Evaluation of the prognostic and predictive value of HER family mRNA expression in high-risk early breast cancer: A Hellenic Cooperative Oncology Group (HeCOG) study

The aim of the study was to evaluate the prognostic ability of the transcriptional profiling of the HER family genes in early breast cancer, as well as to investigate the predictive value of HER2 mRNA expression for adjuvant treatment with paclitaxel. RNA was extracted from 268 formalin-fixed paraffin-embedded (FFPE) tumour tissue samples of high-risk breast cancer patients enrolled in the randomised HE10/97 trial, evaluating the effect of dose-dense anthracycline-based sequential adjuvant chemotherapy with or without paclitaxel. The mRNA expression of all four HER family members was assessed by kinetic reverse transcription-polymerase chain reaction (kRT-PCR). The overall concordance between kRT-PCR and IHC/FISH for HER2 status determination was 74%. At a median follow-up of 8 years, multivariate analysis showed that EGFR and HER2 mRNA expression was associated with reduced overall survival (OS). HER3 and HER4 mRNA level had a favourable prognostic value in terms of OS and disease-free survival (DFS), respectively. Adjusting for HER2 mRNA expression, OS and DFS did not differ between treatment groups. These data indicate that EGFR as well as HER2 are prognostic factors of worse clinical outcomes, whereas HER3 and HER4 gene transcription is associated with better prognosis in high-risk early breast cancer. However, HER2 mRNA expression did not predict clinical benefit from paclitaxel. Kinetic RT-PCR represents an alternative method for evaluating the expression of HER family members in FFPE breast carcinomas.

Keywords: HER family; mRNA; kRT-PCR; prognostic value; predictive value; breast cancer

Adjuvant chemotherapy improves disease-free survival (DFS) and overall survival (OS) in early-stage breast cancer (EBCTCG, 2005) with taxanes representing active agents in such a treatment. However, chemotherapy is associated with potentially life-threatening side effects. Therefore, the identification of reliable prognostic factors as well as biological markers that might have the ability to predict therapeutic response is crucial. So far, no biomarkers have been identified that can reliably predict clinical benefit from paclitaxel in breast cancer patients.

The human epidermal growth factor receptor (HER) family comprises of four homologous members: ErbB-1 (epidermal growth factor receptor (EGFR) or HER1), ErbB-2 (HER2) for which no ligand has been described so far, ErbB-3 (HER3) which is characterised by its impaired kinase activity, and ErbB-4 (HER4) (Mosesson and Yarden, 2004). There is an extensive literature on the role of the HER family in breast cancer and particularly that of HER2, which is considered to be a key oncogene in breast carcinogenesis. Overexpression or amplification of HER2 occurs in 15–30% of breast carcinomas and is considered to confer a more aggressive biology and an unfavourable impact on the course of the disease (Slamon et al, 1987). Moreover, it has been suggested that HER2 overexpression or amplification in breast cancer predicts greater sensitivity to anthracycline-containing chemotherapy (Gennari et al, 2008) and resistance to the CMF regimen (Gusterson et al, 1992). HER2 may also identify patients who are likely to benefit from higher doses of adjuvant chemotherapy.

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Received 1 August 2008; revised 7 October 2008; accepted 11 October 2008; published online 4 November 2008

British Journal of Cancer (2008) 99, 1775 – 1785. doi:10.1038/sj.bjc.6604769 www.bjcancer.com
Published online 4 November 2008
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regarding the response to taxane-based chemotherapy is consid-
ered controversial and the results of the studies have been
conflicting, so far (Pritchard et al, 2008). Although some trials have
supported an interaction between HER2 overexpression and
taxane activity (Konecny et al, 2004; Hayes et al, 2007), others
have failed to demonstrate such an association (Gonzalez-Angulo et al, 2004). Only a limited number of studies have evaluated the
effect of taxane-containing regimens with respect to HER2 status
in the adjudiant setting (Martin et al, 2005; Kostopoulos et al, 2006; Hayes et al, 2007).

