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HER-2 Amplification Impedes the Antiproliferative Effects of Hormone Therapy in Estrogen Receptor-positive Primary Breast Cancer

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ABSTRACT

In experimental models, human epidermal growth factor receptor-2 (HER-2) amplification leads to estrogen independence and tamoxifen resistance in estrogen receptor (ER)-positive human breast cancer cells. Some but not all reports suggest an association between HER-2 positivity and hormone independence in breast cancer patients. This study aimed to evaluate the antiproliferative effects of endocrine therapy in HER-2-positive/ER-positive primary human breast cancer.

The effect on proliferation (Ki67) of hormone therapy was assessed at 2 weeks and/or 12 weeks in biopsies from 115 primary breast cancers with ER-positive tumors. The patients took part in one of 3 neoadjuvant trials of hormonal therapy with a SERM (tamoxifen or idoxifene) or an aromatase inhibitor (anastrozole or vorozole). HER-2 status was assessed by immunocytochemistry and fluorescence *in situ* hybridization (FISH). Fifteen patients were defined as HER-2 positive by both immunohistochemistry and FISH, with the remaining 100 patients HER-2 negative. Geometric mean Ki67 levels were substantially higher in HER-2-positive than HER-2-negative tumors (27.7% *versus* 11.5%, respectively; $P = 0.003$). In HER-2-negative patients, Ki67 was reduced by 62 and 71% at 2 and 12 weeks, respectively ($P < 0.0001$ for both), but HER-2-positive patients showed no significant fall. The proportional change in Ki67 was significantly different between HER-2-positive and -negative patients ($P = 0.014$ at 2 weeks; $P = 0.047$ at 12 weeks). Mean ER levels were lower in the HER-2-positive patients ($P = 0.06$) but the change in Ki67 was impeded even in those with high ER. Apoptotic index was reduced by 30% at 2 weeks in the HER-2-negative group. However, there were no statistically significant differences in apoptotic index between the groups. It is concluded that ER-positive/HER-2-positive primary breast carcinomas show an impeded antiproliferative response to endocrine therapy that nonetheless may vary between individual treatments. This together with high baseline proliferation is likely to translate to poor clinical response.

INTRODUCTION

The *HER-2*[2] proto-oncogene encodes a M_r 185,000 glycoprotein that has substantial homology with the other three members of the family, HER-1 (also known as epidermal growth factor receptor), HER-3, and HER-4. At least six ligands have been identified that bind differentially to these latter three proteins (1), which act as transmembrane receptors. However, none have been identified for HER-2, which appears to exert its biological activity by heterodimerizing as a preferred partner to one of the other three family members after their activation by ligand binding. The heterodimerization leads to autophosphorylation as a result of intrinsic tyrosine kinase activity and thereby triggers a cascade of signaling to the nucleus and subsequent gene activation.

Transfection of the *HER-2* gene into breast (and other) cancer cells leads to aggressive growth characteristics (such as increased proliferation and growth in soft agar and nude mice models) and to enhanced tumorigenicity and metastatic potential (2, 3). These properties can be reversed by monoclonal antibodies directed against HER-2.

The *HER-2* gene is amplified in ~20% of human breast cancers. In almost all cases, this amplification is associated with increased protein expression. Patients with such overexpressing/amplified tumors have a poorer prognosis than those with HER-2-negative tumors (4, 5). There is some evidence that HER-2 status is a predictor for response/resistance to specific chemotherapeutic agents. Of particular importance, positive HER-2 status defines those patients most likely to respond to the humanized monoclonal antibody to HER-2, trastuzumab (Herceptin; Refs. 6).

Transfection of the *HER-2* gene to achieve amplification in ER-positive human breast cancer cells also results in acquisition of estrogen-independent growth that is resistant to therapy with the antiestrogen tamoxifen (2). Several studies have reported that HER-2 positivity is associated with resistance to hormonal therapy (7–9). Despite being of relatively small size, the results of the GUN Trial (9) have been of particular concern, because these indicated that HER-2 overexpression predicted worse outcome for patients on adjuvant tamoxifen than those untreated. Our own study[3] showed a statistically significant adverse interaction between HER-2 positivity and outcome from adjuvant tamoxifen therapy. The point estimate was for a worse outcome for those HER-2-positive patients receiving tamoxifen (relative risk, 1.14), but the CIs were wide (0.75–1.73) and the relative risk for ER-positive HER-2-positive patients was 0.80 (0.39–1.64). Thus, although this indicated reduced benefit, and possibly no benefit, for tamoxifen, there was no clear evidence of enhanced disease progression.

