HER-2 Amplification and Topoisomerase IIα Gene Aberrations as Predictive Markers in Node-positive Breast Cancer Patients Randomly Treated Either with an Anthracycline-based Therapy or with Cyclophosphamide, Methotrexate, and 5-Fluorouracil

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ABSTRACT

Purpose: The purpose of this study is to evaluate HER-2 and topoisomerase IIα (topo IIα) as candidates for predicting the activity of anthracyclines in the adjuvant treatment of breast cancer patients.

Experimental Design: In this study, we evaluated HER-2 and topo IIα gene aberrations by fluorescence in situ hybridization in a series of 430 primary breast cancer samples. Samples came from node-positive breast cancer patients randomly treated either with one of two anthracycline-based regimens [full-dose epirubicin-cyclophosphamide (HEC) and moderate-dose epirubicin-cyclophosphamide (EC)] or with cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) in the context of a Phase III adjuvant therapy trial. Event-free survival comparisons were performed between the three study arms in the subgroups of HER-2-amplified and nonamplified tumors. An exploratory analysis was also performed to evaluate the predictive value of topo IIα in the cohort of HER-2-amplified patients.

Results: HER-2 amplification was observed in 73 of the 354 evaluable samples (21%), whereas topo IIα amplification was found in 23 of the 61 evaluable HER-2-amplified tumors (38%). The three event-free survival comparisons were CMF versus HEC, CMF versus EC, and EC versus HEC. Hazard ratios (HRs) and 95% confidence intervals (CIs) were as follows: (a) CMF versus HEC, HR = 1.42 for HER-2-amplified tumors (95% CI, 0.54–3.76; P = 0.48) and 0.84 for HER-2-nonamplified tumors (95% CI, 0.49–1.44; P = 0.53); (b) CMF versus EC, HR = 1.65 for HER-2-amplified tumors (95% CI, 0.66–4.13; P = 0.29) and 0.66 for HER-2-nonamplified tumors (95% CI, 0.39–1.10; P = 0.11); and (c) EC versus HEC, HR = 0.93 for HER-2-amplified tumors (95% CI, 0.31–2.77, P = 0.90) and 1.33 for HER-2-nonamplified tumors (95% CI, 0.82–2.14; P = 0.25).

Conclusions: HER-2 could have a predictive value for the activity of anthracycline-based regimens in the adjuvant therapy of breast cancer patients. The predictive value of HER-2 would most likely be related to the concomitant amplification of the topo IIα gene.

INTRODUCTION

Individual trials (1–5), as well the Oxford meta-analysis (6), have demonstrated that an anthracycline-based therapy is more effective than a CMF3 regimen in the adjuvant therapy of breast cancer patients, although the absolute disease-free survival and OS benefits related to the use of anthracyclines do not exceed 10% and are even <5% in case of node-negative breast cancer (1–6). In the last 7 years, some retrospective studies have suggested that the benefit of anthracyclines would be confined mainly to those patients with HER-2/neu overexpression in the primary tumor (7–13).

In these retrospective studies, HER-2 has been evaluated

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The abbreviations used are: CMF, cyclophosphamide, methotrexate, and 5-fluorouracil; OS, overall survival; FISH, fluorescence in situ hybridization; IHC, immunohistochemistry; topo IIα, topoisomerase IIα; EFS, event-free survival; HEC, full-dose epirubicin-cyclophosphamide; EC, moderate-dose epirubicin-cyclophosphamide; DAPI, 4′,6-diamidino-2-phenylindole-dihydrochloride; HR, hazard ratio; CI, confidence interval.

3 The abbreviation used are: CMF, cyclophosphamide, methotrexate, and 5-fluorouracil; OS, overall survival; FISH, fluorescence in situ hybridization; IHC, immunohistochemistry; topo IIα, topoisomerase IIα; EFS, event-free survival; HEC, full-dose epirubicin-cyclophosphamide; EC, moderate-dose epirubicin-cyclophosphamide; DAPI, 4′,6-diamidino-2-phenylindole-dihydrochloride; HR, hazard ratio; CI, confidence interval.
mainly by IHC. Our group has reported that the interaction between HER-2 status and anthracycline efficacy may change radically according to the anti-mRNA-2 antibody used in the IHC assay (11). Moreover, when we evaluated the degree of interlaboratory agreement with IHC, we observed that 61 of 109 HER-2-positive samples in one center were classified as HER-2 negative in a second laboratory (14).