In the light of clinical data suggesting that HER2 can be useful as
a predictive marker for both trastuzumab and chemotherapy,
standardisation and determination of the HER2 status in tumours has
become more important. HER2 can be analysed at the DNA, the
mRNA or the protein level. Various techniques are available, each
with advantages and disadvantages. For practical reasons,
immunohistochemistry (IHC) using an anti-HER2 antibody is
currently the method of choice for HER2 testing. However, major
drawbacks of IHC are that the results are not quantitative, the
interpretation is significantly influenced by several technical
factors and the inter-observer variation is quite large. Although
these discrepancies are improved by the use of standardised IHC
tests (such as the HercepTest), it is generally recommended that
(2+) HER2 immunostaining needs to be further validated by
fluorescence in situ hybridisation (FISH) analysis (Ellis et al, 2000;
Mass et al, 2000; Birner et al, 2001; Bartlett et al, 2003). Despite
efforts to standardise these assays, substantial intra- and inter-
laboratory variability of the results still exist. Kinetic reverse
transcription – polymerase chain reaction (kRT – PCR) has recently
been suggested as an alternative technique for the detection and
quantification of HER2 expression. kRT – PCR is simple, relatively
fast and produces reliable, quantitative and reproducible results.
Moreover, it can easily be standardised, reduces inter-observer variability and does not require experienced pathologists for
interpretation. However, a disadvantage of this technique is the
specific requirements for handling of the tissue specimens to
preserve the integrity of RNA. Recent studies have shown that the
small amounts of degraded RNA in archival formalin-fixed
paraffin-embedded (FFPE) tissues can be successfully amplified
and detected using kRT – PCR techniques (Gjerdrum et al, 2004).
EGFR is overexpressed in several human tumours and is also
considered to initiate a variety of important steps during the
malignant transformation. It has been estimated that 45% of
human breast carcinomas overexpress EGFR (range, 14 – 91%)
(Klijn et al, 1992). So far, there are no widely accepted criteria for
the determination of EGFR status. EGFR overexpression has been
associated with oestrogen receptor (ER) and progesterone receptor
(PR) negativity (Pawlowski et al, 2000; Ferrero et al, 2001; Tsuutsui et al, 2002; Biache et al, 2003). Moreover, there may be an
association between EGFR positivity and high histological grade or
lymph node involvement, although not all existing studies are in
agreement (Pawlowski et al, 2000; Ferrero et al, 2001; Witton et al,
2003; Rampaul et al, 2004; Tsaida et al, 2007). Currently, the
prognostic significance of EGFR in breast cancer patients remains
unclear. In addition, HER3 overexpression has been documented
in 20 – 30% of invasive breast carcinomas. The prognostic
significance of HER3 expression is also poorly documented and
the available data are conflicting (Quinn et al, 1994; Travis et al,
1996; Pawlowski et al, 2000). With respect to the HER4 receptor,
the current evidence suggests that it is characterised by
antiproliferative activity (Naresh et al, 2006). HER4 overexpression
has been reported as a favourable prognostic factor in the
literature (Pawlowski et al, 2000; Suo et al, 2002; Witton et al,
2003). The HER family represents therefore an attractive area for
the application of targeted therapies in breast cancer and
significant treatment advances have been made, so far. As trans-
signalling is now considered an essential feature of HER family
function, the role of lateral signalling partners is also becoming
increasingly important.

In this study, we assessed the prognostic significance of HER
family mRNA expression using kRT – PCR, in a series of high-risk
early breast cancer patients, treated with dose-dense anthracy-
cline-based sequential adjuvant chemotherapy with or without
paclitaxel, within the context of a randomised phase III clinical
trial. Furthermore, we investigated whether HER family mRNA
expression in the tumour could possibly identify patients who are
likely to benefit from the addition of paclitaxel to adjuvant
chemotherapy.

MATERIALS AND METHODS

Patients

Formalin-fixed paraffin-embedded tissue blocks of primary breast
cancer were retrospectively collected from 268 patients who were
part of the Hellenic Cooperative Oncology Group (HeCOG) 10/97
trial population. The basic patient and tumour characteristics are
shown in Table 1. The HE10/97 trial randomised a total of 595
high-risk (T1-3N1M0 or T3N0M0) breast cancer patients in the
period 1997 – 2000, to receive either 4 cycles of epirubicin (E)
followed by 4 cycles of intensified CMF (cyclophosphamide,
methotrexate and 5-fluorouracil) combination chemotherapy
(E-CMF) or 3 cycles of epirubicin followed by 3 cycles of paclitaxel
(T) and 3 cycles of intensified CMF (E-T-CMF). Chemotherapy
cycles were administered every 2 weeks and patients received
granulocyte-colony stimulating factor (G-CSF) support. The trial
was approved by the Bioethics Committee of the Aristotle
University of Thessaloniki and patients provided written informed
consent prior to enrolment. All participating patients also gave
written informed consent for research use of their biologic
material. The results of the HE10/97 study have been recently
reported (Fountzilas et al, 2005).

Pathologic determinations

Primary tumour diameter and axillary nodal status were obtained
from the histopathological report. ER and PR status was assessed by
IHC, whereas relative information was provided by the
participating institutions according to their own reference
laboratories. Tissue paraffin sections stained for ER/PR were
considered as positive even when only a small number of
neoplastic cells displayed nuclear immunoreactivity. Histological
grade was evaluated according to the Scarff, Bloom and
Richardson system.

Molecular and immunohistochemical studies

Owing to the logistical and organisational barriers arising from the
retrospective nature of the study, collection of FFPE tumour tissue
samples was possible in less than half of the patients enrolled in
the HE10/97 prospective clinical trial. RNA was isolated from 268
FFPE tumour tissue samples employing an experimental method
based on proprietary magnetic beads from Siemens Healthcare
Diagnostics (Cologne, Germany). For all tumour samples included
in the analysis the number of malignant cells represented at least
75% of all nucleated cells per section, as verified by haematoxylin
– eosin staining. Kinetic RT – PCR was applied for the assessment of
the expression of the EGFR, HER2, HER3, and HER4 genes using
gene-specific TaqManTM-based primer/probe sets. Forty cycles of
nucleic acid amplification were applied and the cycle threshold
(Ct) values of the target genes were identified. Ct values were
normalised by subtracting the Ct value of the housekeeping gene
RPL37A from the Ct value of the target gene (ΔCt). RNA results
were then reported as 40–ΔCt values, which would correlate
proportionally to the mRNA expression level of the target gene.