There are further reports finding no significant interaction between HER-2 positivity and hormone sensitivity (10, 11). In the CALGB 8541 trial, tamoxifen significantly improved disease-free survival and overall survival irrespective of HER-2 status, although the data from this study are complicated by the combined usage with cyclophosphamide/doxorubicin/fluorouracil, which itself may have an interaction with HER-2 (10). Elledge *et al.* (11) found no evidence for a poorer response of HER-2-positive patients to tamoxifen in 205 patients with advanced disease. They cited the important issue that because there is a negative association between ER and HER-2 status, studies that failed to rigorously exclude ER-negative patients may have overestimated the effects of HER-2 expression on tamoxifen response.

The current study examines the relationship of HER-2 positivity with hormone resistance by assessing the impact of HER-2 status on the antiproliferative effects of presurgical endocrine therapy in 115 patients known to have ER-positive tumors. Patients from three studies involving the SERMs, tamoxifen and idoxifene, and the aromatase inhibitors, anastrozole and vorozole, were pooled to provide sufficient power, given the expected low prevalence of the HER-2-positive/ER-

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[2] The abbreviations used are: HER-2, human epidermal growth factor receptor-2; CI, confidence interval; AI, apoptotic index; SERM, serum estrogen response modifier; ER, estrogen receptor; PgR, progesterone receptor; FISH, fluorescence *in situ* hybridization.

[3] Dowsett, M., Houghton, J., Iden, C., Salter, J., Farndon, J., A'Hern, R., and Baum, M. Oestrogen receptor, progesterone receptor, EGF receptor and c-erbB2 status for discriminating primary breast cancer patients benefiting from adjuvant tamoxifen therapy. Lancet, submitted for publication, 2001.

positive phenotype. Positive immunohistochemical HER-2 status was confirmed (or refuted) by FISH to avoid the uncertainties that have been ascribed to the use of immunohistochemistry alone (12). Importantly, the measurement of changes in proliferation enabled us to assess the effects of therapy that might be undetected by clinical measurements; the latter will classify a tumor that is growing more slowly as the result of treatment as a nonresponder.

PATIENTS AND METHODS

Presurgical Studies

Tissue samples were selected for assessment of HER-2 status from three presurgical studies of hormonal therapy in postmenopausal patients if: (*a*) they were ER positive; and (*b*) a pretreatment biopsy and an additional biopsy at 2 weeks and/or 12 weeks after starting therapy were available. A total of 115 patients were eligible by these criteria. Each of the studies was multicenter, but all study biopsy tissues after fixation and blocking were sent to the Royal Marsden Hospital for staining. The primary end point of each of the studies was the change in the proliferation marker Ki67 after ~2 weeks (see below for range of time for individual trials). The core-cut biopsies were in all cases taken with a 14-gauge needle; the precision of measurements of proliferation and apoptosis when using this size of core-cut has been published earlier by our group (13). These precision data on Ki67 were used to power one of the studies (IMPACT), whereas the others were powered informally by comparison with earlier published studies with Ki67 as their primary end point. The details of the individual studies are as follows.

Idoxifene Study. The principal results (excluding HER-2 status) of this trial have been published elsewhere (14). The study was multicenter (12 centers), randomized, and double-blind in postmenopausal women with primary operable breast cancer. Patients received 40 mg/d p.o. idoxifene or placebo for ~14 days prior to surgery to a maximum of 21 days. A core-cut biopsy was taken prior to treatment, and a representative sample of the tumor was obtained at surgery. Seventy-seven patients were enrolled. Only the 40 patients in the idoxifene-treated arm were studied. Four of these were withdrawn or excluded from analysis, 3 patients had protocol violations/deviations, and 1 patient had an adverse event necessitating treatment cessation. Of the 36 remaining patients, 30 had ER-positive tumors and were therefore eligible. Two of those had *in situ* disease, and 1 had insufficient tissue for HER-2 analysis in their pretreatment biopsy, leaving 27 for study. The mean duration of treatment was 16.5 days.

