Abstract. Background: Only a subset of breast cancer patients respond to the HER2 inhibitor trastuzumab, and methods to identify responders are needed. Patients and Methods: We studied 28 patients with metastatic breast cancer that had amplified human epidermal growth factor receptor 2 (HER2) genes in their primary tumour and were treated with a combination of trastuzumab and chemotherapy. Plasma was collected and amplification of the HER2 gene in circulating DNA and the amounts of the extracellular domain (ECD) of HER2 were measured just before first treatment (n=28) and just before second treatment three weeks later (HER2 DNA (n=22), HER2 ECD (n=23)). Results: Pretreatment levels of HER2 gene amplification and HER2 ECD did not correlate to clinical parameters. However, 9 out of 22 patients had a more than a 14% (2 × SD) reduction in HER2 gene amplification following treatment and showed improved response (p=0.02), and overall survival (p=0.05). HER2 ECD kinetics did not correlate to clinical data. Conclusion: We suggest that a decrease in HER2 gene amplification in the plasma predicts a more favourable response to trastuzumab.

Human epidermal growth factor receptor 2 (HER2) is one of four receptors of the epidermal growth factor (EGF) system. It is a characteristic of HER2 that it is overexpressed in approximately 20-30% of invasive breast tumours by a molecular mechanism most frequently involving an amplification of the gene on chromosome 17. The presence of HER2 overexpression in breast cancer is associated with a poor prognosis (1, 2). Trastuzumab is a humanized monoclonal antibody directed against HER2, which binds the extracellular domain of HER2 thereby blocking its activity. Treatment of breast cancer patients with trastuzumab has been shown to increase response rates and survival of these patients with HER2-positive metastatic disease. The beneficial effect of trastuzumab was observed not only when used in combination with chemotherapy (3-6), but also when used as a single agent (7, 8). Traditionally, the overexpression of HER2 protein has been identified by immunohistochemistry (IHC) and amplification of the gene has been determined with fluorescence in situ hybridization (FISH) (9, 10). In a subset of breast cancer patients there is an increased proteolytic release of the extracellular domain of HER2 (HER2 ECD). A commercial kit (HER2/neu kit from Bayer) is available for measuring the HER2 ECD released to the blood. The usefulness of HER2 ECD measurements in predicting the efficacy of trastuzumab treatment of breast cancer patients has been shown (11, 12).

Tumour DNA is present in the blood of patients with a number of cancer types (13). Measurement of circulating tumour DNA has been suggested to have prognostic value in several types of cancer, such as malignant melanoma (14), prostate cancer (15), non small cell lung cancer (16) and colorectal cancer (17).

In the present study, we investigated amplification of the HER2 gene using plasma DNA from breast cancer patients with an amplified HER2 gene in the primary tumour and studied its relationship to survival and response to trastuzumab treatment.

Patients and Methods

Patients. Women with HER2-positive, locally advanced or metastatic breast cancer who started first-line treatment with trastuzumab in combination with chemotherapy were eligible for the study. Patient characteristics are indicated in Table I. HER2 positivity was determined first by immunohistochemistry using HercepTest (Dako, Glostrup, Denmark) and this result was verified by FISH testing using Vysis Path-Vysion HER-2/neu DNA probe kit (Abbott, Abbott Park, IL, USA) according to the procedures previously described for both methods (18). Patients entered the study in a consecutive manner and all patients were diagnosed with an amplified HER2 gene in the primary tumour as determined with the methods described above.
The chemotherapy regimens were docetaxel 100 mg/m² at day 1 every three weeks combined with trastuzumab 8 mg/kg loading and 6 mg/kg maintenance dose at day 1 every three weeks, or i.v. vinorelbine 35 mg/m² at day 1 and 8 every three weeks concomitant with trastuzumab as described above. Tumour response was assessed every third cycle by clinical examination, X-ray, computerized tomography (CT) or magnetic resonance imaging (MRI) using the WHO criteria (19).

For both the HER2 DNA and HER2 ECD assays, 28 patients entered the study and a pre-treatment plasma sample was obtained. One patient died before the second blood sample could be taken. From four patients, it was not possible to get a second blood sample, and from one patient there was only sufficient plasma from the second blood sample for the HER2 ECD assay. Thus, a plasma sample was obtained for HER2 ECD and gastrin (GAST) DNA, where GAST DNA is used as the reference gene. The ratio between the two genes was calculated and used as a measure of HER2 gene amplification. Real-time PCR was conducted with a Lightcycler Instrument with Lightcycler version 1.5 Software (Roche, Basel, Switzerland). The imprecision of the assay was measured by inclusion of internal controls in each analytical run and a coefficient of variation (interassay variation) of 17.4% (a control DNA sample was used with a mean value of the HER2/GAST ratio of 2.7 (13 independent runs)).