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the expression of the EGFR, HER2, HER3, and HER4 genes using
In short, each FFPE slide (5 μm thick) was deparaffinised in xylol and ethanol, the pellet was washed with ethanol and dried at 55°C for 10 min. The pellet was then lysed and proteinised overnight at 55°C with shaking. After addition of a binding buffer and the magnetic particles (Siemens Healthcare Diagnostics, Cologne, Germany) nucleic acids were allowed to bind to the particles for 15 min at room temperature. On a magnetic stand, the supernatant was aspirated and the beads were washed several times with a washing buffer. After addition of an elution buffer and incubation for 10 min at 70°C, the supernatant was aspirated on a magnetic stand without touching the beads. After normal DNAse I treatment for 30 min at 37°C and inactivation of DNAse I the RNA

| Table 1 | Basic patient and tumour characteristics |
|---------|------------------------------------------|
| Age (years) | Median (range) | 51 (22–76) | 52 (28–76) | 51 (22–76) |
| Number of nodes removed | Median (range) | 20 (4–59) | 21 (5–59) | 20 (4–53) |
| Number of positive nodes | Median (range) | 6 (0–54) | 7 (0–54) | 6 (0–49) |
| Positive nodes categories | | | | |
| 0 nodes | 3 (1) | 2 (2) | 1 (1) |
| 1–3 nodes | 58 (22) | 22 (19) | 36 (24) |
| 4–9 nodes | 122 (46) | 57 (49) | 65 (43) |
| >9 nodes | 85 (32) | 36 (31) | 49 (32) |
| Menopausal status | | | | |
| Premenopausal | 136 (51) | 55 (47) | 81 (54) |
| Postmenopausal | 132 (49) | 62 (53) | 70 (46) |
| Type of operation | | | | |
| Modified radical mastectomy | 215 (80) | 95 (81) | 120 (79) |
| Breast-conserving surgery | 53 (20) | 22 (19) | 31 (21) |
| Interval from operation | | | | |
| <2 weeks | 42 (16) | 16 (14) | 26 (17) |
| 2–4 weeks | 126 (47) | 64 (55) | 62 (41) |
| >4 weeks | 100 (37) | 37 (32) | 63 (42) |
| Tumour size | | | | |
| ≤2 cm | 81 (30) | 34 (29) | 47 (31) |
| 2–5 cm | 136 (51) | 62 (53) | 74 (49) |
| >5 cm | 51 (19) | 21 (18) | 30 (20) |
| Histology | | | | |
| Invasive ductal | 190 (71) | 85 (73) | 105 (70) |
| Invasive lobular | 33 (12) | 13 (11) | 20 (13) |
| Mixed | 30 (11) | 12 (10) | 18 (12) |
| Other | 10 (4) | 4 (3) | 6 (4) |
| Unspecified | 2 (1) | 1 (1) | 1 (1) |
| Unknown | 3 (1) | 2 (2) | 1 (1) |
| Grade | | | | |
| I–II | 135 (50) | 48 (41) | 87 (58) |
| III–Undifferentiated | 132 (49) | 68 (58) | 64 (42) |
| Unknown | 1 (0.4) | 1 (1) | 0 (0) |
| ER/PR status (IHC) | | | | |
| Negative | 58 (22) | 26 (22) | 32 (21) |
| Positive | 206 (77) | 89 (76) | 117 (77) |
| Unknown | 4 (1) | 2 (2) | 2 (1) |
| HER2 overexpression (IHC) | | | | |
| No | 164 (61) | 64 (55) | 100 (66) |
| Yes | 64 (24) | 30 (26) | 34 (23) |
| Unknown | 40 (15) | 23 (20) | 17 (11) |
| EGFR overexpression (IHC) | | | | |
| No | 201 (75) | 95 (81) | 106 (70) |
| Yes | 40 (15) | 15 (13) | 25 (17) |
| Unknown | 27 (10) | 7 (6) | 20 (13) |

Patient characteristics are well balanced between the two arms, with the exception of grade (P=0.010), a difference also observed in the prospective clinical trial.
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quality and quantity was checked by measuring absorbance at 260 and 280 nm. RNA was then used in RT–PCR. The primer/probe sets used for amplification of the target genes were the following:

- **EGFR Primer CTCTTGCCCCAAGTGTTAAGCGGAAT**
  - Forward Primer CGCAAGTGTTAAGCGGAAT
  - Reverse Primer CGTAGATTATGAGGTAGTCTG
  - HER2 Probe ACCAGGACCCACAGGCGGG
  - Forward Primer CAGCTTGGCAACACTCTATT
  - Reverse Primer TGCCGTAGTTGCTCTTTT

- **HER3 Probe CTCAMAGGTACTCCCTCCTCCCGGG**
  - Forward Primer CGGTTATGCATCCGACATACAC
  - Reverse Primer GAACCGAGCCCACTGGAAAGG

- **HER4 Probe CAGACTGCCCTTGGCTGAGTATTCC**
  - Forward Primer GAGGCTGCTGAGGCACTTAAAGG
  - Reverse Primer GAGTAACAGATGCTCCTAGTCTATT

Human reference total RNA pooled from 10 human cell lines (Stratagene, La Jolla, California, USA) was used as a positive control. RNA-free DNA extracted from tumour tissues was used as a negative control.

