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Specialist Interest Articles

HER-2 Oncogene Amplification and Overall Survival of Breast Carcinoma Patients

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DNA was extracted from tumour samples of 77 patients with primary breast carcinoma and HER-2 proto-oncogene amplification was assessed. Prognostic indices such as number of positive lymph nodes, tumour size and histological grading were strongly associated with overall survival. No statistically significant correlation between amplification of HER-2 and overall survival was observed. In addition, prognostic indices, HER-2 amplification and disease-free interval was not correlated. Analysis of HER-2 amplification alone is not a useful guide in the management of patients with mammary carcinoma.
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INTRODUCTION

TUMOUR GENESIS and progression can be assumed to be gradual processes during which a cell escapes from a balanced molecular state. Several genes are affected in the development of a variety of human tumours, including proto-oncogenes which control cell growth and biological development. Proto-oncogenes can be turned into dominant cancer genes or oncogenes. Amplification of certain proto-oncogenes in subsets of primary breast tumours supports the notion that they are involved in the development of this disease. Amplification of INT-2 [1], c-MYC [2, 3] and HER-2 [3–9] has been observed. In addition, the frequency of rare c-H-RAS-1 alleles was significantly higher in breast cancer patients compared with an unaffected population [10]. Amplification of HER-2 in human breast cancer was reported to vary between 10% [11] and 40% [5] of the tumours.

The management of breast cancer depends on an understanding of the prognostic factors indicating which patients may be at increased risk for relapse and death. Several indices, such as histopathological grading, ovarian hormonal status, age, number of positive lymph nodes and steroid receptors, are well accepted prognostic factors. HER-2 gene amplification was found more frequently in oestrogen receptor negative patients [9, 12]. Amplification of HER-2 was a significant predictor of both overall survival and time to relapse [5]. In another study no significant correlation between breast cancer recurrence and HER-2 amplification was found [8]. Results from Ali *et al.* [11] are consistent with the lack of a strong association between increased copy number of HER-2 proto-oncogene and the aggressiveness of the tumours [11].

To investigate this issue further, we have studied a well-documented group of patients for HER-2 proto-oncogene amplification in relation to different clinicopathological variables.

PATIENTS AND METHODS

Patients and tumour tissues

Tumour samples were obtained at random from 77 patients who underwent surgery for primary breast carcinoma at the First Department of Surgery, Vienna University, Austria, from 1985 to 1986. Tissue samples that were used for analysis were dissected free of non-tumorous material and examined by frozen section. The tumour samples were frozen in liquid nitrogen immediately after surgical removal and stored in liquid nitrogen until DNA was extracted.

The patients did not receive any therapy before surgery which was done by modified radical mastectomy in 43 patients and by wide local excision in 30 patients. Tumorectomy was done in 4 patients. Adjuvant therapy was radiation (2 patients), ^{192}Ir (9), chemotherapy (7), tamoxifen (19), radiation/tamoxifen (1), chemotherapy/tamoxifen (7), ^{192}Ir/tamoxifen (4) and ^{192}Ir/chemotherapy (9). 19 patients received no adjuvant therapy. Tumours analysed in this study were of various histological subtypes (WHO) (Table 1).

Cytosol and nuclear fractions

All procedures were done at 4°C. Tissue samples were homogenised ("Ultra-Turrax") in 50 mmol/l phosphate buffer pH 7.5. The cytosol fraction was obtained by centrifugation at 50 000 *g* for 1 h. The pellet was resuspended in 20 mmol/l HEPES buffer containing 1 mmol/l PMSF pH 7.2. The suspension was centrifuged at 1000 *g* for 10 min and the pellet containing the nuclear fraction was used for extraction of high molecular weight DNA.

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Table 1. Histological subtypes

Histological subtypes	Single copy	Amplified
Lobular invasive	9	2
Ductal *in situ*	0	1
Ductal invasive		
NOS	41	16
Mucoid colloid	2	0
Tubular	0	1
Papillary	0	1
Comedo type	0	1
Medullar	0	1
Glycogen rich	0	1
Lipid secreting	1	0
Total	53	24

NOS = not otherwise specified.

