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Prevalence of Amplification of the Oncogenes c-*myc*, *HER2/neu*, and *int*-2 in One Thousand Human Breast Tumours: Correlation with Steroid Receptors

Els M.J.J. Berns, Jan G.M. Klijn, Iris L. van Staveren, Henk Portengen, Erica Noordegraaf and John A. Foekens

The frequency of oncogene amplification described in the literature shows a large fluctuation, which could be attributed to the study of relatively small series of tumours, to selection of subgroups of patients, or, especially in retrospective studies, to selection of tumour material from the tumour-bank. To address this question, we have studied amplification of c-*myc*, *HER2/neu* and *int-2/bcl*-1 genes in a series of 1052 collected human breast tumours. The retrospective and prospective subgroups in this collected series of tumours were of equal size. c-*myc* was amplified in 17.1%, *HER2/neu* in 18.7% and *int-2/bcl*-1 in 14.1%, of all breast cancer specimens studied. In the retrospective subgroup the prevalence of amplification was 18.1% for c-*myc*; 22.6% for *HER2/neu* and 11.6% for *int-2/bcl*-1, whereas in the prospective subgroup an incidence of amplification of 16.1%, 15.1% and 16.3% for c-*myc*, *HER2/neu* and *int-2/bcl*-1, respectively was observed. *HER2/neu* amplification was negatively correlated with oestrogen receptor (ER) and progesterone receptor (PR) status ($P < 0.0001$; for both), c-*myc* amplification was more prevalent in the PR-negative subpopulation ($P < 0.05$) and *int-2/bcl*-1 amplification was positively correlated with ER status ($P < 0.001$).
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INTRODUCTION

MUTATIONS (including amplifications and deletions) of (onco-) genes have been proposed to play a role in the initiation and progression of breast cancer [1]. The mutation in the target gene may influence the expression levels or specific properties of the protein product of this gene which may be a transcription factor, a signal transducer, a growth factor or its receptor. Molecular markers will likely prove valuable in clinical decision-making. Assessment of the value of these parameters as a predictor of response to therapy or as possible point of action for new treatment modalities, is important.

In breast cancer, the prevalence of amplification of the c-*myc* gene (a nuclear phosphoprotein which presence seems to be required for proliferation while its absence may be obligatory for complete withdrawal from the cell-cycle), has been reported to range from 1–41% [2–11]. The *int*-2 gene, which encodes a protein homologous to fibroblastic growth factor (FGFs, a family involved in angiogenesis, tissue induction and cell migration) [12, 13], has been reported to be amplified between 9 and 23% [7, 14–18]. The *hst*, *int*-2 and the *bcl*-1 loci (on chromosome 11q13) are usually present in amplification units. However, there is little evidence of expression of *int*-2 [19]. There are numerous reports on the amplification of *HER2/neu* (c-*erb*B-2) gene, which encodes a transmembrane protein (185 kD) with a phosphotyrosine kinase domain and which is closely related but distinct from the epidermal growth factor (EGF-R; c-*erb*B-1) gene [20–22]. The amplification data range between 8 and 46% [23–26]. The broad range of these reported oncogene amplifications could be attributed either to the study of relatively small series of patients, patient selection (especially in retrospec-

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tive studies), or to the selection of tumour material from a tumour-bank, in which relatively large tumours may be over-represented. In addition, there is also no consensus whether these oncogene amplifications are related to steroid-hormone receptor levels. Because selection of a patient population, or the application of tumour material from tumour banks may bias the data on oncogene amplification, we have analysed amplification of c-*myc*, *HER2/neu* and *int-2/bcl-1* genes and the relation between amplification and the steroid-hormone receptor status in a series of 1052 breast cancer tissues (collected for retrospective and prospective analysis; the size of these subgroups was equal).