Data regarding EGFR and HER2 protein expression using IHC were available in 241 and 228 patients, respectively (Table 1). EGFR was assessed at the Department of Pathology of the Metaxas Cancer Hospital, Athens, as described earlier (Tzaida et al., 2007). HER2 was determined at the Department of Pathology of the Hygeia Hospital, Athens, with additional analysis of cases with an IHC score of 2 + by FISH, as described earlier (Kostopoulos et al., 2006).

**Statistical analysis**

Categorical data are presented as counts and corresponding percentages, whereas continuous data are presented as medians and ranges. For all receptors, the median was the pre-specified cutoff point and its distinguishing ability for patient prognosis was tested by means of the log-rank test. In case of no distinguishing ability of the median in terms of OS, the plan was to proceed with an exploratory analysis to test if the 25th and 75th percentiles were more appropriate cutoffs. Exploratory analysis was performed in a subgroup of the sample and validated in the rest of the patients. If the 25th and 75th percentiles were not validated as appropriate cutoff points, exploration would continue from the 10th to the 90th percentiles. In case a conclusion was reached on a cutoff point through exploration, subsequent analysis would initially be performed excluding the corresponding gene and repeated including it at the optimal cutoff (ability to distinguish OS significantly in the whole sample). We present the results of the analysis including this gene, only in cases where the results were not altered significantly. Comparison of categorical data between groups of patients was performed using the \( \chi^2 \)-test. Variables included in the comparisons were involved lymph nodes (0–3 vs \( \geq 4 \)), histological grade (good or moderate vs poor or undifferentiated), ER and PR status (positive vs negative), tumour size (\( \leq 2 \) vs \( 2–5 \) vs \( > 5 \) cm), histology (ductal vs lobular vs other) and age (\( < 50 \) vs \( > 50 \) years). Continuous data were compared using the Mann–Whitney test, or the Kruskal–Wallis test in case of more than two groups. Correlations among the receptors were assessed using the Spearman's Correlation Coefficient Method.

Overall survival was measured from time of chemotherapy initiation to patient's last contact or death. Disease-free survival was measured from time of chemotherapy initiation to patient's last contact or disease progression. Cases of disease progression, deaths from any cause without verified relapse and second cancers were treated as events in the estimation of DFS (Hudis et al., 2007). Survival was estimated using the Kaplan–Meier method. Comparisons between groups of patients, as defined by receptor cutoffs, were performed using the log-rank test. Multivariate Cox analysis including age (\( < 50 \) vs \( > 50 \) years), involved lymph nodes (0–3 vs \( \geq 4 \)), histology (ductal vs lobular vs other), histological grade (good or moderate vs poor or undifferentiated), size (\( \leq 2 \) vs \( 2–5 \) vs \( > 5 \) cm), ER/PR status (positive vs negative), hormone therapy (yes vs no), radiotherapy (yes vs no), EGFR (\( \geq 75 \)th percentile vs \( < 75 \)th percentile), and HER2, HER3, HER4 (\( > \)median vs \( < \)median) was performed. Variable selection was performed based on the likelihood ratio test with an exclusion criterion set at 0.10. The final model was adjusted for the group of randomisation (E-T-CMF vs E-CMF). Interaction between paclitaxel containing chemotherapy and the genes of interest was also considered. Level of significance was \( \alpha = 0.05 \) for all tests. Results of this study were presented according to reporting recommendations for tumour marker prognostic studies (McShane et al., 2005). The statistical analysis was conducted using SPSS 11 for Windows.

**RESULTS**

**Normalised mRNA expression of HER family receptors**

The distribution of tumour samples according to the normalised expression of mRNA encoding for HER family receptors is shown in Figure 1. The median value for EGFR was 32.95 (range, 24.85–36.11), for HER2 35.56 (range, 30.32–40.98), for HER3 34.63 (range, 28.3–37.1), and for HER4 31.79 (range, 24.67–35.43).

**Concordance between kinetic RT–PCR and IHC**

The total number of tumours with data available from both IHC and kRT–PCR was 240 and 228 for EGFR and HER2, respectively. For EGFR, 39 of the 240 tumours (16%) were IHC positive, whereas 59 tumours (24.5%) were kRT–PCR positive. For HER2, 64 of the 228 tumours (28%) were IHC/FISH positive, whereas 113 tumours (49.5%) had HER2 mRNA expression above the median, as assessed by kRT–PCR. For these tumours, we found a statistically significant association between the evaluations obtained by the two methods, for the EGFR (Mann–Whitney test, \( P < 0.001 \)) and the HER2 (Kruskal–Wallis test, \( P < 0.001 \)) receptors. The observed overall concordance between the determination of HER2 by kRT–PCR and IHC/FISH was 74%. The levels for sensitivity and specificity were 92 and 67%, respectively. The overall agreement between kRT–PCR and IHC for EGFR was 75%. Sensitivity and specificity were 49 and 80%, respectively (Table 2).