Vorozole-Tamoxifen (VorTam) Study. The principal results (excluding HER-2 status) of this study are being published elsewhere (15). Postmenopausal patients with ER-positive primary breast cancer were recruited from five hospitals and randomized to treatment with vorozole 2.5 mg/day p.o. or tamoxifen 20 mg/day p.o. until surgery. Patients were scheduled for surgery after 12 weeks treatment, although some who were considered unsuitable for surgery continued on therapy thereafter. Treatment was not blinded to the clinician or the patient, but the laboratory was blinded to the randomization until completion of analyses. Patients were assessed at 4, 8, and 12 weeks. If there was evidence of progressive disease at 4 or 8 weeks, the patient was to be withdrawn from the study. Core-cut biopsies were taken before and after 2 weeks therapy, and at surgery a representative sample of the excision tumor was obtained. In the situation where surgery was not performed, a core-cut biopsy was taken at 12 weeks. Fifty-three patients were recruited to the study, of whom 26 received vorozole and 27 received tamoxifen. Forty-two of these (22 vorozole and 20 tamoxifen) were assessable for changes in Ki67: 2 withdrew, 1 patient's samples were lost, and 8 had insufficient tissue in the pretreatment or 2-week sample. One patient on tamoxifen had insufficient tissue left in her pretreatment core-cut for HER-2 assessment, leaving 41 patients for study (21 vorozole and 20 tamoxifen). In these 41, both pretreatment and 3-month samples were available, but 2-week samples were available and suitable for analysis from only 31 women (18 tamoxifen and 13 vorozole).

IMPACT Study. This study is on-going and is a multicenter, double-blind, randomized comparison of anastrozole 1 mg/day p.o., tamoxifen 20 mg/day p.o., or the combination of anastrozole plus tamoxifen. Treatment was given after diagnosis, and surgery was scheduled to be 12 weeks later. Core-cut biopsies were taken before and after 2 weeks of treatment, and a representative sample was taken from the tumor at surgery. In patients not proceeding to surgery, a further core-cut was taken at 12 weeks. This is a parallel study to the large ATAC (Arimidax, Tamoxifen Alone or Combined) adjuvant trial (16). The study initially aimed to recruit 150 patients, having been powered on expected changes in Ki67 at 2 weeks. It has since been increased to 330 patients to provide sufficient power for comparisons of clinical response. All patients had ER-positive primary breast cancer. This study was performed on the first 51 patients with repeat biopsy samples. A total of 47 patients were assessable for HER-2. All patients had a pretreatment and 12-week biopsy, and 43 also had a 2-week biopsy. The randomization code for these patients has not been broken, and response data have not been collated.

Tumor Samples

All core-cut and excision biopsy samples were fixed in 10% neutral buffered formalin and embedded in paraffin wax at the local pathology laboratory. In the majority, embedded blocks were sent for processing and analysis to the Royal Marsden Hospital, where histological sections (3-μm thick) were cut onto positively charged slides and dried overnight. In other cases, similar sections were prepared in the local laboratory and then sent to the Royal Marsden Hospital.

Analytical Methods

Analyses of samples from all three trials were performed using the same immunohistochemical methods. Other than the HER-2 methodology, these have been described in detail elsewhere. In brief, measurement of cell proliferation used the MIB1 mouse monoclonal antibody to Ki67 (13). At least 1000 cells were scored in a total of 10 high-powered fields, and the results were expressed as the percentage of positive cells. Apoptotic cells were identified using the terminal deoxynucleotidyltransferase-mediated nick end labeling assay as described previously (13). The number of apoptotic cells was assessed in a total of 3000 malignant cells and expressed as a percentage as the AI. ER staining was by the Novocastra 6F11 mouse monoclonal antibody, and PgR staining was by the Novocastra 1A6 mouse monoclonal antibody (17). ER and PgR expression was assessed semiquantatively by assessing the intensity (scored 0–3) and percentage of cells positive at each intensity in 10 high-powered fields. The two measurements were multiplied and summed to give an H-score varying from 0 to 300. Samples were considered positive for either receptor if the score exceeded 20. For all four of these markers, analyses were conducted on all pretreatment and on-treatment samples. In each case, all samples from the same patient were included in the same assay batch.

All pretreatment samples were assessed for HER-2 by immunohistochemical analysis using the DAKO HercepTest kit with strict adherence to the manufacturer's instructions. Samples scored as 2+ or 3+ were considered positive. In brief, 5-μm-thick, paraffin-embedded sections were dewaxed and taken to water. Sections were placed in epitope retrieval solution at 95–99°C in a water bath for 40 min. After epitope retrieval, sections were allowed to cool in the solution for 20 min at room temperature before endogenous peroxidase activity was blocked with hydrogen peroxide for 5 min. After a brief rinse in wash buffer, sections were incubated in prediluted primary antibody for 45 min, washed in buffer, and then incubated in prediluted visualization reagent for 30 min. After an additional wash in buffer, peroxidase activity was demonstrated with diaminobenzidine for 10 min before counterstaining with Mayer's hematoxylin and mounted in a resinous mountant.

