between c-myc overexpression and amplification. This reduces the significance of c-myc amplification as shown by multivariate analysis. The Kaplan–Meier curves (Figures 1A and 2A) also show that patients with c-myc overexpression or amplification have a smaller probability for longer DFS than patients without either of them. The difference in DFS effect was greater for c-myc overexpression than for c-myc amplification.

Statistic analysis for OS

Overall survival is negatively influenced by the following variables: c-myc amplification and overexpression, tumour size,

Table 4 Cox univariate and multivariate analysis[a] for the overall survival of 152 primary breast cancer patients

Factor	Univariate *P*-value	Multivariate *P*-value	Relative risk	95% confidence intervals
c-myc amplification	<0.0001	0.0006	3.10	2.18–4.41
c-myc overexpression	0.0095	NS	–	–
Cathepsin-D	NS	NS	–	–
c-erbB-2 amplification	NS	NS	–	–
c-erbB-2 overexpression	0.0021	0.024	1.62	1.01–2.59
Oestrogen receptor	0.0006	0.0053	0.48	0.32–0.71
Progesterone receptor	0.0041	0.025	0.52	0.29–0.94
Differentiation grade	0.042	NS	–	–
Lymph node status	0.0081	0.0043	3.30	2.18–4.98
Tumour size	0.0021	0.015	1.84	1.15–2.94
Menopausal status	NS	NS	–	–
Age	NS	NS	–	–

[a]Cox regression analysis; NS, not significant ($P < 0.05$); median follow-up 60 months.

Table 5 Univariate and multivariate analysis[a] for locoregional recurrence of 152 primary breast cancer patients

Factor	Univariate *P*-value	Multivariate *P*-value	Relative risk	95% confidence intervals
c-myc amplification	0.0024	NS	–	–
c-myc overexpression	0.0075	NS	–	–
Cathepsin-D	0.0016	0.0067	4.2	2.52–6.99
c-erbB-2 amplification	0.0022	0.0091	2.7	1.50–4.86
c-erbB-2 overexpression	NS	NS	–	–
EsR	0.0063	0.019	0.32	0.20–0.52
PgR	0.012	NS	–	–
Differentiation grade	NS	NS	–	–
Lymph node status	NS	NS	–	–
Tumour size	0.018	NS	–	–
Menopausal status	NS	NS	–	–
Age	NS	NS	–	–

[a]Cox regression analysis; NS, not significant ($P < 0.05$); median follow-up 60 months.

c-erbB-2 overexpression, lymph node status and differentiation grade (Table 4). Again, the role of oestrogen and progesterone receptors is protective, whereas age, menopausal status, CD and c-erbB-2 amplification do not seem to influence OS. The Kaplan–Meier survival curves (Figures 1B and 2B) show the reduced probability of patients with c-myc amplification or c-myc overexpression for OS in contrast to those without the two markers. Using multivariate analysis we proved that OS can be predicted by combining the variables: c-myc amplification, lymph node involvement and oestrogen receptor. The prediction is improved if progesterone receptor, tumour size and c-erbB-2 overexpression are taken into account as well.

Analysis for LRR

We observed that 27% and 28% of patients with c-myc amplification and overexpression respectively developed locoregional recurrence, while only 9% and 8% of patients without c-myc amplification and overexpression respectively had locoregional recurrence (Figures 1C and 2C). In Cox univariate analysis, c-myc amplification, c-myc overexpression, CD concentration, c-erbB-2 amplification and tumour size have a positive effect on locoregional recurrence, while oestrogen and progesterone receptors have a negative effect (Table 5). The Kaplan–Meier curves (Figures 1C and 2C) also show that patients with c-myc amplification or overexpression have a greater chance of developing LRR than patients without them. Multivariate analysis suggests that high CD concentration is the most important variable for LRR. The positive correlation between CD concentration and c-myc amplification and overexpression, reported previously by our group (Scorilas et al, 1993), reduces in multivariate analysis the role for c-myc determination for prediction of disease course.