**Relationships among HER family receptors mRNA expression**

A positive correlation was found between HER2 and HER3 mRNA levels (\( r = 0.224, P < 0.001 \)). No association was demonstrated between HER2 and the other two family members. Moreover, HER3 and HER4 mRNA values were positively correlated to each other (\( r = 0.444, P < 0.001 \)) and negatively correlated to EGFR (\( r = -0.143, P = 0.019 \) and \( r = -0.125, P = 0.043 \), respectively).

**Association of HER family receptors mRNA expression with clinicopathological parameters**

EGFR mRNA expression was inversely related to the presence of ER (\( P = 0.044 \)). HER2 was positively associated with the number of involved lymph nodes (\( P = 0.013 \)). HER3 mRNA expression was associated with ER positivity (\( P = 0.017 \)), whereas HER4 was associated with histopathological grade I + II (\( P = 0.001 \)) and ER and PR positivity (\( P < 0.001 \) and \( P < 0.001 \), respectively). Furthermore, EGFR mRNA expression was inversely associated with ductal histology (\( P = 0.029 \)), whereas that of HER2 was positively associated with ductal histological type (\( P = 0.001 \)).
Survival status of the patients was updated in October 2007. The median follow-up time was 95.5 months (95% CI: 92.4–98.6, range, 7–117 months). During this time, 87 patients had developed a relapse and 61 patients had died. The 3-year OS was 93% (95% CI: 90–96%), whereas the 5-year OS was 85% (95% CI: 81–90%). The 3-year DFS was 80% (95% CI: 75–84%), whereas the 5-year DFS was 74% (95% CI: 68–79%).

For each of the HER family receptors, three cutoff points (25th, 50th and 75th percentiles) were assessed for prognostic value. In the majority of cases, the median (50th percentile) was the optimal cutoff point. However, in the case of EGFR the 75th percentile was

Table 2 Evaluation of HER2 and EGFR by kRT–PCR compared with IHC

| HER2 (IHC/FISH) | NPV/PPV % | Sensitivity % | Specificity % |
|----------------|-----------|---------------|---------------|
| 0, 1+, 2+/FISH(−) | 96/52     | 92            | 67            |
| 2+/FISH(+) 3+   | 5 (8%)    |               |               |
| HER2 (kRT-PCR) | Below median | 110 (67%) | 5 (8%)   | 96/52     | 92 | 67 |
|                | Above median | 54 (33%) | 59 (92%) |           |   |    |

| HER3 mRNA expression | 50th percentile | 25th percentile | 75th percentile |
|----------------------|-----------------|-----------------|-----------------|
|                      | 32              | 28              | 38              |

| HER4 mRNA expression | 50th percentile | 25th percentile | 75th percentile |
|----------------------|-----------------|-----------------|-----------------|
|                      | 32              | 28              | 38              |

Egfr (IHC) N = 240

| Negative | Positive |
|----------|----------|
| 161 (80%) | 20 (51%) |
| 40 (20%)  | 19 (49%)  |

NPV, negative predictive value; PPV, positive predictive value.

Prognostic value of HER family receptors mRNA expression

Survival status of the patients was updated in October 2007. The median follow-up time was 95.5 months (95% CI: 92.4–98.6, range, 7–117 months). During this time, 87 patients had developed a relapse and 61 patients had died. The 3-year OS was 93% (95% CI: 90–96%), whereas the 5-year OS was 85% (95% CI: 81–90%). The 3-year DFS was 80% (95% CI: 75–84%), whereas the 5-year DFS was 74% (95% CI: 68–79%).

For each of the HER family receptors, three cutoff points (25th, 50th and 75th percentiles) were assessed for prognostic value. In the majority of cases, the median (50th percentile) was the optimal cutoff point. However, in the case of EGFR the 75th percentile was
the best threshold, allowing us to distinguish two populations of significantly different prognosis. Using the 75th percentile, patients whose tumours had increased EGFR mRNA expression had significantly reduced OS (22 out of 67 deaths in EGFR-positive vs 38 out of 200 deaths in EGFR-negative patients, log-rank $P = 0.022$) (Figure 2A1). The hazard ratio (HR) for death in EGFR-positive patients was 1.83 (95% CI: 1.08–3.09, $P = 0.024$). The median value was used as a cutoff point for HER2, HER3 and HER4. A significant association between HER2 mRNA overexpression and reduced OS was demonstrated (39 out of 134 deaths in HER2-positive vs 22 out of 134 deaths in HER2-negative patients, log-rank $P = 0.024$) (Figure 2A2). The HR for death in HER2-positive patients was 1.81 (95% CI: 1.07–3.05, $P = 0.027$). In contrast, HER3 mRNA expression was not found to be significant in the subgroup of ER-negative patients ($P = 0.61$–1.81) (Figure 3). In the subgroup of ER-positive patients, respect to the DFS, the interaction was also non-significant ($P = 0.86$ for OS and $P = 0.654$ for DFS).