Because of the existing difficulties in standardization of the IHC procedures, we have decided to address the issue of HER-2 as a predictive marker by using FISH to evaluate the HER-2 status.

FISH is an expensive and time-consuming technique whose accuracy in selecting HER-2-positive patients has been documented (15, 16). Accordingly, we gathered primary breast cancer samples from node-positive breast cancer patients randomly treated with either an anthracycline-based adjuvant therapy or CMF in the context of a multicenter Phase III trial, and we evaluated HER-2 amplification by FISH. The primary study aim was to investigate the interaction between anthracycline efficacy and HER-2 status when the latter is evaluated by FISH.

A second exploratory analysis was carried out in the present study because of some data suggesting that: (a) the HER-2 status does not interfere in vitro with the degree of sensitivity to anthracyclines (17, 18); and (b) the presumed predictive value of HER-2 would be related mainly to the concomitant amplification of the topo IIa gene, which occurs in about 40% of HER-2-amplified breast cancer patients (19). Moreover, it has been recently reported that in breast cancer cell lines, HER-2 activation increases the topo IIa enzyme activity directly, thus leading to increased sensitivity to topo IIa inhibitors (20).

Topo IIa is the molecular target of anthracyclines. Recently, it has been reported that HER-2-amplified, topo IIa-amplified breast cancer cells are extremely sensitive to anthracyclines, whereas HER-2-amplified, topo IIa-nonamplified cells show an intermediate degree of sensitivity to the same drugs (21).

Accordingly, in the present study, we have also evaluated topo IIa gene aberrations by FISH in the subgroup of patients with HER-2 gene amplification. The aim of this second analysis was to explore in vivo the concept that HER-2 amplification would be indirectly implicated in the degree of sensitivity to anthracyclines through the association with topo IIa gene amplification. No topo IIa gene evaluation was carried out in HER-2-nonamplified tumors because of previous data suggesting that in this subgroup of patients, no topo IIa gene aberrations are observed (19).

**PATIENTS AND METHODS**

**Clinical Study**

The prospective clinical trial on which the present study is based has already been published (22). In brief, pre- and postmenopausal patients (patient age, ≈70 years) with radically resected, histologically confirmed, node-positive breast cancer were randomly allocated to one of the following treatment arms: (a) CMF every 4 weeks × 6 cycles (oral cyclophosphamide version); (b) EC × 8 cycles [epirubicin (60 mg/m² day 1) and cyclophosphamide (500 mg/m² i.v. day 1), q 3 weeks]; and (c) HEC × 8 cycles [epirubicin (100 mg/m² day 1) and cyclophosphamide (830 mg/m² i.v. day 1), q 3 weeks]. Patients were stratified according to the participating institution, number of histologically positive axillary nodes, and menopausal status. After the end of chemotherapy, postmenopausal patients with estrogen receptor-positive tumors or tumors with unknown estrogen receptor status received tamoxifen (40 mg/day) for 5 years. Locoregional radiotherapy was performed at the end of chemotherapy in all patients treated with breast-conserving surgery and in mastectomy-treated patients according to specific radiotherapy guidelines from each participating center.

**Predictive Marker Study**

**Tumor Sample Collection**

A list of all patients entered into the clinical study was submitted to each participating center. Each center had to provide the operational office with one paraffin-embedded sample of the primary tumor. Samples were sent by the participating center to the operational office by conventional mail. Once samples were received, they were classified and stored at room temperature until the predictive markers analysis was carried out.

**HER-2 Evaluation by FISH**

The evaluation of HER-2 status by FISH was carried out at the pathology department of the Jules Bordet Institute.