HER-2 amplification

DNA was extracted with materials obtained from Oncor (Gaithersburg, Maryland). In brief, the nuclear fraction was treated with a buffer containing proteinase K at 60°C for 3–12 h. After three extractions with phenol/chloroform 1/1 followed by two chloroform extractions, high molecular weight DNA was precipitated with 2.5 volumes of ethanol. DNA was spooled and dissolved in buffer 10 mmol/l Tris/HCl, 1 mmol/l EDTA pH 7.4.

DNA was digested and hybridised by methods described previously [9]. The RNA probe was derived from an *Eco*RI/*Bam*HI cDNA clone [13]. Hybridisations with this probe were done at 52°C for 12–18 h. The membranes were washed twice in 2 × sodium saline citrate (SSC), 0.1% sodium dodecyl sulphate (SDS) for 10 min and once with 0.1 × SSC at 53°C for 40 min. Autoradiography was done by exposing the membranes to "Amersham-MP" films with intensifying screen at −70°C for 2–3 days. Exposure times were chosen so that the single-copy signal of placenta (control) DNA was in the linear range of the film (up to 1.5 extinction units). After densitometry the HER-2 probe was washed off the membranes by incubation in 0.4 mol/l NaOH at 42°C for 40 min followed by neutralisation in 0.2 mol/l HCl pH 7.5, 0.1 × SSC, 0.1% SDS for 15 min and finally by incubation in 0.1 × SSC, 0.1% SDS at 65°C for 40 min. The amounts of DNA applied to each lane were controlled by hybridisation with a probe for the arginase gene [14]. The degree of amplification (A) of HER-2 in tumour DNA (i) was calculated: $A = X_i/X_p$, where X_i is the ratio of the HER-2 sample signal to the arginase sample signal and X_p is the ratio of the HER-2 placenta signal to the arginase placenta signal.

Amplification was classified as moderate (2 to 5 fold) or high (greater than 5 fold) with placenta DNA as single-copy control.

Protein and DNA assay

Protein was measured with Coomassie Brillant Blue G-250 (Bio-Rad). DNA was assayed by fluorimetry (Höfer TKO-100, Höfer, San Francisco) with Hoechst 33256 as dye.

Statistics

Univariate tests by Mantel [15] were used as well as Cox's multiple regression for censored survival data, adjusting a prognostic factor for all other factors included in the model. All P values obtained by univariate tests or by the regression model were accompanied by corresponding estimates of the strength of a factor—i.e. by hazard ratios or estimates of relative risk of the unfavourable to the more favourable level of each factor.

RESULTS

DNA from 77 primary human mammary carcinomas was isolated and analysed for amplification of HER-2. The results obained with 10 tumour DNAs are shown in Fig. 1. Amplification was found in 24/77 (31%) primary breast tumours. Moderate amplification was seen in 21/77 (27%) tumour DNAs, of which two results are shown in lanes 3 and 9 of Fig. 1. Higher amplification was observed in 3 cases (lanes 2 and 11, Fig. 1). Single copies were found in 53/77 (69%) primary breast tumours (lanes 1, 4, 5, 7, 8 and 10, Fig. 1).

A 2 fold increase in hybridisation signal was used as a measure of gene amplification. For statistical analysis tumour samples containing 2 and more than 2 copies were combined and classified as amplified. With this definition 31% of tumour samples were amplified.

On each filter, control DNA that did not contain amplified HER-2 was run (lane 6, Fig. 1). Lambda DNA digested with *Hin*dIII was used as a molecular weight marker (23 kb to 2.2 kb). The sizes of the hybridising fragments are indicated in Fig. 1.

Analysis of follow-up of the patients by Kaplan–Meier estimates, with the meaning of the status indicator reversed, gave 40.5 months for the median and 35 and 44 months for lower and upper quartiles, respectively. During the observation period of 51 months, 21 patients relapsed and 16 patients died from the mammary carcinomas.