Measurement of HER2 ECD. The amount of HER2 ECD circulating in the blood was measured with HER2/neu assay (Bayer, Leverkusen, Germany). Plasma (200 μl) was analysed with the ADVIA Centaur HER-2/neu immunoassay kit according to the supplier’s instructions. This assay is a sandwich immunoassay that is FDA approved to determine the changes in HER2 ECD during trastuzumab treatment.

Results

HER2 DNA in plasma of patients with metastatic breast cancer. The ratio between HER2 and GAST DNA in the plasma DNA isolated from breast cancer patients just prior to the start of treatment with trastuzumab combined with chemotherapy is presented in Figure 1. The mean of the HER2/GAST ratio of controls without cancer was assigned the value 1 (SD=0.07) (Figure 1). The HER2/GAST ratio was above 1.14 (2 × SD above the mean of the controls) in 50% (14 out of 28 patients). This thus defines two groups of patients with either an elevated or a normal HER2 DNA status. Correlation of the HER2 DNA status (elevated or normal) immediately before the first treatment with trastuzumab and chemotherapy to time to progression (TTP) or overall survival (OS) demonstrated no significant association. The patients were classified into four groups according to response: complete response, CR; partial response, PR; no change, NC; and progressive disease, PD. One patient obtained a CR, fourteen a PR, eight showed NC, four had PD, and one patient was not evaluable as she died in febrile neutropenia after the first treatment with vinorelbine and trastuzumab. There was no statistically significant correlation between HER2 DNA status (elevated or normal) before trastuzumab treatment and response.

Kinetics of HER2 DNA in the plasma of breast cancer patients treated with trastuzumab. From 22 of the 28 patients it was possible to analyse the HER2 DNA and GAST DNA levels both immediately before and three weeks after the first treatment. This enabled us to determine the kinetics of the HER2/GAST DNA ratio following treatment. In 9 patients, the level of

Table I. Patient characteristics at baseline for women with metastatic HER2-positive breast cancer undergoing treatment with trastuzumab and chemotherapy.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>Median (range) 56 (33-71)</td>
</tr>
<tr>
<td>Hormone receptor status</td>
<td>Positive 10, Negative 17, Unknown 1</td>
</tr>
<tr>
<td>Performance status</td>
<td>0 18, 1 8, 2 2, 3 0, 4 0</td>
</tr>
<tr>
<td>Site of metastasis</td>
<td>Lung 7, Liver 8, Bone 15, Soft tissue 19, Other 3</td>
</tr>
<tr>
<td>Treatment</td>
<td>Vinorelbine/trastuzumab 12, Docetaxel/trastuzumab 16</td>
</tr>
<tr>
<td>Menopausal status</td>
<td>Premenopausal 9, Postmenopausal 19</td>
</tr>
</tbody>
</table>

Isolation of DNA from plasma. A volume of 4 ml of blood was drawn into EDTA tubes (Terumo, Leuven, Belgium). The blood sample was centrifuged at 1250×g for 10 minutes and the supernatant (plasma) was transferred to another test-tube. A total of 200 μl of plasma was used for DNA isolation with QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. DNA was redissolved in 50 μl water and stored at −80°C.
HER2 DNA amplification in the plasma DNA decreased by more than 14% (2 × SD of the controls), whereas in 13 patients it did not (Figure 2A and 2B, respectively). The group of patients whose HER2 DNA decreased had a better OS (Figure 3, p=0.05, log-rank test), as well as a better response to treatment (Table II, Pearson’s Chi-square test, p=0.02). There was no statistically significant correlation between the kinetics of HER2 DNA amplification and TTP (p=0.24, log-rank test, data not shown).

HER2 ECD in plasma of patients treated with trastuzumab. The amount of HER2 ECD was determined in the plasma of 28 patients immediately before trastuzumab treatment and in 47 healthy individuals (Figure 4). The supplier of the HER2/neu kit suggests a cut-off value of 15 ng/ml. In 15 out of the 28 patients (54%), there was an HER2 ECD concentration higher than this cut-off value, while none of the controls had values above this cut-off. There was no difference in OS or TTP between the group of patients that
had HER2 ECD >15 ng/ml (n=15) and those below this concentration (n=13), and no correlation with the four response groups (CR, PR, NC, PD). Likewise, there was no correlation to response when the patients were grouped into responders (PR or CR) or non-responders (PD or NC).