**Tissue Specimens and Slide Preparation.** Four-μm sections were cut from archival tissue that had been fixed in buffered formalin and embedded in paraffin. Sections were placed on Superfrost Plus slides (Menzel-Gläser, Germany). Slide-mounted tissue sections were then baked overnight at 65°C and deparaffinized in xylene three times for 10 min each, followed by immersion in 100% ethanol twice for 5 min each. Air-dried tissue sections were subsequently treated in 0.2 M HCl for 20 min at room temperature, washed twice in 2× SSC [1× SSC = 0.15 M NaCl and 0.015 M sodium citrate (pH 7.0)] for 5 min each, and then treated in a solution of 1 M NaSCN for 30 min at 80°C. Slides were then washed twice in 2× SSC and treated in a solution of pepsin (0.5 mg/ml) in 0.9% NaCl (pH 2.0) for 10–20 min at 37°C. After pepsin digestion, tissue sections were washed in 2× SSC as described above and dehydrated in graded alcohol (70%, 90%, and 100% ethanol).

**In Situ Hybridization (According to the Vysis Protocol).**

Slide-mounted tissue sections were denatured in 70% formamide 2× SSC (pH 7.0) at 74°C for 5 min and then immersed in 70% ethanol at room temperature. After dehydration through graded alcohols, slides were air dried and warmed at 45°C. The dual color probe Spectrum Orange HER-2/Spectrum Green CEP17 (PathVysion, catalogue number 30-161060; Vysis, Downers Grove, IL) was then applied (10 μl) on a selected area of the sample (the invasive part of the tumor), and sections were coverslipped and sealed with rubber cement. Hybridization was carried out overnight at 37°C in a moist chamber. Washes were performed at 74°C for 2 min in 0.2× SSC 0.3% NP40. After drying, sections were counterstained with a DAPI (Boehringer Mannheim, Mannheim, Germany) antifade solution and analyzed.

**Fluorescence Microscopy and Scoring Criteria.** A Leica DMBR epifluorescence microscope equipped with a 100 W mercury-arc lamp and ×40 and ×100 objectives was used in...
**Table 1**  Main patient and tumor characteristics in the two subgroups with and without available tumor samples

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. of patients without available formalin-fixed samples N = 347 (%)</th>
<th>No. of patients with available formalin-fixed samples N = 430 (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;50</td>
<td>187 (54)</td>
<td>228 (53)</td>
<td>0.89</td>
</tr>
<tr>
<td>≥50</td>
<td>160 (46)</td>
<td>202 (47)</td>
<td></td>
</tr>
<tr>
<td>Type of surgery</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conservative</td>
<td>125 (36)</td>
<td>155 (36)</td>
<td>0.94</td>
</tr>
<tr>
<td>Mastectomy</td>
<td>222 (64)</td>
<td>275 (64)</td>
<td></td>
</tr>
<tr>
<td>No. of positive nodes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1–3</td>
<td>194 (56)</td>
<td>271 (63)</td>
<td>0.07</td>
</tr>
<tr>
<td>≥4</td>
<td>153 (44)</td>
<td>159 (37)</td>
<td></td>
</tr>
<tr>
<td>ER status&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>201 (58)</td>
<td>275 (64)</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>76 (22)</td>
<td>99 (23)</td>
<td>0.86</td>
</tr>
<tr>
<td>Unknown</td>
<td>70 (20)</td>
<td>56 (13)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> ER, estrogen receptor.

combination with a Triple bandpass filter (DAPI/FITC/tetramethylrhodamine isothiocyanate). Scanned at high magnification (×100), at least 60 hybridized nuclei from invasive parts of tissue sections were counted. Amplification was defined as a ratio of the number of HER-2 signals:centromeric 17 signals of >2. H&E-stained slides were read by one pathologist (D. G.) to select the areas with invasive cancer. Then the hybridized slides were read, and the spots were counted by another observer (D. G.), who was blinded with regard to the correspondent treatment arm and clinical outcome for each evaluated sample.

topo IIα Evaluation by FISH

This marker was evaluated by the laboratory of Cancer Biology, University of Tampere (Tampere, Finland).