Overall survival of patients whose DNA contained only a single copy was not different from that of subjects with proto-oncogene amplification. The lack of association between HER-2 amplification and overall survival was noted both in the univariate test ($P = 0.66$) and the regression model ($P = 0.58$) (Fig. 2). In addition, a relative risk of 1.37 was calculated. Histological subtypes of tumours were compared with HER-2 amplification status (Table 1). No association of specific subtypes and elevated copy numbers of HER-2 could be observed. The

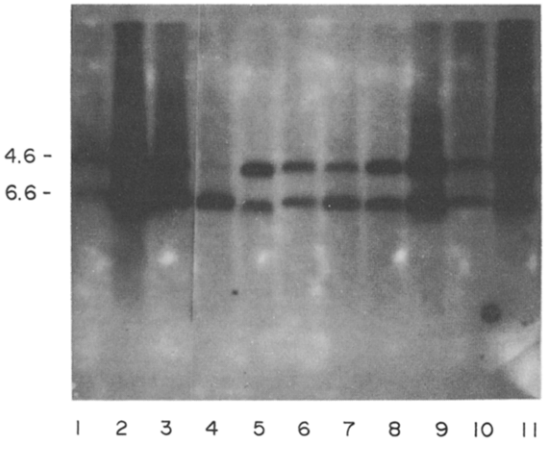

Fig. 1. Analysis of the HER-2 gene in DNA of primary human breast carcinoma. *Hin*dIII digested lambda DNA was used as a molecular weight marker and sizes of the hybridising fragments are indicated. Lanes 1, 4, 5, 7, 8 and 10 = single copy, lanes 3 and 9 = 2–5 copies, lanes 2 and 11 = more than 5 copies and lane 6 = placenta DNA.

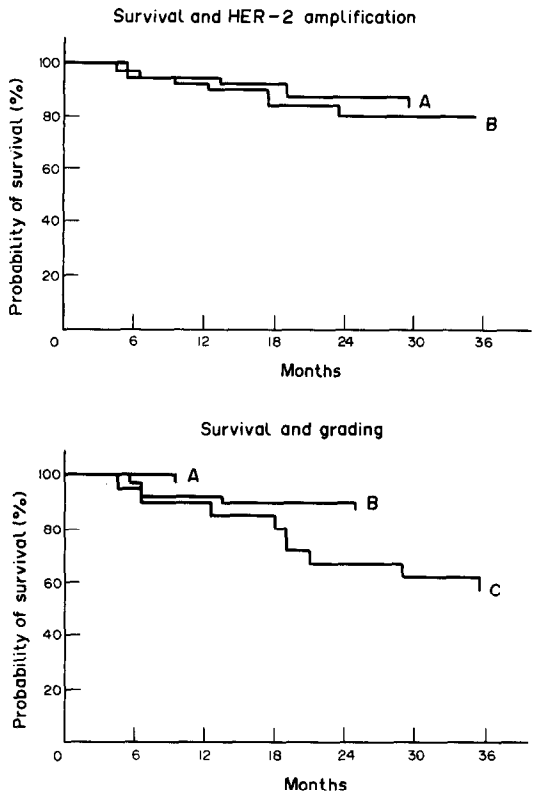

Fig. 2. Association between overall survival, HER-2 amplification and grading. Upper: A = single copy of HER-2 proto-oncogene and B = amplified HER-2 proto-oncogene. $P = 0.66$ in univariate test and $P = 0.58$ in regression model. Lower: A = grade I, B = grade II and C = grade III. $P = 0.013$ in univariate test and $P = 0.0085$ in regression model.

association between histological grading, tumour size and lymph nodes with overall survival was calculated to establish whether these prognostic factors applied to the patients recruited for our investigation. Overall survival of patients with grade III was significantly shorter than that with grades II and I, respectively, and a strong association of histological grading with overall survival was noted in the univariate test (Fig. 2). Furthermore, a strong association between tumour size and overall survival was observed ($P = 0.025$) (Fig. 3). The correlation between number of lymph nodes and overall survival was investigated and again a strong association was found in the univariate test (Fig. 3).