All immunohistochemically positive HER-2 samples were also assessed using the Vysis PathVysion kit, which incorporates a control probe for chromosome 17 as well as the test probe for the *HER-2* gene, according to the manufacturer's instructions. In brief, 4-μm-thick, paraffin-embedded sections were dewaxed, taken to absolute ethanol, and air dried. They were then placed in 0.2 N HCl at room temperature for 20 min, in "pretreatment solution" at 80°C for 30 min, and then underwent a proteolytic digestion at 37°C for 25 min. The sections were then denatured in formamide at 72°C for 5 min before incubation in the PathVysion HER-2/17 probe overnight in the dark at 37°C. The next day, the sections were washed in posthybridization buffer for 2 min at 72°C, air dried in the dark, and then mounted in 4′,6-diamidino-2-phenylindole.

Table 1 *Basic demographics of patients in the clinical trials and distribution of HER-2-positive and -negative tumors among the trials*

Drug/trial	*n*	Age (yr) Mean ± SD	Tumor size (mm) Mean ± SD	HER-2 ICA[a] +ve	HER-2 ICA and FISH +ve	HER-2 ICA or FISH −ve
Vorozole	21	70.0 ± 6.7	26.5 ± 11.5[b]	3	3	18
Tamoxifen	20	72.9 ± 6.2	30.3 ± 11.9[b]	2	1	19
Idoxifene	27	69.2 ± 9.4	24.7 ± 13.8[b]	9	8	19
IMPACT	47	72.4 ± 14.5	37.3 ± 14.5[c]	5	3	44
Total	115	72.2 ± 8.0		19	15	100

[a] ICA, immunohistochemical analysis; +ve, positive; −ve, negative.
[b] Maximum ultrasound diameter.
[c] Maximum caliper diameter.

Statistical Analysis

The data from the three trials were pooled to provide a single set of data on hormonally treated patients. Geometric descriptive statistics are given for Ki67 and AI because of the geometric distribution of the data. Arithmetic descriptive statistics are shown for the ER data, which were normally distributed. All comparative statistics were performed using nonparametric tests, Mann-Whitney for between-group comparisons and Wilcoxon for within-group comparisons.

RESULTS

A total of 115 patients were studied. The basic demographics of this group and the baseline biomarker characteristics are shown in Tables 1 and 2, respectively, the latter according to HER-2 status. Nineteen patients were HER-2 positive by immunocytochemistry, but in 4 of these the gene was not found to be amplified by a factor of at least 2. Thus, 15 patients (13%) were considered HER-2 positive and 100 HER-2 negative. Mean Ki67 levels were more than twice as high in the HER-2-positive as in the HER-2-negative group (27.7% *versus* 11.5% positive cells; $P = 0.003$).

ER levels were lower in the HER-2-positive group than in the negative group ($P = 0.06$). Twenty-six patients had ER <150, of which 9 (35%) were HER-2 positive. In contrast of the 89 patients with ER >150, only 6 (7%) were HER-2 positive. The proportion of PgR-positive tumors was also lower, but not significantly so ($P > 0.20$) in the HER-2-positive group.

In the HER-2-negative group, Ki67 levels fell to a mean 38 and 29% of pretreatment levels after 2 and 12 weeks of treatment, respectively ($P < 0.0001$ for both; Table 3A). Less marked and statistically insignificant falls occurred in the 15 HER-2-positive patients to 75% at 2 weeks ($P = 0.23$) and 71% at 12 weeks ($P = 0.09$). The proportional fall in Ki67 was significantly different between HER-2-positive and -negative groups at both the 2- and 12-week time points ($P = 0.014$ and 0.047, respectively). After both 2- and 12-week treatments, mean Ki67 levels in the HER-2-positive group remained nearly twice as high as that before treatment in the HER-2-negative group (Fig. 1).

Individual changes in Ki67 levels are shown for the HER-2-positive group according to treatment received in Fig. 2. Five of the 15 patients showed falls of 50% or greater at 2 weeks, compared with 46 of 86 in the HER-2-negative group (not significant). In 4 patients, there were increases in Ki67 over the first 2 weeks; 1 was on vorozole, 1 was in the IMPACT trial; and 2 were on idoxifene.

The levels of and changes in Ki67 were also assessed according to PgR status and compared statistically in the HER-2-negative patients (subgroups too small for comparison in the HER-2-positive group; Table 4; Fig. 3). There were highly significant falls in Ki67 at both 2 and 12 weeks for both the PgR-positive and -negative groups. However, the proportional fall was greater for the PgR-positive group, particularly at 12 weeks (80% *versus* 51% fall; $P = 0.0056$). After 12 weeks of treatment, the mean Ki67 level in the HER-2-negative PgR-positive group was only 2.1%.