In addition, mRNA expression of EGFR, HER3 and HER4 was not predictive for benefit from adjuvant treatment with paclitaxel, neither for OS nor for DFS (data not shown).

**DISCUSSION**

In this study, we used kinetic RT–PCR to analyse the transcriptional profiling of the HER family receptor genes, in a comparatively large series of high-risk (predominantly T2–3, node-positive) early breast cancer patients, with a considerably long follow-up of 8 years. Our analysis included gene transcription assessment of all four HER family members. The majority of the clinical pathological studies have focused on protein expression and/or gene amplification of individual HER family receptors. Consequently, the clinical outcome of breast cancer patients with regard to HER family expression as a whole panel remains largely unidentified. In addition, only a small number of trials have evaluated HER family receptors at the mRNA level.

In our patient cohort, the overall concordance between kRT–PCR and IHC/FISH for the determination of HER2 status was good (74%). Our data confirm previous studies demonstrating a substantial agreement between the results of HER2 status evaluation at the mRNA and protein levels (Ginestier et al, 2004; Gjerdrum et al, 2004; Vinatzer et al, 2005). A recent study that compared four different methods of assessment of HER2 status found a good correlation between RT–PCR and IHC, with an overall concordance that varied from 82 to 93% (Ginestier et al, 2004). In another study that assessed HER2 status at the DNA, mRNA and protein levels, the concordance of the RT–PCR with the HercepTest was 86.4% (Vinatzer et al, 2005). Using the 75th percentile as a threshold in our exploratory analysis, the concordance between the two methods was higher (87%). However, the prognostic ability of HER2 mRNA expression was lost, suggesting that the increase in the cutoff point is likely to miss the effect of lower, but potentially biologically important mRNA levels of HER2. With the use of the median value as a threshold and considering the IHC/FISH as the standard technique for HER2 assessment, the kRT–PCR assay was associated with a high level of sensitivity (92%) and satisfactory specificity (67%). The majority of HER2-positive tumours by IHC/FISH were also categorised as HER2-positive by kRT–PCR (92%). However, among cases showing strong protein expression in IHC, 8% displayed low mRNA expression. This observation may be related either to increased mRNA degradation in FFPE tumour blocks or to accumulation of the protein product, due to aberrant catabolism. Our findings suggest that kRT–PCR is an alternative method for evaluating HER family receptors in FFPE breast tumours. However, routine methods of histological fixation and tissue processing could potentially damage or destroy RNA. In addition, dilution of tumour genomic material by nucleic acids from non-neoplastic tissue components is also a potential source of imprecision (Gjerdrum et al, 2004). Furthermore, the required equipment for kRT–PCR is not available in all histopathology laboratories and is quite expensive.

The mRNA expression levels of HER3 and HER4 receptors were positively correlated to each other and negatively correlated to EGFR, in complete agreement with previously reported data (Knowlden et al, 1998; Pawlowski et al, 2000; Bieche et al, 2003). In addition, we demonstrated a positive association between HER2 and HER3 mRNA expression. A similar correlation was described earlier, both at the mRNA and protein level (Bieche et al, 2003; Witton et al, 2003; Sassen et al, 2008). It has been suggested that the HER2/HER3 heterodimer constitutes the most mitogenic dimer in the HER family (Citré et al, 2003). HER2 does not bind to phosphatidylinositol 3-kinase (PI3K) and this function is directly mediated through the HER3 receptor (Prigent and Gullick, 1994). With respect to the relationships with the clinicopathological
Figure 2  (A1) OS (P = 0.022) and B1. DFS (P = 0.076) for patients with EGFR mRNA expression < 75th percentile (N = 200, blue line) and ≥75th percentile (N = 67, red line). (A2) OS (P = 0.024) and B2. DFS (P = 0.026) for patients with HER2 mRNA expression < median (N = 134, blue line) and ≥median (N = 134, red line). (A3) OS (P = 0.026) and B3. DFS (P = 0.135) for patients with HER3 mRNA expression < median (N = 133, blue line) and ≥median (N = 134, red line). (A4) OS (P = 0.010) and B4. DFS (P = 0.001) for patients with HER4 mRNA expression < median (N = 130, blue line) and ≥median (N = 130, red line).
clinical studies

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HER2 status in relation to clinical outcome in breast cancer

PCR (Q-PCR) and RNA expression profiles (RNA-EP) to evaluate yielding comparable prognostic information (Vinatzer assessment of HER2 status as the current standard methods, RT– PCR showed that this technique is clinically as useful in the studies investigating the prognostic value of HER2 using real-time prognostic value for OS in the multivariate analysis. Previous significance in terms of OS and DFS. Moreover, HER2 retained its worse OS when compared to those with either EGFR or HER2 both EGFR and HER2 mRNA overexpression had significantly

