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Clinical Therapy and HER-2 Oncogene Amplification in Breast Cancer: Chemo- vs Radiotherapy

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One hundred and five breast cancer patients with stage T3/4, N±, Mo were treated at random either with a pre- and postoperative chemotherapy (A) (5-drug-combination + tamoxifen) or with a pre- and postoperative radiotherapy (B). Paraffin embedded tissue samples were prepared from tumor material taken by biopsy prior to therapy as well as at surgery from patients of both groups to estimate the HER-2 oncogene copy numbers before and after treatment. In 53 and 50% of the pretherapeutic samples the HER-2 gene was amplified in groups A and B, respectively. In the posttherapeutic group 60% of the chemotherapy and 48% of the radiotherapy patients, respectively, had low or high HER-2 oncogene copy numbers. In addition, HER-2 amplification before and after therapy was estimated in 28 patients. An increase of oncogene copy numbers could be detected in 21% of the chemotherapy patients, and a decrease was noted in 11%. No radiotherapy patient showed a rise, but 11% a loss of copy numbers. Although amplification of HER-2 oncogene was not found to be associated with overall survival as it was in many studies before, it could still be a predictor of clinical outcome and the cause of mammary carcinomas developing into stage T3/4.

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INTRODUCTION

The HER-2 (neu/c-*erb*B-2) oncogene is located on chromosome 17q21 and encodes a 185 kDa glycoprotein with tyrosine kinase activity [1]. It is of interest because it was reported to be amplified in 10–30% of human mammary carcinomas and seems to be associated with overall survival and disease free survival time [1, 2]. Although the situation is still controversial [1, 3], it is thought to be a valuable prognostic parameter [4, 5] with worse prognosis for patients with amplified HER-2.

Tamoxifen is an antiestrogen that blocks the estrogen receptor and thus decreases the growth of estrogen receptor positive mammary carcinomas [6]. It is widely used in endocrine therapy of receptor positive breast cancer patients and is also discussed as a possible agent to prevent the development of breast cancer in high-risk women [7].

The aim of the present study was to investigate, whether HER-2 gene amplification is changed by either radiotherapy or hormone therapy in primary mammary carcinomas with stage T3/4, N±, Mo.

EXPERIMENTAL

Patients

One hundred and five patients with primary breast cancer at stage T3/4, N±, Mo were recruited for this study between 1983 and 1989. They were randomized to two different therapies (A and B) before surgery. Therapy A consisted of chemotherapy (5-drug combination therapy + tamoxifen) while therapy B was performed by radiotherapy. Prior to either therapy a tissue sample was taken by biopsy and embedded in paraffin. Similarly, a tissue sample obtained from the tumor at surgery was embedded in paraffin.

Patients' characteristics and mean receptor statuses are shown in Tables 1 and 2.

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Therapy

The average interval between biopsy and excision of the tumor was 4 months. In this period either a chemotherapy or a radiotherapy was prescribed to all 105 patients.

Chemotherapy patients (therapy A) received one 10-mg tablet of tamoxifen orally t.i.d. and additionally 2 cycles of a chemotherapeutic regimen: on days 1 and 8 1000 mg 5-fluorouracil, 25 mg methotrexate, 500 mg cyclophosphamide i.v.; day 28: 1 mg vincristine, 14 mg/m^2 mitoxantrone i.v. were given, on day 49 the cycle was repeated.

Radiotherapy (therapy B) was done according to the methods of Frischbier *et al.* [8, 9]. A total dose of 45 Gray with a single dose of 2 Gray delivered over a period of 4–5 weeks was given to the internal mammary and supraclavicular lymph node areas using cobalt 60 beams.