The degree of fall in Ki67 after 2 weeks treatment was significantly related to the ER H-score: for the total set of 101 tumors with pretreatment and 2-week values, $r = 0.302$ and $P = 0.002$; for HER-2 negative tumors, $r = 0.264$ and $P = 0.04$. The data are displayed in Fig. 4, with the regression line drawn for the HER-2-negative tumors. The regression line for HER-2-positive tumors was not significantly different, but 12 of the 15 HER-2-positive tumors plotted were above the HER-2-negative regression line. There was a cluster of 6 HER-2-positive tumors with relatively high ER values. The change in Ki67 in these 6 HER-2-positive tumors was compared with the 43 others with ER >200. The proportional reduction in Ki67 was significantly lower in the HER-2-positive tumors than the HER-2-negative tumors in this high ER subgroup ($P = 0.02$).

No significant changes in AI were found in the HER-2-positive group (Table 3B). In the HER-2-negative group, AI levels fell to a mean 77% of pretreatment levels after 2 weeks ($P = 0.0005$) and recovered to 88% of pretreatment levels by 12 weeks, a value not significantly different from pretreatment. However, the proportional changes in AI at 2 and 12 weeks were not significantly different between the HER-2-negative and -positive groups.

DISCUSSION

Several recent papers have suggested that HER-2 positivity of breast carcinomas may be indicative of resistance to hormonal (predominantly tamoxifen) therapy, but the data are by no means conclusive (7–11). The heterogeneity of the published data may in part result from the ER status of the tumor not being considered; there is an inverse relationship between ER positivity and HER-2 positivity, such that only ~50% of HER-2-positive tumors are ER positive in contrast

Table 2 *Levels of ER, PgR, Ki67, and apoptosis in the HER-2-positive and -negative patients before treatment*

	ER, H-score Mean ± SE	PgR No. of patients +ve[a] (%)	Ki67 % Geometric mean (95% CI)	AI % Geometric mean (95% CI)
HER2−	194 ± 6	68 (69%)	11.5 (9.3–14.1)	0.87 (0.76–1.00)
n	100	99	100	88
HER2+	152 ± 17	7 (47%)	27.7 (21.5–35.7)	1.09 (0.76–1.58)
n	15	15	15	14
HER2− *vs.* HER2+	$P = 0.06$	$P > 0.20$	$P = 0.003$	$P > 0.20$

[a] +ve, positive.

Table 3 *Changes in Ki67 (A) and AI (B) according to HER-2 status*

Mean values at 2 (2w) and 12 (12w) weeks are shown as absolute percentages and as a proportion of the baseline percentage. "Pre" describes pretreatment.

	A. Ki67				
	Ki67 (%)			Ki67 (ratio)	
	Pre	2w	12w	2w:pre	12w:pre
HER2−					
Geometric mean	11.5	4.7	3.2	0.38	0.29
95% CI	9.3, 14.1	3.6, 6.1	2.3, 4.5	0.30, 0.48	0.21, 0.39
Total no.	100	86	81	86	81
Pre *vs.* on treatment		$P < 0.0001^a$	$P < 0.0001^a$		
HER2+					
Geometric mean	27.7	20.9	21.8	0.75	0.71
95% CI	21.5, 35.7	13.9, 31.4	11.0, 43.2	0.51, 1.10	0.48, 1.04
Total no.	15	15	7	15	7
Pre *vs.* on treatment		$P > 0.10^a$	$P = 0.09^a$		
HER2− *vs.* HER2+	$P = 0.003^b$			$P = 0.014^b$	$P = 0.047^b$
	B. AI				
	AI (%)			AI (ratio)	
	Pre	2w	12w	2w:Pre	12w:Pre
HER2−					
Geometric mean	0.87	0.70	0.81	0.77	0.88
95% CI	0.76, 0.99	0.61, 0.81	0.70, 0.94	0.67, 0.88	0.75, 1.04
Total no.	88	76	67	76	67
Pre *vs.* on treatment		$P = 0.0005^a$	$P > 0.10^a$		
HER2+					
Geometric mean	1.09	1.11	1.37	1.02	1.02
95% CI	0.79, 1.52	0.75, 1.65	0.77, 2.43	0.81, 1.27	0.51, 2.05
Total no.	14	13	6	13	6
Pre *vs.* on treatment		$P > 0.10^a$	$P > 0.10$		
HER2− *vs.* HER2+	$P > 0.10^b$			$P > 0.10^b$	$P > 0.10^b$

[a] Wilcoxon.
[b] Mann-Whitney.