MATERIALS AND METHODS

Patients and tissues

Human breast cancer tissue specimens were randomly drawn from what was left of a pool of frozen samples (stored in liquid nitrogen), which were originally submitted to the laboratory for steroid receptor analysis. Human breast cancers collected between 1978 and 1988 were defined as belonging to the retrospective subgroup. From 1989 onwards, an aliquot of the first homogenate was saved for DNA isolation from all tumours sent to our laboratory for the assessment of steroid hormone receptor status. These cancers, which thus were unselected, were defined as belonging to the prospective subgroup.

Receptor assay

About 0.4–0.8 g of tumour tissue was pulverised and homogenised as recommended by the EORTC for processing breast tumour tissue for cytosolic ER and PR determinations. Part of the homogenate was used for DNA analysis. After centrifugation of the remaining homogenate, for 30 min at 100 000 *g* at 4°C, the supernatant fraction (cytosolic extract) was used for ER and PR determinations, either with enzyme immunoassays (ER-EIA and PgR-EIA kits, Abbott Laboratories, Abbott Park, Illinois) or with radioligand binding assays as described before [27] and as recommended by the EORTC [28]. All values of 10 fmol steroid receptor per mg protein and above were considered receptor positive.

DNA analysis

An aliquot of the total tissue homogenate was used for DNA isolation. Southern blotting of *Eco*R1 digested DNA was performed by standard techniques [29]. Briefly, DNA was digested, size fractionated on a 0.6% agarose gel and transferred to a nylon membrane (Hybond N$^+$, Amersham, Buckinghamshire, UK) and hybridised overnight at 65°C with randomly primed [30], ^{32}P-dATP labelled probes (specific activity of 1–2 × 10^9 cpm/μg DNA). *HER2/neu* (p*HER*2-436-1, ATCC 59296), c-*myc*, (an *Eco*R1-Cla 1 human exon 3-specific c-*myc*), and IGF-1-R (plGF-9-R.8, ATCC 59295). The possibility of multiple copy numbers of chromosomes was ruled out since we also tested for (chromosome specific control genes): p53, c-Ha-*ras* and c-*fes*. Probes were all obtained from the American Type Culture Collection (Rockville, Maryland, USA). *Bcl-1*, (a 2.1 kb Sst-1 fragment), and *int-2*; SS6 (a 0.9 kb SacI–SacI fragment), was a gift from Gordon Peters and Clive Dickson (ICRF, London, UK). After washing at high stringency (0.3 × sodium saline citrate), autoradiography with intensifying screens was performed for 4 h to 2 days at −70°C using Kodak XAR-5 films. Autoradiograms were scanned with a Bio-Rad Video densitometer 620. The *IGF*-1-R (two gene copies per cell) probe was used as an internal control for the amount of DNA loaded per lane. The *IGF*-1-R was amplified in 2% of the cases studied (Berns *et al.* Sporadic amplification of the IGF-1 receptor gene in human breast tumors, *Cancer Res*, accepted). Amplification was defined as more than two copies of the gene per cell.

Table 1. Oncogene amplification in human primary breast cancer: retrospective and prospective study

	c-*myc*	*HER2/neu*	*Int-2/bcl-1*
All samples	17.1	18.7	14.1
Retrospective	18.1	22.6	11.6
Prospective	16.1	15.1	16.3

(%)

Statistical analysis

The associations between oncogene amplification and receptor status were studied by the Pearson χ^2 analyses. A P level of 5% was chosen as a criterion for significance.

RESULTS

Prevalence of (onco)-gene amplification

Oncogene copy numbers were studied in DNA isolated from homogenates of 1052 human breast cancer samples. 77 samples were not evaluable due to very low recovery of DNA or because the DNA could not be digested with *Eco*R1. This indicates that DNA isolation and subsequent digestion from frozen tumour powders is successful in 93%. In this study gene copy numbers of the amplified c-*myc* gene varied between 3 and 18; of the amplified *HER2/neu* gene between 3 and 43; and of the amplified *int-2/bcl-1* gene between 3 and 18 copies. Co-amplification of c-*myc* and *int-2/bcl-1* was observed in 25 cases and co-amplification of *HER2/neu* and *int-2/bcl-1* was observed in 23 cases. In the retrospective series a negative relation between *HER2/neu* and c-*myc* amplification was observed ($P < 0.0001$). It is interesting to note that, in one cell line, the *HER2/neu* gene has been identified as a cellular target gene for negative regulation by c-*myc*.