At the time of topo IIα evaluation, investigators in Tamperere were blinded with regard to the correspondent treatment arm and clinical outcome for each evaluated sample. Two-color FISH of topo IIα was carried out with a previously characterized PAC probe and a chromosome 17 centromeric probe (p17H8), which was used as a reference to determine the overall copy number of chromosome 17 (19, 21). In brief, the sections were deparaffinized and incubated in 0.1 M Tris-HCl (pH 7.3) in a temperature-controlled microwave oven (92°C for 15 min). Enzymatic digestion was carried out with FFPE digestion enzyme (Zymed; 10–30 min at room temperature). The slides were washed with PBS and dehydrated in graded dilutions of ethanol. The probe mixture consisting of topo IIα and 17 centromere probes (30 ng and 10 ng of probe DNA, respectively) was applied to the slides (5–10 μl/slide) under coverslips. The slides were codenatured on a hot plate (94°C for 3 min), followed by overnight hybridization at 37°C. After hybridization, the slides were stringency washed with 0.5× SSC (5 min at 75°C), followed by three washes in PBS 0.2% Tween 20. The topo IIα probe was detected with anti-digoxigenin rhodamine (diluted 1:300; Roche-Boehringer, Mannheim, Germany). Nuclei were counterstained with 0.1 μM DAPI in an antifade solution (Vectorshield; Vector Laboratories, Burlingame, CA). FISH was evaluated using an Olympus BX50 epifluorescence microscope equipped as described previously (19, 21). Signals from at least 50–500 nonoverlapping nuclei with intact morphology were evaluated to determine the mean number of signals/cell for each probe. Both absolute copy numbers and the relative copy number ratio (ratio between mean number of topo IIα signals and the mean number of chromosome 17 centromere signals) were determined. Control hybridizations to nonmalignant breast tissue and to normal peripheral blood lymphocytes were carried out to ascertain equal hybridization efficiencies of topo IIα and the chromosome 17 centromere. Amplification of topo IIα was defined as a relative copy number ratio of ≥1.5, and deletion of topo IIα was defined as a ratio of ≤0.8.

**Statistical Analysis**

EFS was the outcome used to assess treatment efficacy and was defined as the interval elapsed between the date of randomization and the date of documented disease relapse, second primary tumor, or death. EFS distribution was estimated by the Kaplan-Meier method or by using Cox modeling. No OS analysis was done in the subgroup of patients identified by the investigated biological markers because of the small number of events observed in each treatment group.

The patients evaluated in the present retrospective study (predictive markers study) were a subgroup of the study population entered into the prospective clinical trial. To evaluate the representativeness of this subgroup, main patient and tumor characteristics, including those factors with prognostic significance in the univariate analysis performed on the 777 patients entered into the clinical study, were compared in the two distinct groups (i.e., the 347 patients not evaluated in the present study because of the lack of a formalin-fixed tumor sample and the 430 patients entered into the present study). Patient and tumor characteristics were all categorical, and χ² tests for heterogeneity were used to compare both groups.

The different cohorts of patients identified by HER-2 were examined with regard to differences in the magnitude of treatment effect on patient outcome by estimating HRs and the corresponding 95% CIs and p-values for no effect. HRs were calculated for the following comparisons: (a) CMF versus HEC (comparison 1); (b) CMF versus EC (comparison 2); and (c) EC versus HEC (comparison 3). For comparisons 1 and 2, a HR of >1 has to be interpreted as a poorer EFS distribution for patients treated with CMF, whereas for comparison 3, a HR of >1 has to be interpreted as a poorer EFS distribution for patients treated with EC. To test the main study hypothesis that the therapeutic benefit from the anthracycline-based therapy compared with CMF was greater in HER-2-positive patients than in HER-2-negative patients, an interaction term combining treatment arm and HER-2 status was added to the Cox proportional hazards model. Its significance was tested using a likelihood ratio test.