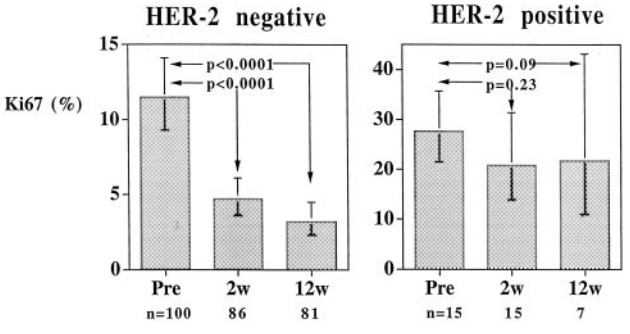

Fig. 1. Geometric mean levels (±95% CI) of Ki67 before and during therapy according to HER-2 status. *Bars*, SD. *Pre*, pretreatment; *2w*, 2 weeks; *12w*, 12 weeks.

to ~75% of the whole population.[3] In essence, and as noted by Elledge *et al.* (11), much of the reported hormonal insensitivity of HER-2-positive tumors could result from ER negativity rather than HER-positivity *per se*. That study also showed trends indicating a modestly shorter time to treatment failure for ER-positive, HER-2-positive tumors than ER-positive, HER-2-negative tumors treated with tamoxifen.

An additional factor may be that many of the studies of this relationship have been conducted in adjuvant trials (9, 10). In these, individual response is impossible to categorize, and large numbers of ER-positive/HER-2-positive patients are required to establish statistically significant differences between groups. To date, no adjuvant trial that has assessed this relationship has had the statistical power to exclude a benefit from tamoxifen in this group. In studies that have been conducted in patients with metastatic disease, the biological measurements of HER-2 status have invariably been conducted on the primary lesion, which has a temporal, topological, and possibly biological dissociation from the metastatic sites in which response is assessed.

Lastly, many different antibodies and staining protocols have been used to characterize HER-2 status of the disease. These are known to give very different results and to relate variably to HER-2 amplification (12).

In this study, we have attempted to minimize these problems by: (*a*) selecting an entirely ER-positive group of patients; (*b*) conducting FISH analysis to confirm any immunocytochemically detected overexpression; and (*c*) conducting the study in primary disease in which the response can be assessed in the same lesion as the HER-2 measurement. A novel aspect of the study was the assessment of the biological response, *i.e.*, change in proliferation, rather than clinical response of the tumor. A strength of this is that biological changes in the determinants of tumor growth (*i.e.*, proliferation or cell death) may be identifiable that may not be reflected as a clinical response. This principle is illustrated by xenograft studies in which a 50% reduction

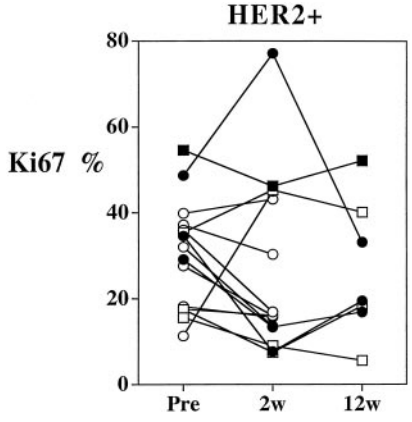

Fig. 2. Individual changes in Ki67 in the HER-2-positive group according to treatment received: ○, idoxifene; ●, vorozole; □, IMPACT; ■, tamoxifen.

Table 4 *Changes in Ki67 according to combined HER-2 and PgR status*[a]

Mean values at 2 (2w) and 12 (12w) weeks are shown as absolute percentages and as a proportion of the baseline percentage. "Pre" describes pretreatment.

	Ki67 (%)			Ki67 (ratio)	
	Pre	2w	12w	2w:pre	12w:pre
HER2−, PgR−					
Geometric mean	14.3	7.1	7.2	0.46	0.49
95% CI	10.2–20.1	4.3–11.6	4.0–12.9	0.33–0.65	0.30–0.80
Total no.	31	29	26	29	26
Pre *vs.* on treatment		$P = 0.0019$[b]	$P = 0.0036$[b]		
HER2−, PgR+					
Geometric mean	10.4	3.8	2.1	0.34	0.20
95% CI	8.0–13.5	2.8–5.1	1.4–3.2	0.25–0.47	0.15–0.32
Total no.	68	57	54	57	54
Pre *vs.* on treatment		$P < 0.0001$[b]	$P < 0.0001$[b]		
PgR− *vs.* PgR+	0.159[c]			0.125[c]	0.0056[c]
HER2+, PgR−					
Geometric mean	29.9	25.9	26.3	0.87	0.82
95% CI	19.8–45.0	12.5–53.5	14.1–48.8	0.41–1.83	0.48–1.40
Total no.	8	8	4	8	4
HER2+, PgR+					
Geometric mean	25.5	16.3	17.0	0.64	0.58
95% CI	17.0–38.2	10.2–25.9	1.10–272.5	0.48–0.85	0.17–1.97
Total no.	7	7	3	7	3