DNA-preparation

Two 10 μm paraffin embedded sections of mammary cancer tissue essentially free of stroma were deparaffinized with 1.5 ml isooctane. Each sample was vortexed thoroughly, incubated at 70°C for 5 min and spinned down at 12,000 *g* for about 5 min. The used isooctane was decanted and replaced by 1.5 ml of fresh one. After four cycles of extraction the samples were dried in a SpeedVac concentrator and incubated with 65 μl proteinase K lysis buffer (1 mM $CaCl_2$, 0.5% Tween 20, 10 mM Tris–HCl pH 8.0 with 20 μg proteinase K) at 56°C for 4 h. The enzyme was denatured by boiling the samples for 20 min at 98°C [10].

PCR reaction

The DNA extraction supernatant (0.5–1.0 μl) was vortexed with 49.5 and 49.0 μl of reaction mix, respectively, (0.2 mM dNTP, 0.5 μM of each primer: Dif1, Dif2, PC03, KM38, 2 U/50 μl Taq polymerase, 100 mM KCl, 4 mM $MgCl_2$, 20 mM Tris–HCl pH 8.4, 0.001% gelatine G2500). Each mix contained one pair of primers for the HER-2 oncogene and another one for the β globin gene which served as reference gene in differential PCR. The primers for the HER-2 gene, Dif1 as sense-primer (5′ CCTCTGACGTCCATCATCTC3′) and Dif2 as antisense-primer (5′ ATCTTCTGCTGCCGTCGCTT3′), and for the β globin—reference gene, PC03 (5′ ACACAACTGTGTTCACTAGC3′) and KM38 (5′ TGGTCTCCTTAAACCTGTCTT3′) were used as published previously [11].

DNA was amplified in a Thermocycler using the following parameters:

	Denaturation	*Annealing*	*Extension*
Start cycle:	2 min at 94°C	2 min at 55°C	2 min at 72°C
30 cycles:	30 s at 94°C	30 s at 55°C	1.5 min at 72°C
Last cycle:			5 min at 72°C

In every PCR run a normal placenta DNA was used as single copy control and another tube containing reaction mix without DNA as negative control.

Electrophoresis and densitometry

The PCR products were separated by agarose gel electrophoresis using a 3% gel in 1 × TAE (1.5% NusieveGTG, 1.5% low melting agarose by BIO-RAD®). The gels were stained with ethidium bromide in 1 × TAE (2 mg/l). Each gel contained one lane with the single copy control and another one with the negative control.

A Hirschmann elscript 400 densitometer was used for scanning the Polaroid® negatives (type 55). The oncogene copy number was estimated from the ratio of peak areas, using the placenta ratio for normalization. Class definition was according to Slamon *et al.* [1], i.e. "single copy" (amplification up to 1.50), "low copy" (amplification 1.51–5.00) and "high copy" (amplification higher than 5.00).

Validation of the PCR method

The validity of the quantitative PCR method was examined by comparing HER-2 amplification data obtained by dot blot and PCR systems using identical DNA samples extracted from frozen tissue as published previously [10]. Linear regression analysis resulted in a slope of 0.98 and an intercept of 0.05 with a correlation coefficient of 0.81 (data not shown).

Reproducibility was checked by estimating single copy DNA from placental tissue and amplified DNA samples of ovarian cancer tissues using the same block on separate occasions. The PCR experiments resulted in a coefficient of variation of 12% in terms of absolute figures and the same degree of amplification was observed when 20 experiments were evaluated.

Statistics

Statistical analysis of data was carried out by Wilcoxon scores, Kruskal–Wallis tests, Chi-square tests and Kaplan–Meier analysis.

RESULTS

DNA could be successfully extracted from paraffin embedded tissue samples of 91/105 breast cancer patients out of which 56 samples were pretherapeutical and 63 posttherapeutical, respectively. 28 patients provided samples to both groups.

In the pretherapeutic group 27/56 (48%) samples showed single copy HER-2 oncogene whereas in 29/56 (52%) samples the HER-2 gene was found to be amplified. HER-2 copy numbers were balanced between patients that underwent therapy A and B, respectively (Table 3). In the posttherapeutic group 27/63 (43%) patients had single copy HER-2 gene and 36/63 (57%) amplified oncogene, respectively. The mean standard variation of all amplification results was 12%.