The Cox proportional hazards model also included those factors with prognostic significance (P < 0.05) in the univariate analysis performed on 777 patients entered into the clinical trial. The following list of putative prognostic factors for EFS was evaluated in the univariate analysis: age, menopausal status, surgery type, histological grade, histological type, tumor clinical size, tumor pathological size, number of involved ipsilateral axillary nodes, and estrogen and progesterone receptor status. Surgery type (mastectomy versus breast-conserving surgery)
and the number of involved axillary nodes (1–3 or ≥4) were the only factors with prognostic significance in the univariate analysis. Accordingly, these two factors were introduced into the Cox proportional hazards model. Moreover, a third factor (i.e., use of adjuvant tamoxifen, yes or no) was introduced in the Cox model because of previous data suggesting a negative interaction between tamoxifen treatment and HER-2 overexpression (23). All reported p-values are two tailed.

RESULTS

Clinical Study

Detailed results regarding the clinical trial have been extensively reported (22). The allocated treatment for the 777 eligible patients was as follows: (a) CMF, 255 patients; (b) EC, 267 patients; and (c) HEC, 255 patients. Between 41% and 44% of the patients were postmenopausal. A total of 67%, 63%, and 64% of the patients were treated with mastectomy in the CMF, EC, and HEC arms, respectively. Between 39% and 41% of the patients had four or more involved ipsilateral axillary nodes.

The main patient characteristics were well balanced between the three study arms, and no statistically significant differences in their distributions were found. The median relative dose intensities were 0.89, 0.95, and 0.90 for CMF, EC, and HEC, respectively. The median number of cycles delivered was six for CMF and eight for both the EC and HEC treatment groups.

At a median study follow-up of 74 months, no differences between CMF and HEC were observed with regard to EFS and OS [EFS, HR = 1.08 (95% CI, 0.81–1.44), P = 0.61; OS, HR = 1.07 (95% CI, 0.75–1.51), P = 0.72]. No differences with regard to EFS were observed between CMF and HEC in the subgroups of patients identified according to the following factors: (a) age (<50 or ≥50 years); (b) number of positive axillary nodes (1–3 or ≥4); (c) tumor size (pT1 or >pT1); (d) estrogen receptor and progesterone receptor status (positive or negative); and (e) histological grade (1, 2, or 3).

As far as the CMF versus EC comparison is concerned, the following results were observed for EFS and OS: (a) EFS, HR = 0.84 (95% CI, 0.64–1.12), P = 0.24; and (b) OS, HR = 0.82 (95% CI, 0.59–1.14), P = 0.24.

The results of the EC versus HEC comparison suggest an increased benefit associated with the use of the latter [EFS, HR = 1.28 (95% CI, 0.96–1.69), P = 0.09; OS, HR = 1.31 (95% CI, 0.93–1.83), P = 0.12].

Predictive Marker Study

Collection of Tumor Samples

A total of 625 paraffin-embedded samples of primary tumors were collected between September 1996 and December 1998, corresponding to 80% of the clinical study population (777 eligible patients). The 625 blocks were collected from 12 of the 14 centers participating in the clinical study; all centers that entered at least 10 patients into the clinical study contributed to the collection of tumor samples. Of the 625 blocks, 430 were fixed in formalin and appropriate for FISH evaluation. The remaining 195 samples were fixed in boiun, making the evaluation of HER-2 by FISH not feasible. Of the 430 patients with available formalin-fixed samples, representing 55% of the clinical trial population, 148 were treated with CMF, 137 were treated with HEC, and 145 were treated with EC.

To explore whether the subgroups of patients evaluated in the present study was representative of the original clinical trial population, main patient and tumor characteristics were compared between this subgroup (n = 430) and the subgroup without available samples (n = 347).

According to the results shown in Table 1, it can be concluded that no detectable selection bias occurred, although an increased but nonstatistically significant prevalence of tumors with 1–3 positive nodes were found in the subgroup of patients with available tumor samples. When EFS was compared between the two groups of patients with and without available tumor samples, no statistically significant difference was found (P = 0.26, log-rank test), with 2- and 5-year EFS rates of 85% and 70%, respectively, for the cohort with available samples and 86% and 65%, respectively, for the other cohort.