[a] PgR unavailable on one HER-2− patient.
[b] Wilcoxon.
[c] Mann-Whitney.

in Ki67 combined with a doubling of apoptosis can be associated with only stable disease (18). Thus, these biological end points may be more sensitive of antitumor effects than clinical response. Nonetheless, it is important to note that overall a significant relationship between change in proliferation, as measured by Ki67, after 2 weeks and clinical response has been found in two of our previous (15, 19) studies, and this has been confirmed recently by an independent group (20). Additionally, Miller *et al.* (21) have reported a significant relationship between change in the related proliferation marker KiS1 and clinical response to tamoxifen after 3 months.

Patients from three clinical trials had to be pooled to derive enough ER-positive/HER-2-positive patients for a study with sufficient statistical power. A strength of the study was that each of the individual trials had their pathological assessments conducted by identical techniques in the same laboratory, but because a number of different endocrine therapies were also pooled, the study cannot exclude the possibility that there may be differences between certain endocrine therapies and their interaction with HER-2. Additionally, over half of the patients that were HER-2 positive came from the idoxifene study; thus, the data may reflect interactions with SERMs rather than with estrogen deprivation. SERMs act by competitive binding to ER (α and β) and lead to conformational changes in it which differ from those elicited by estradiol. The resultant activated receptor is thought to have estrogen agonist or antagonist effects, according to the particular levels of transcriptional coactivators or corepressors in the cell (22). In model human breast cancer systems, idoxifene was found to have less agonist activity than tamoxifen, but the clinical significance of this, if any, is not known (23).

Anastrozole and vorozole are potent third-generation aromatase inhibitors that lead to near complete estrogen deprivation in postmenopausal women (24). These compounds differ from SERMs in that their mode of action is simply to reduce levels of ligand to the ER. Some data indicate that ER-dependent transcriptional activity in a HER-2-positive breast cancer cell line can be repressed by estrogen withdrawal but not by tamoxifen (25), which would suggest that a differential sensitivity to these agents might exist in breast cancer.

It is widely recognized that HER-2-positive tumors generally have a more aggressive phenotype than HER-2-negative tumors as reflected by the patients' poor prognosis (4, 5), and it has been reported previously that proliferation is higher in the HER-2-positive group (26). The data presented here demonstrated that this extends to the ER-positive subgroup of patients. The finding that those patients whose tumors were PgR positive had a greater reduction in Ki67 than those who had PgR-negative tumors is consistent with the widely recognized finding of a greater likelihood of a clinical response to hormone therapy in patients with PgR-positive cancers (27). These data provide further support for changes in Ki67 being related to clinical outcome.

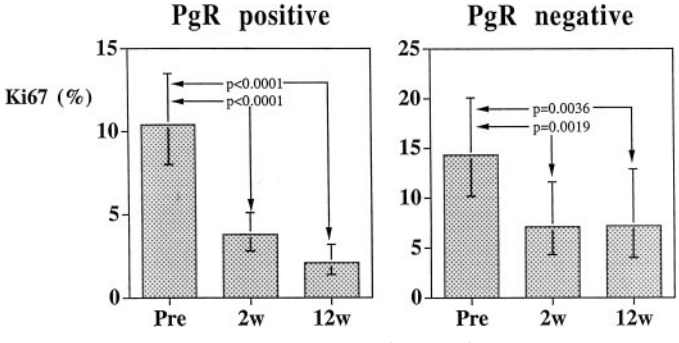

Fig. 3. Geometric mean levels (±95% CI) of Ki67 before and during therapy in HER-2-negative tumors according to PgR status. *Bars,* SD. *Pre,* pretreatment; *2w,* 2 weeks; *12w,* 12 weeks.

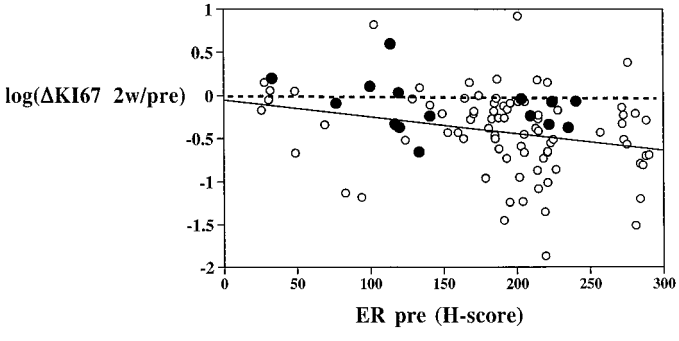

Fig. 4. Relationship between proportional change in Ki67 after 2 weeks (plotted as log base 10) and ER H-score. Smaller ○, HER-2 negative; larger ●, HER-2 positive. The *dotted line shows* the level of no change. The *solid line* shows the linear regression line for HER-2-negative tumors ($r = 0.264$; $P = 0.04$).