The percentages of oncogene amplifications are depicted in Table 1, showing that there are differences in frequencies of amplification of *HER2/neu* and *int-2/bcl-1* between the overall series ($n = 1052$) and the retrospective group ($n = 517$) and the prospective ($n = 535$) group of tumours. The incidence of amplification of *HER2/neu* was significantly higher in the retrospective group when compared with the prospective group ($P < 0.005$). The incidence of *int-2/bcl-1* amplification was higher in the prospective group when compared with the retrospective group, but this difference was not statistically significant ($P < 0.06$). The incidence of c-*myc* amplification was similar in both subgroups.

Relation between oncogene amplification and steroid hormone receptors

We next examined the relation between oestrogen and progesterone receptor levels and c-*myc*, *HER2/neu* and *int-2/bcl-1* amplification. The relationships are given in Tables 2 and 3. *HER2/neu* amplification was more prevalent in both ER and PR-negative tumours ($P < 0.0001$; both groups). c-*myc* amplification was related to PR-negative tumours ($P < 0.05$), whereas amplification of *int-2/bcl-1* was related to ER-positive tumours ($P < 0.001$).

Table 2. Relation between oncogene amplification and ER status

	c-*myc* amplified	c-*myc* normal
ER-positive *	106 (16.5)	536 (83.5)
ER-negative	35 (18.5)	154 (81.5)
	HER2/neu amplified	*HER2/neu* normal
ER-positive †	90 (14.4)	535 (85.6)
ER-negative	55 (30.9)	123 (69.1)
	Int-2/bcl-1 amplified	*Int-2/bcl-1* normal
ER-positive ‡	95 (17.4)	450 (82.6)
ER-negative	10 (6.3)	149 (93.7)

(%)
* Not significant.
† $P < 0.0001$.
‡ $P < 0.001$.

Table 3. Relation between oncogene amplification and PR status

	c-*myc* amplified	c-*myc* normal
PR-positive *	84 (15.3)	464 (84.7)
PR-negative	56 (21.1)	209 (78.9)
	HER2/neu amplified	*HER2/neu* normal
PR-positive †	70 (13.2)	461 (86.8)
PR-negative	70 (27.5)	184 (72.4)
	Int-2/bcl-1 amplified	*Int-2/bcl-1* normal
PR-positive ‡	77 (16.6)	387 (83.4)
PR-negative	25 (11.1)	200 (88.9)

(%)
* $P < 0.05$.
† $P < 0.0001$.
‡ Not significant.