We reanalysed our data using the mean + 2 SD (12.4 ng/ml) calculated based on the results obtained for the controls (n=47) included in our study as the cut-off value. As with the cut-off value from the manufacturer, this did not result in any statistically significant correlation to OS, TTP or response in our cohort of patients.

In 23 out of the 28 patients receiving trastuzumab combined with chemotherapy, a plasma sample obtained both immediately before and three weeks after start of this treatment was available for testing. According to the recommendations of the supplier of the HER-2/neu kit, a pre-treatment value above 15 ng/ml (seen in 12 out of the 23 patients) combined with a more than 15% increase (seen in 1 out of these 12 patients) indicates progression of the disease during trastuzumab treatment. In our 23 patients, progression was only predicted in 1, although 9 patients (39%) actually progressed. Statistical analysis showed that the assay did not predict response in our patients. Similar results were obtained if we used the cut-off value of 12.4 ng/ml (mean + 2 SD of the controls in our study) instead of the 15 ng/ml suggested by the manufacturer.

Discussion

To our knowledge, we present the first attempt to measure the level of HER2 gene amplification in the blood of breast cancer patients and to use this as a predictor of response to treatment with trastuzumab in combination with chemotherapy.

The HER2 inhibitor trastuzumab has proven to be useful in the treatment of metastatic breast cancer, and a beneficial effect has been observed when patients were either treated with trastuzumab alone or with trastuzumab in combination with conventional chemotherapy (3, 6, 7). However, only one-third of the patients respond to trastuzumab given as monotherapy, and two-thirds respond to trastuzumab given together with conventional chemotherapy (5, 20). Methods are needed that are able to identify the responding patients. We demonstrate that in women with metastatic breast cancer with a primary tumour displaying an amplified HER2 gene, the amplified HER2 DNA can be detected in the free DNA circulating in the blood in 50% of the patients. Furthermore, a reduction in this circulating HER2 DNA following treatment with trastuzumab combined with chemotherapy is associated with response to treatment, as well as to an improved OS. However, we only found HER2 amplified DNA in approximately half of the patients before treatment, and as all the patients had a primary tumour with HER2 DNA amplification, this suggests that the patients differ with respect to the release of tumour DNA into the circulation. The mechanism responsible for this is not known. However, in our patients, the amount of amplified HER2 DNA before treatment showed no correlation to OS or response.
The ECD of HER2 is released from the cells after proteolytic cleavage and can be detected in the bloodstream of a subset of patients with metastatic breast cancer (21). A commercial kit is available for measuring the HER2 ECD in the blood and it has been shown that in patients with a HER2 ECD level above 15 ng/ml and an elevation of the HER2 ECD concentration after treatment with trastuzumab of 15% above the baseline level (i.e. the amount measured before treatment with the drug) correlates to progression of the disease (12, 22, 23). However, in our group of patients, we only observed one patient where progression was predicted based on these criteria and no association between HER2 ECD and survival or response to treatment was observed.

We established a cut-off level by analysing HER2 DNA in the plasma of controls without cancer under the same conditions as the patient samples. However, use of this cut-off level also did not predict either response or survival. Our group of patients was treated with trastuzumab together with either docetaxel or vinorelbine, whereas the patients in the study by Koestler et al. (12) were treated with either trastuzumab alone or in combination with one of four different chemotherapeutic agents. Thus, it is a possibility that there is a difference in the performance of the HER2 ECD assay in predicting the response of patients treated with trastuzumab in combination with different types of chemotherapy. In another study that used a reduction to 77% of the baseline level of HER2 ECD as the cut-off level, a correlation to OS was observed (n=99) (11). However, using this cut-off level did not reveal any correlation to OS, TTP or response in our patient group (data not shown). Our patient cohort was relatively small, and this might explain why the HER2 ECD assay was unable to predict the outcome of the treatment. However, despite the limited size, the method based on the kinetics of circulating amplified HER2 DNA was able to show predictive value even in this limited number of patients.

Our results show that a decrease of HER2 gene amplification in the plasma can predict response and OS. This suggests that it might represent a new method that could prove useful in monitoring trastuzumab treatment of breast cancer patients as early as three weeks after the first treatment. This method offers the advantage that the amplified HER2 DNA in the blood represents the situation at the time of treatment. This might offer an improvement as compared to the traditional diagnostic principles, which are most often only based on a biopsy of the primary tumour usually taken at an earlier time point. However, our patient cohort was relatively small and to confirm the potential of such a method, additional studies need to be performed with a larger number of patients included.

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