As well as changes in proliferation, changes in apoptosis may account for a change in growth rate. Measurement of AI during treatment was therefore also conducted in this study. However, its interpretation in a group of patients treated with different endocrine agents must be cautious because we have reported recently that during the first 2 weeks of treatment, tamoxifen appears marginally to enhance AI, but aromatase inhibitors significantly reduce apoptosis (15). Overall, there was a significant decrease in AI in the HER-2-negative patients at 2 weeks of 30%. This occurs at the same time as, but is quantitatively less important than, the 62% fall in Ki67. This reduction in AI may result from the movement of HER-2-negative cells out of the cell cycle by hormonal therapy, thus possibly leading to a partial resistance to apoptosis. In contrast to the differential effects on Ki67 in HER-2-positive and -negative tumors, there were no significant differences in AI. Together with previous data linking clinical response with change in proliferation (19, 20, 28), this study is consistent with the view that changes in proliferation are the dominant factor in determining a response to endocrine therapy.

The current data indicate clearly that there is a significantly greater reduction in Ki67 for the HER-2-negative breast cancer patients than the HER-2-positive patients at both 2 weeks and 12 weeks after starting treatment. This greater effect on proliferation indicates strongly that there is a greater likelihood of clinical response in the HER-2-negative group. It is notable that there was a trend to a reduction in Ki67 in the HER-2-positive group, and that 5 of 15 patients showed >50% reduction in proliferation. We have demonstrated previously in a study of the variability of Ki67 in the same tumor that a >50% change in Ki67 in an individual tumor is statistically significant with a CI of 95% (13). It is particularly notable, however, that the mean Ki67 after 2 and 12 weeks in the HER-2-positive group is between 5 and 10 times that at the same time points in the ER-positive/PgR-positive/HER-2-negative group, and that the levels early in treatment in the HER-2-positive group remain substantially above the mean pretreatment level in the HER-2-negative group. Thus, although there may be a benefit to the patient from a slowing of proliferation in these tumors, it seems likely that this will translate to a measurable clinical response in only a minority of the patients. In 4 patients, there was an increase in Ki67 over the first 2 weeks of treatment. These patients received three different treatments such that there is no possibility of associating this with a possible early flare reaction to a particular agent.

It has been noted previously that in those patients who have ER-positive and HER-2-positive cancers, the mean concentration of ER in the tumor is lower than in those tumors that are HER-2-negative (11). Thus, it was important to consider the possibility that the poorer biological response to endocrine therapy might be associated with lower ER values rather than the HER-2 positivity *per se*. This analysis was particularly significant in this study, because we found that the concentration of ER related positively to a reduction in Ki67 levels. Miller *et al.* (21) have noted previously that clinical response to neoadjuvant tamoxifen is most likely in patients with the highest ER levels, and it has been known for many years that in the advanced disease setting, patients with high levels of ER have a much greater chance of an endocrine response than those with low ER-positive disease (29). Our data have confirmed that ER levels are lower in HER-2-positive/ER-positive cancers than in HER-2-negative/ER-positive tumors. We were, however, able to establish that in a group of HER-2-positive patients that had high tumor ER levels, the change in Ki67 was also subnormal. Thus, although lower ER levels may explain part of the hormonal resistance in HER-2-positive tumors, HER-2 positivity itself also appears to play a role. It will be important to establish the mechanism of hormonal resistance because this may be expected to help direct appropriate alternative therapy; if the effect is attributable to ligand-independent ER phosphorylation, a compound such as ICI 182,780, which disrupts ER, might be helpful. Alternatively, if the effect is attributable to direct growth factor-enhanced proliferation, a drug such as Herceptin might be more effective.

Changes in PgR are also observable during the hormonal therapy of breast cancer as a result of the estrogen-dependent expression of that protein. Theoretically, it may have been instructive to have assessed the influence of HER-2 on these changes that occur through the variable targeting of PgR to 26S proteasomal degradation (30). However, this analysis was not undertaken because of the profoundly different time-dependent effects of the SERMs and aromatase inhibitors on PgR expression (14, 15).

In conclusion, we have demonstrated that HER-2-positive/ER-positive tumors have a significantly poorer antiproliferative response to hormonal therapy. These data support a quantitative but not absolute resistance to such therapy for most patients with tumors of this type.

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