HER-2 Amplification

Table 2 summarizes the results related to the HER-2 status of the 430 patients with available formalin-fixed samples.

In 76 of the 430 patients, the HER-2 status by FISH was not evaluable because of technical problems (i.e., lack of the invasive component, lack of signal after hybridization, or detachment of the tissue specimen after pretreatment).

In the remaining 354 patients, no statistically significant difference was observed in the prevalence of HER-2-amplified tumors among the three study arms (P = 0.45, χ² test for homogeneity).

Interaction between HER-2 Amplification and Treatment Efficacy

CMF versus HEC. Fig. 1 summarizes the results of the EFS comparison between CMF and HEC in the two cohorts of HER-2-amplified and -nonamplified patients. It can be observed that although the number of evaluated patients is modest, the difference in point estimates of the HRs suggests that in HER-2-amplified patients, HEC is more effective than CMF (HR = 1.42), whereas in the HER-2-nonamplified cohort, the superiority of the anthracycline-based regimen seems to be lost (HR = 0.84). However, statistical significance is not reached in either of the two subgroups, and the CIs of the two HRs overlap.

Table 2: Prevalence of HER-2 amplification

<table>
<thead>
<tr>
<th>Study arms</th>
<th>Overall N = 430 (%)</th>
<th>CMF N = 148 (%)</th>
<th>HEC N = 137 (%)</th>
<th>EC N = 145 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonevaluable</td>
<td>30 (7%)</td>
<td>20 (14%)</td>
<td>26 (19%)</td>
<td>76 (53%)</td>
</tr>
<tr>
<td>Evaluable</td>
<td>118 (52%)</td>
<td>117 (78%)</td>
<td>119 (87%)</td>
<td>354 (59%)</td>
</tr>
<tr>
<td>Amplified (ratio &gt; 2)</td>
<td>28 (24%)</td>
<td>20 (17%)</td>
<td>25 (21%)</td>
<td>73 (21%)</td>
</tr>
<tr>
<td>Nonamplified</td>
<td>90 (76%)</td>
<td>97 (83%)</td>
<td>94 (79%)</td>
<td>281 (79%)</td>
</tr>
</tbody>
</table>
**CMF versus EC.** Fig. 2 reports the results of the EFS comparison between CMF and EC in the two distinct subgroups of HER-2-amplified and -nonamplified patients.

As for the CMF versus HEC comparison, it can be seen that in HER-2-amplified patients, EC has a better observed efficacy than CMF (HR = 1.65), whereas in the HER-2-nonamplified subgroup, CMF seems to be more effective than the anthracycline-based regimen (HR = 0.66). Nevertheless, no statistical significance is reached, and the CIs of the two HRs overlap.

**EC versus HEC.** The results of the EFS comparison between EC and HEC are reported in Fig. 3. The analysis has been performed in the two distinct cohorts of HER-2-amplified and -nonamplified patients. Point estimates of the HRs are more homogeneous than those for the other two comparisons. In HER-2-amplified tumors, the two anthracycline-based regimens seem to be almost equally effective (HR = 0.93), whereas in HER-2-nonamplified tumors, HEC is associated with a better observed efficacy than EC (HR = 1.33), as reported in the prospective clinical trial. It must be emphasized that a limited number of patients have been evaluated, and no statistical significance has been reached in the two subgroups of patients analyzed.

**Interaction between topo IIα Amplifications and Treatment Efficacy in HER-2-amplified Tumors**

Topo IIα gene aberrations were evaluated only in HER-2-amplified tumors because in a published study, no aberrations...
tions were found in a series of >100 HER-2-nonamplified tumors (19).4

Of the 73 HER-2-amplified tumors, 61 were evaluable for topo IIa amplification by FISH. topo IIa amplification was found in 23 of the 61 tumors (38%; CMF = 8 cases, HEC = 5 cases, and EC = 10 cases). No topo IIa amplification was found in the remaining 38 HER-2-amplified tumors (CMF = 15 cases, HEC = 13 cases, and EC = 10 cases). In the 38 tumors without topo IIa amplification, topo IIa deletion was found in 8 cases (CMF = 4 cases, HEC = 3 cases, and EC = 1 case).