DISCUSSION

Amplification of HER-2 in primary breast tumours may be of significance in view of its homology with the receptor for epidermal growth factor [16, 17]. Epidermal growth factor has an important role in the growth of mammary epithelium [18]. Increased levels of HER-2 proto-oncogene transcripts were found in primary breast tumours that possessed increased copy numbers of the gene [6]. The HER-2 oncogene encodes a 185 kD transmembrane phosphoprotein with tyrosine kinase activity [16]. Specific antibodies to the 185 kD HER-2 protein reverse the malignant phenotype of cells in a rat model [19, 20], suggesting a role for this oncogene in human breast tumours.

In our study the HER-2 gene was amplified in 31% of human primary breast carcinomas. The frequency of amplification of HER-2 photo-oncogene in our panel of primary human breast tumours was greater than that we reported previously [9]. Amplification of HER-2 proto-oncogene was observed in 10–40% of breast tumours from different groups of patients [5–7, 11, 12, 21]. In addition, the level of amplification of this proto-oncogene varied between different groups of patients studied by different laboratories, with a 2 to 40 fold increase in the copy number of HER-2 proto-oncogene. HER-2 amplification has been correlated with prognosis of breast cancer patients [5, 7, 8, 12]. In another study [11], however, no evidence was found for the prognostic significance of HER-2 amplification in human breast carcinoma.

Our data showed a strong association of well accepted clinicopathological indices (number of positive lymph nodes, histopathological grading and tumour size) with overall survival. The same variables have been identified previously [22–24]. However, there was no association between the amplification of HER-2 proto-oncogene and overall survival. HER-2 gene amplification was found more frequently in oestrogen receptor negative and progesterone receptor negative breast tumours than in receptor positive cases [9]. However, no correlation between HER-2 proto-oncogene amplification and oestrogen receptor content was observed in another study [5]. Oestrogen receptor status is an acknowledged index that correlates with the aggressiveness of the tumour. In our study patients with a negative oestrogen receptor status had shorter overall survival.

Our findings support the lack of association between the amplification of proto-oncogene and a clinically poor prognosis of mammary carcinoma. The discrepancy between our study, a recent investigation [11] indicating a lack of evidence for the prognostic significance of HER-2 amplification in human breast carcinoma and previous investigations [5, 7, 8, 12] is not fully

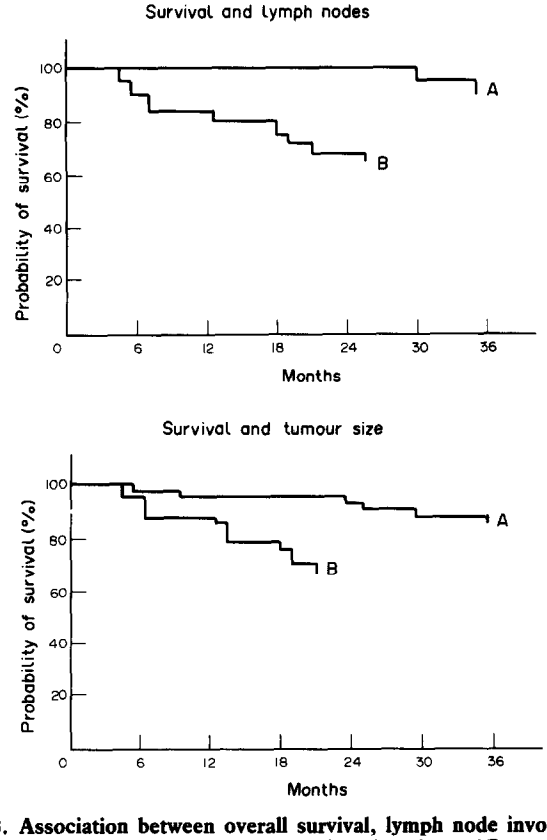

Fig. 3. Association between overall survival, lymph node involvement and size of tumour. Upper: A = no lymph nodes and B = more than 3 lymph nodes. $P < 0.001$ in univariate test and $P = 0.0058$ in regression model. Lower: A = under 2 cm, and B = over 2 cm. $P = 0.025$ in univariate test and $P = 0.275$ in regression model.