Table 1. General patients' characteristics

	Chemotherapy	Radiotherapy
Mean diameter of tumor (cm)	9.2 ± 1.8	7.2 ± 2.8
Positive lymphe nodes (%)	77	79
Negative lymphe nodes (%)	21	23
Mean age at operation (years)	60.6 ± 14.4	60.8 ± 9.7
Menarche (years)	13.6 ± 1.5	13.7 ± 1.7
Age at menopause (years)	43.9 ± 15.2	44.7 ± 15.6
Premenopausal (%)	23	22
Perimenopausal (%)	9	8
Postmenopausal (%)	68	70
Number of children	1.4 ± 1.3	1.6 ± 1.7

Table 3. HER-2 amplification in pre- and posttherapeutical breast cancer samples

		HER-2 amplification					
		SC		LC		HC	
			%		%		%
Pretherapeutic	CT	16	47	18	53	0	0
(n = 56)	RT	11	50	11	50	0	0
Posttherapeutic	CT	15	38	24	60	1	2
(n = 63)	RT	12	52	11	48	0	0

CT = chemotherapy; RT = radiotherapy; SC = single copy HER-2 gene (<1.51 copies), LC = low copy HER-2 gene (1.51–5.00 copies); HC = high copy HER-2 gene (>5.00 copies).

A comparison of pretherapeutic with posttherapeutic data shows that there is very little difference in HER-2 amplification in the radiotherapy group (Table 3). It is noteworthy that the percentage of samples with amplified HER-2 oncogene increased in the chemotherapy group even yielding one sample with high copy oncogene.

Looking in more detail at the 28 patients from whom tissue samples were taken by biopsy prior to therapy as well as at surgery, changes in HER-2 amplification can be observed (Table 4). In 1/9 (11%) patients who were treated by radiotherapy the class of HER-2 amplification decreased whereas it remained unchanged in 8/9 (89%) cases. In the chemotherapy group HER-2 amplification class stayed constant in only 13/19 (68%) of mammary carcinomas. It increased in 4/19 (21%) of the cases and decreased in 2/19 (11%) of the patients. This trend was not statistically significant because of the small sample numbers.

Statistical tests did not show any significant correlation between HER-2 copy numbers and steroid receptor levels, disease free interval and overall survival time (Fig. 1).

DISCUSSION

The present study shows the influence of a combined tamoxifen plus cytotoxic chemotherapy on HER-2 copy numbers. It is important to note that the tissue samples were derived from stage T3/4 mammary carcinomas. This might be the reason for the high percentage (about 50%) of amplified samples (Table 3), whereas previous studies reported HER-2 amplification between 10–30 % [1, 2]. The high incident of amplified HER-2 in the present study suggests that this oncogene is involved in the biology and the progression of mammary tumors.

Lack of association of HER-2 amplification with overall-survival in the present investigation is in contrast to previous reports [1]. In addition, no correlation between HER-2 amplification and disease free interval was observed recently [12]. The discrepancy between results of the present study, a recent report [12] and previous observations [1] is not fully understood at present. But the importance of protooncogene products in the regulation of cell proliferation is continually reaffirmed.

It was also interesting to note that therapy A seemed to cause an increase of HER-2 copy numbers (Table 3 and 4). This trend was not statistically significant, which might be due to the small number of patients. Similar effects have already been noted in expression of HER-2 RNA: tamoxifen enhances the expression of HER-2 in estrogen-receptor positive cells *in vitro* [13]. The concordance between HER-2 amplification and overexpression could be detected as published earlier [14]. Radiotherapy obviously does not influence HER-2 amplification. A decrease in oncogene amplification as occurred in three samples (Table 4) could be caused by clonal selection of polyclonal tumors.