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In the prognostic analyses, we found a shorter OS in patients with increased EGFR mRNA expression, using the 75th percentile as a cutoff point. In addition, the prognostic significance of EGFR for OS was maintained in the multivariate analysis. EGFR is generally considered to be a negative prognostic factor in breast cancer (Pawlowski et al, 2000; Tsutsui et al, 2002; Witton et al, 2003; Tzaida et al, 2007), but up to now, no definitive association between EGFR expression and survival has been demonstrated. The role of EGFR in HER2-mediated cellular transformation is not fully elucidated. Experiments have provided some evidence for a synergistic interaction of these receptors in cellular transformation and induction of mammary tumours (DiGiovanna et al, 1998). Moreover, interactions between EGFR and HER2 with respect to the prognosis of breast cancer patients have been reported (DiGiovanna et al, 2005). Similarly, in our study, patients with both EGFR and HER2 mRNA overexpression had significantly worse OS when compared to those with either EGFR or HER2 overexpression.

Prognostic value of HER family mRNA in early breast cancer

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Table 3 Multivariate analysis (N = 260)

| Parameter       | OS          | DFS          |
|-----------------|-------------|--------------|
|                 | HR 95% CI   | P-value      | HR 95% CI   | P-value      |
| EGFR            |             |              |             |              |
| <75th percentile| 1.71 1.00–2.93 | 0.050       | 1.52 0.95–2.44 | 0.079       |
| ≥75th percentile| 1.84 1.07–3.17 | 0.027       |              |              |
| HER2            |             |              |             |              |
| <Median         | 1            |              | 1            |              |
| ≥Median         | 2.42 1.08–5.34 | 0.032       | 2.70 1.38–5.28 | 0.004       |
| Grade           |             |              |             |              |
| I–II            |              |              | 1.52 0.96–2.41 | 0.071       |
| III-Undifferentiated | 1.52 0.96–2.41 | 0.071       |              |              |
| Adjuvant hormone |             |              |             |              |
| No              | 0.48 0.23–0.99 | 0.50        | 0.52 0.27–0.99 | 0.046       |
| Yes             |              |              |              |              |
| Group of randomisation | | | | |
| E-T-CMF         | 0.85 0.51–1.41 | 0.526       | 0.93 0.60–1.45 | 0.928       |
| E-CMF           |              |              |              |              |

parameters, our findings are in accordance with previous studies evaluating the expression of HER family members in breast cancer, either at the mRNA or the protein level.

In the prognostic analyses, we found a shorter OS in patients with increased EGFR mRNA expression, using the 75th percentile as a cutoff point. In addition, the prognostic significance of EGFR for OS was maintained in the multivariate analysis. EGFR is generally considered to be a negative prognostic factor in breast cancer (Pawlowski et al, 2000; Tsutsui et al, 2002; Witton et al, 2003; Tzaida et al, 2007), but up to now, no definitive association between EGFR expression and survival has been demonstrated. The role of EGFR in HER2-mediated cellular transformation is not fully elucidated. Experiments have provided some evidence for a synergistic interaction of these receptors in cellular transformation and induction of mammary tumours (DiGiovanna et al, 1998). Moreover, interactions between EGFR and HER2 with respect to the prognosis of breast cancer patients have been reported (DiGiovanna et al, 2005). Similarly, in our study, patients with both EGFR and HER2 mRNA overexpression had significantly worse OS when compared to those with either EGFR or HER2 overexpression.

With regard to HER2, we confirmed its negative prognostic significance in terms of OS and DFS. Moreover, HER2 retained its prognostic value for OS in the multivariate analysis. Previous studies investigating the prognostic value of HER2 using real-time RT–PCR showed that this technique is clinically as useful in the assessment of HER2 status as the current standard methods, yielding comparable prognostic information (Vinatzer et al, 2005). A recent study (Bergqvist et al, 2007) used quantitative real-time PCR (Q-PCR) and RNA expression profiles (RNA-EP) to evaluate HER2 status in relation to clinical outcome in breast cancer patients. Analyses of relapse-free survival and OS on the basis of 5 and 10 years follow-up indicated that, in contrast to IHC/chromogenic in situ hybridisation, both Q-PCR and RNA-EP analyses yielded significant results after 10 years of follow-up. These findings suggest that both Q-PCR and RNA-EP are of similar, or even superior, prognostic value compared with the current standard techniques.

The prognostic value of HER3 remains up to now unclear and the available data are contradictory. A number of studies evaluating HER family receptors have indicated a negative prognostic value of HER3 in breast cancer patients (Bieche et al, 2003; Witton et al, 2003; Sassen et al, 2008). In contrast, our present study showed a positive association between HER3 mRNA expression and OS. Moreover, in the multivariate analysis HER3 maintained its prognostic value for OS. Other studies support the positive prognostic ability of HER3 as well (Quinn et al, 1994; Pawlowski et al, 2000; Lee et al, 2007). It has also been shown that a naturally occurring secreted form of the human HER3 receptor is a potent negative regulator of neuregulin-stimulated HER family receptor activation (Lee et al, 2001).