DISCUSSION

Breast cancer is the most common form of malignancy among women today. It would be beneficial for patients to have tools available that could more reliably predict the rate of recurrence in primary breast cancer, in addition to the classical prognostic factors. In recent years, many biological markers have been studied for their correlation with prognosis (McGuire et al, 1991; Gasparini et al, 1992; Osborne et al, 1992; Foekens et al, 1996; Nass and Dickinson, 1996; Thor and Yaudell, 1996). The myc family of nuclear proto-oncogenes plays critical roles during cell growth, differentiation and transformation. Although the molecular mechanisms underlining myc-mediated cellular transformation are still under investigation, evidence is accumulating that c-myc transcriptionally controls the expression of a diverse group of genes, and that its deregulation leads to a cellular imbalance in the expression of genes that control both proliferation and death.

Amplification of the c-myc locus in breast cancer tissue has been observed in many studies (Berns et al, 1992*a*, 1992*b*; Borg et al, 1992; Watson et al, 1993; Lonn et al, 1995). The reported frequency of amplification varies greatly (from 4% to 52%) in these studies, but the overall mean appears to be about 20%. There is also considerable variability in the predictive value of c-myc amplification and correlation with other prognostic markers of breast cancer. Some reports indicate that c-myc amplification is predictive for shortened relapse-free and/or overall survival (Berns et al, 1992*a*; Borg et al, 1992; Lonn et al, 1995), while Berns et al (1995) upon concurrent examination of c-erbB-2, c-myc and int-2 showed that c-myc was the only oncogene whose amplification was significantly related with the rate of relapse.

In addition, a number of studies have examined c-myc expression in breast cancer at both the mRNA and protein levels. Northern analysis indicated that c-myc mRNA expression was elevated compared to that observed in normal breast tissue in 70% (Escot et al, 1986) or 45% (Garcia et al, 1989) of breast tumours.

Immunohistochemistry also has been frequently used to examine the relative levels of myc protein in mammary tumour specimens (Pavelic et al, 1992; Pietilainen et al, 1995). Tulchin et al (1996), using immunohistochemistry, have shown continuity of c-myc expression during tumour progression, whereas Bland et al (1995), while studying the co-expression of c-myc with other oncogenes, report that co-expression of c-myc, Ha-ras and c-fos function as a strong prognostic correlate for recurrence and survival. Variation in results throughout the literature is not surprising given the broad range of sample size, composition and follow-up, as well as inconsistencies in experimental and statistical methodology.

In the present study, the distribution of c-myc amplification and overexpression, c-erbB-2 amplification and overexpression and high CD concentration (Table 1) are in agreement with data reported in the literature. With respect to the prognostic value of c-erbB-2 for DFS and OS, we observed a discriminative power only for its overexpression, which was reduced in multivariate analysis (Tables 3 and 4). More important is our finding concerning the clinical impact of c-myc amplification and overexpression. This study (Tables 3 and 4) suggests that both are highly significant for DFS and OS of patients, albeit in multivariate analysis only c-myc overexpression for DFS and c-myc amplification for OS remain statistically significant. For ER the best predictors of the new markers examined are high CD concentration, c-myc amplification and c-myc overexpression (Table 2), but following multivariate analysis, only the CD remains relevant, which suggests that the prognostic power of the three variables is not additive. In general, the prognostic relevance of c-myc amplification and overexpression overlap as we reported (Scorilas et al, 1993) and the determination of one of them only is sufficient. The association of LRR with high CD concentration, c-erbB-2 amplification and c-myc amplification and overexpression is also important. Nevertheless, their impact is not additive and following multivariate analysis only high CD concentration remains statistically significant and can be exploited as a marker for modification of patient treatment (Table 5). The present study also reveals that for ER and LRR, c-erbB-2 amplification and overexpression have smaller prognostic value from the c-myc amplification and overexpression, although for the former these values are additive (Table 2 and 5). For ER the only new variable which has significance in multivariate analysis is high CD (Table 2). The observation, which to the best of our knowledge has not been reported previously, is the positive association between c-myc and LRR. Therefore, we can propose that patients showing c-myc amplification or overexpression have a tendency for locoregional recurrence, which could be exploited as a marker for modification of patients' treatment.

In conclusion, c-myc amplification can be used as a prognosticator for overall survival, whereas c-myc overexpression for relapse-free survival of breast cancer patients. The best of the new predictors for early relapse remains high CD concentration, while c-myc amplification and overexpression manifest a tendency for locoregional recurrence.

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