understood at present. But the importance of proto-oncogene products in the regulation of cell proliferation is continually being reaffirmed. Transcriptional regulation of c-MYC oncogene expression by oestrogen in hormone-responsive human breast cancer cells has been described [25]. Studies in human mammary tumour cell lines revealed that gene amplification could not be detected by southern blot or DNA dot-blot analysis in all tumour cell lines in which HER-2 transcript was increased [26]. Similarly, overexpression did not always correlate with amplification in all mammary tumour tissue samples as well as cells grown *in vitro* [27]. But other explanations are possible for the discrepancies between our study, Ali *et al.* [11] and previous reports. In addition, factors such as genetic and environmental can contribute to the evolution of breast carcinoma.

The degree of relative HER-2 copy numbers that we observed was similar to that described previously [5, 9]. We used only tumour tissue samples that were dissected free of non-tumorous material. This was controlled by frozen sections. Therefore, dilution by non-tumorous tissue should not be responsible for possible underestimation of relative HER-2 copy numbers and the discrepancy with previous reports [5, 7, 8, 12].

HER-2, c-MYB, c-RAS-Ki, INT-2 and c-MYC have been described as occasionally amplified in primary breast cancers [28]. No significant correlation with HER-2 amplification and any tumour characteristics, such as size, axillary nodes, stage, oestrogen or progesterone receptor or recurrence, was noted. However, significant correlations with amplification of one or more proto-oncogenes were observed with recurrence of clinically advanced tumours and early recurrence of tumours of stages I–III. Detailed analysis indicated that this mainly reflected recurrence of stage III tumours which are already known to be at high risk of recurrence [28].

Breast cancer cells seem to use multiple genetic mechanisms in their progression and metastasis. Analysis of HER-2 alone is not a useful guide. At present, assessment of proto-oncogene amplification offers no additional information beyond that obtained with acknowledged prognostic guides. Further studies are necessary to delineate the interaction of particular clinical indices such as amplification of oncogenes in tumours with other factors contributing to the aggressiveness of mammary carcinoma.