Regarding the prognostic significance of HER4, a positive association of HER4 mRNA expression with both OS and DFS was shown. Furthermore, HER4 retained its prognostic power for DFS in the multivariate analysis. Other studies have also supported the favourable prognostic role of HER4 in breast cancer both at the mRNA and the protein level (Pawlowski et al, 2000; Suo et al, 2002; Witton et al, 2003). This positive effect is most likely associated with an inhibitory effect on growth and differentiation signalling. In cell line experiments, when HER2-positive cancer cells were transfected to overexpress HER4, a reduction in proliferation and an increase in apoptosis was observed (Sartor et al, 2001). More recent studies have further increased our knowledge regarding HER4-associated apoptosis (Naresh et al, 2006).

With respect to the prognostic power of the combined expression profile of all four HER family receptors, we found that the combination of low EGFR, low HER2, high HER3, and high HER4 mRNA expression was associated with a significantly longer OS and DFS, compared to any other combination. This finding suggests that it is the co-expression pattern, rather than the expression of individual family members, that should be taken into account when evaluating the prognosis of the patients and making individualised therapeutic decisions. Moreover, it has been demonstrated that binding of specific ligands to the extracellular domain allows for receptor homo- or heterodimerisation resulting in activation of the cytoplasmatic catalytic function, which leads to receptor autophosphorylation. This autophosphorylation triggers a complex series of signal transduction pathways, such as phosphatidylinositol 3-kinase-Akt, Ras-Raf-MEK-mitogen-activated protein kinase-dependent pathway, PLC–PKC, and JAK/STAT. These pathways affect essential tumorigenic processes, such as proliferation, differentiation, migration, inhibition of apoptosis, and enhanced survival. Therefore, apart from the co-expression of the receptors, the expression of ligands, as well as the cross-talk on different levels among the signal transduction pathways, might also be important.

In this study, we also investigated the predictive ability of the gene transcription of the HER family receptors in tumours of high-risk breast cancer patients. The patients had participated in the randomised HE10/97 trial evaluating the effect of anthracycline-based dose-dense sequential adjuvant chemotherapy, with or without paclitaxel (Fountzilas et al, 2001). Moreover, long-term follow-up was available. Patient characteristics were well balanced between the two arms with the exception of grade, a difference also observed in the prospective clinical trial (Fountzilas et al, 2005). The unbalance concerning histological grade is an important issue, as it may have an impact on the results. However, since a multivariate analysis was performed, including both grade and
randomisation arm, all presented results take into account this unbalance.

To the best of our knowledge, this is the first study evaluating the effect of a taxane-containing regimen vs a non-taxane treatment, according to HER2 status at the mRNA level. In the HE10/97 clinical trial, the addition of paclitaxel had no influence in DFS and OS. In our patient cohort, the interaction between HER2 mRNA expression in the tumours and the addition of paclitaxel was not significant. In the entire HE10/97 trial, the hazard of death was significantly reduced when patients with negative hormonal receptor status were treated with paclitaxel. There is evidence that ER positivity may represent a negative predictive factor for the response to chemotherapy in breast cancer (Berry et al, 2006). In our patient cohort, performing an exploratory analysis based on ER status, no significant HER2/paclitaxel interaction was found in either ER-positive or ER-negative patients. Therefore, no predictive ability of HER2 mRNA expression for paclitaxel was established in our study.

Recently, investigators from the CALGB 9344 randomised adjuvant trial (Henderson et al, 2003) reported that patients with HER2-positive tumours derived significant benefit from the addition of paclitaxel to a doxorubicin–cyclophosphamide regimen regardless of ER status, whereas there was no additional benefit in HER2-negative, ER-positive patients. Therefore, no predictive ability of HER2 mRNA expression for paclitaxel was established in our study. In conclusion, the present study suggests that EGFR as well as HER2 mRNA overexpression are prognostic factors of worse clinical outcome in high-risk operable breast cancer patients, whereas HER3 and HER4 mRNA overexpression are both associated with a better prognosis. The combined expression profile of the HER family receptors, and not the isolated HER2 < median value HER2 ≥ median value HER2 ≥ median value HER2 < median value Figure 3 OS (A1 and A2) and DFS (B1 and B2) for patients treated with (red line) or without (blue line) paclitaxel, according to HER2 mRNA expression. A1 and B1 (HER2 < median): 55 (41%) E-T-CMF and 79 (59%) E-CMF-treated patients. A2 and B2 (HER2 ≥ median): 62 (46%) E-T-CMF and 72 (54%) E-CMF-treated patients.
expression of individual members, is likely to be more important when assessing the prognosis of the patients. Furthermore, on the basis of our findings, HER2 gene transcription does not predict greater sensitivity to paclitaxel-based adjuvant chemotherapy. In addition, kinetic RT–PCR represents a valid alternative method for detection and quantification of HER family receptor gene expression in FFPE breast tumour tissues.

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ACKNOWLEDGEMENTS

We thank Ms Georgia Vourli (MSc) for the statistical analysis, Ms Evita Fragou and Ms Dimitra Katsala for monitoring the study, Ms Maria Moschoni for coordinating the data management, Ms Thalia Spinari for tissue sample collection, and Ms Inke Feder and Mr Patrick Maass for excellent technical assistance.
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