Assuming that no topo IIa amplifications existed in HER-2-nonamplified tumors, the prevalence of topo IIa amplification in a population of 354 node-positive breast cancer patients is estimated to be 6%.

Figs. 4–6 report the EFS curves of HER-2-amplified patients for the following comparisons: (a) CMF versus HEC; (b) CMF versus EC; and (c) EC versus HEC, respectively. EFS is shown by treatment arm and by topo IIa status (amplified versus nonamplified).

Although the number of patients evaluated is extremely small, the results seem to support the concept that the improved efficacy

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4 J. Isola, personal communication.
of HEC over CMF in HER-2-amplified patients is confined to the subgroup of topo II\textsubscript{H9251}-amplified tumors (Fig. 4). Of the five patients with HER-2-amplified, topo II\textsubscript{H9251}-amplified tumors receiving HEC, four patients remain disease free, whereas one has relapsed after 60 months from the date of randomization (data not shown in the curves of Fig. 4, which describe the first 4 years of follow-up). In Fig. 5, it can be observed that EC seems to be better than CMF only in the subgroup of HER-2-amplified, topo II\textsubscript{H9251}-amplified tumors, whereas CMF seems to be better than EC in the cohort of HER-2-amplified, topo II\textsubscript{H9251}-nonamplified patients. Fig. 7 reports the EFS curves of HER-2-amplified patients for the comparison of CMF versus anthracycline-based therapy. In this analysis, EFS for patients receiving anthracyclines has been calculated independently of the epirubicin dose delivered. This analysis allows us to increase the number of patients evaluated in the anthracycline-based arm and supports the concept that topo II\textsubscript{H9251}, rather than HER-2, could be the true candidate to predict the efficacy of anthracyclines in early breast cancer patients receiving adjuvant therapy.

**DISCUSSION**

Different retrospective studies have suggested that an anthracycline-based adjuvant therapy has the highest efficacy in the subgroup of breast cancer patients with HER-2 over-expression (7–13).

IHC has been the most widely used technique in these retrospective studies to evaluate HER-2 on archival samples of the primary tumor (7–13).

Because of some known problems existing with the evaluation of HER-2 by IHC (14, 24–26), we decided to investigate the predictive value of HER-2 by FISH, which has been demonstrated to be a reliable technique for HER-2 evaluation (15, 16, 27). Moreover, it has been reported recently that in an unselected population of 900 stage I–III breast cancer patients, IHC does not consistently discriminate cases likely to have a poor prognosis, whereas FISH provides superior prognostic information in distinguishing high-risk from low-risk patients (15).

This study, to the best of our knowledge, is the first to investigate the predictive value of HER-2 by FISH in a population of early breast cancer patients randomly treated with either an anthracycline-based therapy or CMF. Its results seem to support the concept of an interaction between HER-2 status and anthracycline efficacy.

In particular, in the cohort of HER-2-amplified patients, results suggest that the anthracycline-based adjuvant therapy is more effective than CMF, whereas the latter seems to be at least as effective as anthracyclines in HER-2-nonamplified patients. It is worth noting that even when a suboptimally dosed anthracycline-based regimen such as EC [epirubicin (60 mg/m\textsuperscript{2}) q 3 weeks] was compared with CMF in the subgroup of HER-2-amplified tumors, the anthracycline-based regimen appeared to be the most effective, although in the whole study population entered into the clinical trial, there was a trend favoring CMF over EC.

Nevertheless, because of the limited number of patients analyzed in the present study, no firm conclusions can be drawn regarding the predictive value of HER-2 in the adjuvant therapy of breast cancer.

An additional aim of this study was to explore the potential value of topo II\textsubscript{H9251} gene amplification as a marker of hypersensitivity to anthracyclines. There are several in vitro experiments as well as some recently reported clinical data supporting the investigation of topo II\textsubscript{H9251} in this specific context (11, 19–21, 28, 29).