DISCUSSION

Published oncogene amplification data in breast cancer show a broad range. This could be attributed to the huge divergence in the amount of tumour samples studied, selection of tumours from tumour banks or a choice of subgroups of patients (e.g. patient groups of different geographical or genetic composition or patients which show early metastasis). We have included these variables in our study, which describes the analysis of oncogene amplifications (c-*myc*, *HER2/neu* and *int-2/bcl-1* genes) and their relation with steroid-hormone receptor status in a large series of breast cancer tissues. Our data show that there are differences in percentage of *HER2/neu* and *int-2/bcl-1* amplification in both retrospectively or prospectively processed tumours. *HER2/neu* is more prevalent in the retrospective series whereas *int-2/bcl-1* is more current in the prospectively processed series. *HER2/neu* has been reported to be "tentatively" related to tumour size. Tumours obtained from the tumour bank were initially likely of larger size, since these were remainders of tumour samples, originally processed for routine ER and PR analysis. Therefore, the observed higher prevalence of *HER2/neu* amplification in the retrospectively processed tumours, when compared with the unselected prospectively processed tumours, may be explained by a relationship between *HER2/neu* amplification and tumour size. At present, the techniques of screening for breast cancer allow the detection of (very) small tumours. The data from this study indicate that the part of *HER2/neu* amplification as predictor for clinical outcome of breast cancer (which is still under debate) may change in the near future. We found no difference in the incidence of c-*myc* amplification between the retrospective and the prospective series of processed tumours. However, in a specific subset of patients, patients which develop metastasis, we have observed that the frequency of c-*myc* amplification was increased about two times to 33% (Berns *et al.* submitted). In the present study we observed a significant inverse relationship between *HER2/neu* amplification and ER or PR (which has also been observed by others, [24]) and between *int-2/bcl-1* amplification and ER-positivity, also shown by Borg *et al.* [31]. On the other hand, c-*myc* amplification was found more prevalent in PR-negative tumours. This was not reported in studies using a small series of tumours and was only observed in a relatively larger series ($n = 292$) studied by Adnane *et al.* [7]. There are few reports on the combined oncogene amplifications of c-*myc*, *HER2/neu* and *int-2* and their relation with ER and PR, in a large series of tumours. Data on oncogene amplification and receptor status have been described by Garcia *et al.* [32], in a series of 125 prospectively collected primary breast cancer samples and by Adnane *et al.* [7] in a series of 292 tumours. The latter report describes an interesting difference in incidence of both c-*myc* amplification (9.3% vs. 20.8%) and *int-2/hst* amplification (21.5% vs. 15.6%) in two patient-populations, living in different parts of France. This indicates that there may even be a geographical difference in certain oncogene amplifications in breast cancer patients.

We conclude that the selection of a specific patient population, the application of tumour material from tumour banks or the use of prospectively or retrospectively collected tumour material may bias the data on prevalence of oncogene amplification.

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Expression of c-*erb*B2, TGF-β1 and pS2 Genes in Primary Human Breast Cancers

C.G. Delvenne, R.A. Winkler-Gol, M.J. Piccart, J. Hustin, D. Michaux, G. Leclercq, J.M. Nogaret and Ph. Autier

The presence of c-*erb*B2, TGF-β1 and pS2 mRNAs was examined in primary breast tumours. The c-*erb*B2 mRNA was overexpressed in 34% of the tumours. There was a positive, statistically significant correlation between c-*erb*B2 gene overexpression and nodal status. TGF-β1 mRNA was detected in 84% of the tumours, regardless of their clinical status. When possible, the c-*erb*B2 and TGF-β1 proteins were identified immunohistochemically on frozen sections from the same tumours. For TGF-β1, the mRNA and immunohistochemical results were divergent in 6 cases, 5 of which did contain clearly detectable mRNA but did not stain with the antibody. The pS2 mRNA was detected in 22% of the tumours and in the BT474 cell line. There was a significant correlation between the presence of pS2 mRNA and of oestrogen receptors. No statistically significant correlation was observed between pS2 and TGF-β1 genes expression and the clinical parameters of the tumours.
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INTRODUCTION

THE c-*erb*B2 oncogene is coding for an EGF-receptor-related transmembrane growth factor receptor with tyrosine kinase activity [1]. This gene is amplified and overexpressed in a number of adenocarcinomas, such as mammary adenocarcinomas [2]. It appears that in human tumours the amplified gene is not mutated [3]. The overexpression of the normal c-*erb*B2 protein is highly oncogenic in the NIH3T3 assay [4]. Slamon and his coworkers [5] first showed that c-*erb*B2 gene overexpression has a prognostic value. Since than, numerous studies have addressed this question and the use of c-*erb*B2 in predicting relapse-free or overall- survival is controversial. The data have been recently reviewed and discussed by Perren [6].

TGF-β1 is a growth factor with growth inhibitory properties for breast cancer cells [7, 8]. The presence of TGF-β1 mRNA and protein in the tumour could thus be a positive factor as far as the evolution of the disease is concerned.

The pS2 cDNA was cloned as an oestrogen-induced mRNA in the MCF7 mammary adenocarcinoma cell line. Later it was found that the gene is also expressed in the normal gastric