1. Lidereau R, Callahan R, Dickson C, Peters G, Escot C, Ali IU. Amplification of the int-2 gene in primary human breast tumors. *Oncogene Res* 1988, **2**, 285–291.
2. Escot C, Theillet C, Lidereau R, *et al.* Genetic alteration of the c-myc protooncogen (MYC) in human primary breast carcinomas. *Proc Natl Acad Sci USA* 1986, **83**, 4834–4838.
3. Varley JM, Swallow JE, Brammal WJ, Whittaker JL, Walker RA. Alterations to either c-erbB-2 (neu) or c-myc proto-oncogene in breast carcinomas correlate with poor short-term prognosis. *Oncogene* 1987, **1**, 423–430.
4. King CR, Kraus MH, Aaronson SA. Amplification of a novel v-erbB-related gene in a human mammary carcinoma. *Science* 1985, **229**, 974–976.
5. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* 1987, **235**, 177–182.
6. Van de Vijver M, Van de Bersselaar R, Devilee P, Cornelisse C, Peterse J, Nusse R. Amplification of the neu (erbB-2) oncogene in human mammary tumors is relatively frequent and is often accompanied by amplification of the linked c-erbA oncogene. *Mol Cell Biol* 1987, **7**, 2019–2023.
7. Battifora H, Cline MJ, Yokota J. Proto-oncogene abnormalities in human breast cancer: correlations with anatomic features and clinical course of disease. *J Clin Oncol* 1987, **5**, 999–1006.
8. Zhou D, Battifora H, Yokota J, Yamamoto T, Cline MJ. Association of multiple copies of the c-erbB-2 oncogene with spread of breast cancer. *Cancer Res* 1987, **47**, 6123–6125.
9. Zeillinger R, Kury FD, Czerwenka K, *et al.* HER-2 amplification, steroid receptors and epidermal growth factor receptors in primary breast cancer. *Oncogene* 1989, **4**, 109–114.
10. Lidereau R, Escot C, Theillet C, *et al.* High frequency of rare alleles of the human c-Ha-ras-1 proto-oncogene in breast cancer patients. *J Natl Cancer Inst* 1986, **77**, 697–701.
11. Ali IU, Campbell G, Lidereau R, Callahan R. Lack of evidence for the prognostic significance of c-erbB-2 amplification in human breast carcinoma. *Oncogene Res* 1988, **3**, 139–146.
12. Berger MS, Locher GW, Saurer S, *et al.* Correlation of c-erb-2 gene amplification and protein expression in human breast carcinoma with nodal status and nuclear grading. *Cancer Res* 1988, **48**, 1238–1243.
13. Yamamoto T, Ikawa S, Akiyama T, *et al.* Similarity of protein encoded by the human c-erb-B-2 gene to epidermal growth factor receptor. *Nature* 1986, **319**, 230–234.
14. Dizikes GJ, Grody WW, Kern RM, Cederbaum SD. Isolation of human liver arginase cDNA and demonstration of homology between the two human arginase genes. *Biochem Biophys Res Commun* 1986, **141**, 53–59.
15. Mantel N. Evaluation of survival data and two new rank order statistics arising in its consideration. *Cancer Chemother Rep* 1966, **50**, 163–170.
16. Schechter AL, Stern DF, Vaidyanathan L, *et al.* The neu oncogene: an erb-B-related gene encoding a 185,000-M tumor antigen. *Nature* 1984, **312**, 513–516.
17. Bargmann CI, Hung MC, Weinberg RA. The neu oncogene encodes an epidermal growth factor receptor-related protein. *Nature* 1986, **319**, 226–230.
18. Barnes DM, Sato G. Growth of a human mammary tumor cell line in a serum-free medium. *Nature* 1979, **281**, 388–389.
19. Drebin JA, Link VC, Stern DF, Weinberg RA, Greene MI. Down-modulation of an oncogene protein product and reversion of the transformed phenotype by monoclonal antibodies. *Cell* 1985, **41**, 695–706.
20. Drebin JA, Link VC, Weinberg RA, Greene MI. Inhibition of tumor growth by a monoclonal antibody reactive with an oncogene-encoded tumor antigene. *Proc Natl Acad Sci USA* 1986, **83**, 9129–9133.
21. Van de Vijver M, Mooi WJ, Peterse JL, Nusse R. Amplification and over-expression of the neu oncogene in human breast carcinomas. *Eur J Surg Oncol* 1988, **14**, 111–114.
22. Zielinski CC, Kuzmits R, Kubista E, *et al.* Die Bedeutung der Hormonrezeptoren von Mammakarzinomszellen für die Dauer des erkrankungsfreien Intervalls. *Wien klin Wschr* 1985, **97**, 493–497.
23. Zielinski CC, Kubista E, Salzer H, *et al.* Adjuvant chemotherapy combined with Tamoxifen by postmenopausal patients with stage-III breast cancer. *Lancet* 1986, **i**, 1164–1172.
24. Clark GM, McGuire WL, Hubay CA, Pearson OH, Marshall JS. Progesterone receptors as a prognostic factor in stage II breast cancer. *N Engl J Med* 1983, **309**, 1343–1346.
25. Dubik D, Shiu RP. Transcriptional regulation of c-myc oncogene expression by estrogen in hormone-responsive human breast cancer cells. *J Biol Chem* 1988, **263**, 12705–12708.
26. Kraus MH, Popescu NC, Amsbaugh SC, King CR. Overexpression of the EGF receptor-related proto-oncogene erbB-2 in human mammary tumor cell lines by different molecular mechanisms. *EMBO J* 1987, **6**, 605–610.
27. Kury FD, Schneeberger C, Sliutz G, *et al.* Determination of HER-2/neu amplification and expression in tumor tissue and cultured cells using a simple, phenol free method for nucleic acid isolation. *Oncogene* 1990, **5**, 1403–1408.
28. Zhou DJ, Ahuja H, Cline MJ. Proto-oncogene abnormalities in human breast cancer: c-ERBB-2 amplification does not correlate with recurrence of disease. *Oncogene* 1989, **4**, 105–108.