Topo II\textsubscript{H9251} is the target enzyme for topo II\textsubscript{H9251} inhibitors such as anthracyclines. Some years ago, in vitro data demonstrated a direct correlation between the intranuclear topo II\textsubscript{H9251} levels and the degree of sensitivity to anthracyclines (28). Moreover, it has been reported that topo II\textsubscript{H9251} gene aberrations (i.e., amplification, deletion, or both) can be found in 50–80% of HER-2-amplified...
breast cancer samples, whereas no topo IIα gene aberrations are found in HER-2-nonamplified tumors (19). Interestingly, HER-2-amplified, topo IIα-amplified cells were shown to be highly sensitive to anthracyclines, whereas HER-2-amplified, topo IIα-deleted cells displayed a poor degree of sensitivity to anthracyclines (21).

In the clinical setting, only a few experiences have been reported with regard to the predictive value of topo IIα in breast cancer patients (11, 29). In our previous study, in which topo IIα was evaluated by IHC in the same series of patients as the one reported in the present study, results suggested that topo IIα-positive tumors (i.e., >10% of cells with positive staining) derived the highest benefit from the anthracycline-based adjuvant therapy (11). In a second study correlating the efficacy of anthracyclines in metastatic breast cancer with topo IIα status as evaluated by FISH, it was found that all cases experiencing a clinical or radiological complete response (seven patients) had topo IIα gene amplification and that tumors with topo IIα gene deletion were the least responsive to a first-line therapy with anthracyclines (17% response rate; Ref. 29).

The results of the present study support the hypothesis that topo IIα could be the most attractive candidate for predicting the efficacy of an anthracycline-based regimen and that the predictive value of HER-2 could be most likely be related to the concomitant amplification of these two genes located on the same arm of chromosome 17. Nevertheless, our present results must be seen as hypothesis-generating results because of the fairly limited number of patients in which both HER-2 and topo IIα gene amplifications could be evaluated.

Previous experiences and the results of this study support further investigation of topo IIα as a predictive marker for the activity of anthracyclines in breast cancer.

A serious problem in running future research programs evaluating the predictive value of topo IIα is the low prevalence of topo IIα gene amplification or deletion in a population of breast cancer patients (<10% and <5% of patients, respectively). The low prevalence of topo IIα amplification can explain the modest advantage of anthracyclines over CMF in an unselected population of breast cancer patients. On the other hand, it also makes the interpretation of results coming from individual studies difficult because of the extremely modest number of patients in whom a topo IIα gene aberration is found.

Accordingly, only a meta-analysis of individual studies can address the potential predictive value of this marker in breast cancer patients. Such a meta-analysis is now planned and will hopefully collect data on >3000 early breast cancer patients randomly treated with either CMF or an anthracycline-based therapy. In this project, a meta-analysis of clinical data will be performed, whereas HER-2 and topo IIα evaluation will most likely be centralized. Because of the cost and time needed to perform FISH evaluation on nearly 3000 cases, it seems logical to screen all cases for HER-2 by IHC and to perform HER-2 evaluation by FISH only in cases with immunostaining (≥1% of positive cells). Moreover, because of the data suggesting that no topo IIα amplifications are observed in HER-2-nonamplified tumors (19), topo IIα evaluation will be performed only in HER-2-amplified cases.

In conclusion, we have investigated the predictive value of HER-2, as evaluated by FISH, in a population of node-positive breast cancer patients randomly treated with either an anthracycline-based therapy or CMF. Our results, although not conclusive, support the concept that an interaction exists between anthracycline efficacy and HER-2 status. Moreover, the present study, in which topo IIα gene aberrations have also been evaluated, highlights that the interaction between HER-2 and anthracycline could most likely be explained by a concomitant topo IIα gene amplification in patients with HER-2-amplified tumors.

Because of the low prevalence of HER-2 and topo IIα genes aberrations, only a meta-analysis of individual studies comparing an anthracycline-based therapy with CMF in early...
breast cancer patients will fully address the potential predictive value of these two biological markers.

**REFERENCES**