Present data combine to suggest that tamoxifen plus cytotoxic chemotherapy although most beneficial for patients with estrogen receptor positive breast cancer might cause an increase in HER-2 oncogene copy numbers. Further studies are necessary to corroborate this notion.

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Table 2. Effects of therapy on mean receptor content

	Therapy	E2-receptor	Pg-receptor	Ag-receptor
Pretherapeutic	CT	31.7 ± 62	87.8 ± 199	33.0 ± 53
	RT	100.5 ± 130	103.0 ± 293	30.4 ± 24
Posttherapeutic	CT	19.6 ± 55	36.1 ± 98	28.7 ± 36
	RT	68.6 ± 134	76.4 ± 294	19.9 ± 27

E2-receptor = estrogen receptor (fmol); Pg-receptor = progesterone receptor (fmol); Ag-receptor = androgen receptor (fmol); CT = chemotherapy; RT = radiotherapy.

Table 4. HER-2 amplification in patients undergoing chemo- or radiotherapy

	HER-2 amplification					
Therapy	No change		Increase		Decrease	
CT (n = 19)	13	68%	4	21%	2	11%
RT (n = 9)	8	89%	0	0%	1	11%

CT = chemotherapy; RT = radiotherapy.

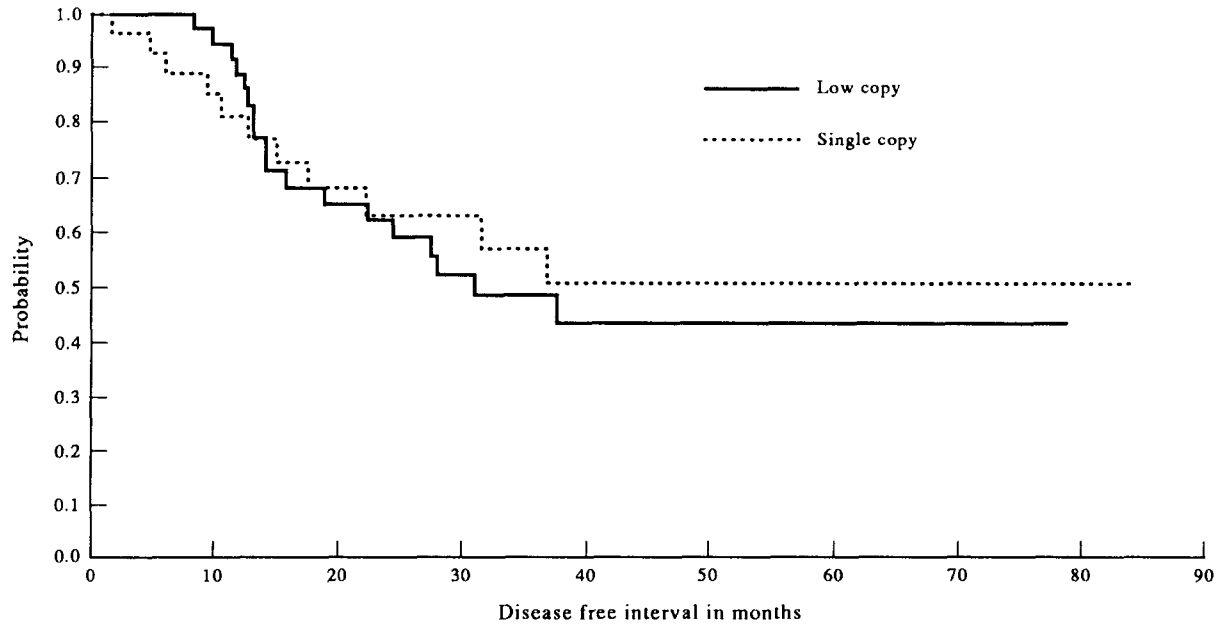

Fig. 1. Association between HER-2 amplification and disease free interval. Single copy = single copy HER-2 oncogene; low copy = low copy HER-2 oncogene.

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