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Serum EGFR levels and efficacy of trastuzumab-based therapy in patients with metastatic breast cancer

Gernot Hudelist[a,e], *Wolfgang J. Köstler*[b], *Daphne Gschwantler-Kaulich*[c], *Klaus Czerwenka*[d], *Ernst Kubista*[a,c], *Ruth Müller*[c], *Samir Helmy*[c], *Mahmood Manavi*[c], *Christoph C. Zielinski*[a,b], *Christian F. Singer*[a,c,*]

[a]*Ludwig Boltzmann Institute for Clinical and Experimental Oncology (CLEXO), Vienna, Austria*
[b]*Division of Oncology, Department of Medicine I and Center for Excellence in Clinical and Experimental Oncology, Medical University of Vienna, Vienna, Austria*
[c]*Division of Special Gynaecology, Department of OB/GYN, Medical University of Vienna, Wuhringer Gurtel 18-20, 1090 Vienna, Austria*
[d]*Division of Gynecopathology, Department of Pathology, Medical University of Vienna, Vienna, Austria*
[e]*Department of OB/GYN, Villach Medical Center, Austria*

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ABSTRACT

The antibody trastuzumab inhibits signal transduction in Her-2/*neu* overexpressing human breast cancer. However, the activation of co-expressed EGFR has also been show to additionally modulate the anti-tumoural effects of this drug. Similar to Her-2/*neu*, the extra cellular binding region of EGFR is believed to be proteolytically released from the cell surface upon receptor activation and can be detected in patients' serum (sEGFR). Considering the biological significance of an interaction between EGFR and Her-2/*neu* signalling in other human malignancies, we have investigated if trastuzumab treatment would affect sEGFR in 33 patients with Her-2/*neu* overexpressing metastatic breast cancer. We detected EGFR expression in 33% of Her-2/*neu* overexpressing breast tumours. In contrast to serum Her-2/*neu* (ECD) levels, which were correlated with the degree of Her-2/*neu* expression ($P = 0.048$, Mann–Whitney test), we did not detect significant differences between sEGFR serum levels in EGFR expressing or non-expressing tumours. Furthermore, sEGFR serum levels were not correlated with clinical parameters such as response or clinical benefit rates, and no association was found between increased sEGFR levels and progression-free survival or overall survival. While we have previously observed a selective and significant decrease of ECD levels in patients who derived a clinical benefit from trastuzumab treatment during the first weeks of treatment, we were unable to find similar alterations in sEGFR concentrations. We therefore conclude that the measurement of systemic sEGFR levels in addition to ECD serum concentrations do not allow the prediction of clinical course of trastuzumab-treated patients more accurately.

1. Introduction

Trastuzumab (Herceptin®) is a recombinant humanized monoclonal antibody targeted at the Her-2/*neu* receptor which is overexpressed in 20–30% of malignant breast tumours [1,2]. When administered as a single agent, trastuzumab has demonstrated overall tumour response rates between 15% [2,3] and 26% [3] in the metastatic setting. In

* *Corresponding author*: Tel.: +43 1 40400 2801; fax: +43 1 406 6749.
E-mail address: Christian.singer@meduniwien.ac.at (C.F. Singer).
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combination with chemotherapy, the antibody leads to prolonged disease-free and overall survival when compared to standard chemotherapeutic treatment regimens [4–6]. Trastuzumab exerts its anti-tumoural effects by binding to the Her-2/*neu* extracellular domain (ECD), which is then followed by internalization and degradation of the receptor protein [7,8], leading to a consecutive induction of G_1 arrest of cell cycle progression [9]. It also prevents shedding of the ECD of Her-2/*neu*, a process that would otherwise lead to the generation of a 95 kD phosphorylated carboxy-terminal membrane-bound fragment with active signalling properties and a 110 kD ECD released in the patients' bloodstream [10,11].

Growing evidence strongly suggests that the biological effects of trastuzumab not only depend on the grade of Her-2/*neu* overexpression, but are also modulated by the presence of the epidermal growth factor receptor (EGFR). This member of the Her family is expressed in 19–67% of breast carcinomas and binding of EGF, its principal ligand [12,13], results in the generation of receptor complexes with other EGFR-family members such as Her-2/*neu*, Her-3 or Her-4 [14,15]. The biological significance of the EGF system has recently been underscored by a study by Kuwada et al. [16]. The study demonstrated that Her-2/*neu* overexpressing colon carcinoma cell lines were more susceptible to the anti-tumoural effects of trastuzumab if they also overexpressed functional EGF/EGFR.

Similarly to Her-2/*neu*, the soluble extracellular binding region of EGFR (sEGFR) is proteolytically released from the cell surface of EGFR-expressing tumour cells and can be accurately quantified in serum via enzyme-linked immunosorbent assays [17,18]. Elevated levels of sEGFR have been detected in patients with invasive or recurrent carcinoma of the cervix when compared to premalignant lesions and healthy controls [19]. Recently, Gregorc et al. [20] were even able to predict response to treatment with the small-molecule EGFR tyrosine kinase inhibitor geftinib (Iressa®) in patients with non-small cell lung cancer. However, studies evaluating the clinical relevance of serum EGFR in breast cancer are still lacking.

Considering the complex interactions between EGFR and HER-2/*neu* receptor signalling, we have investigated the usefulness of sEGFR measurements in predicting the efficacy of trastzumab in patients with metastatic breast cancer (MBC) and have compared it to the soluble Her-2/*neu* (ECD) serum levels.

2. Patients and methods

2.1. Patient population

Trastuzumab (Herceptin®, Roche Pharmaceuticals, Vienna, Austria) ±chemotherapy was administered to all patients for grade II+ or III+ Her-2/*neu* overexpressing metastatic breast cancer between April 2000 and February 2002. Retrospective identification was performed by using pharmacy protocols. Treatment regimens were based on previously published treatment protocols [4,5,21–23]. Four milligram per kilogram was given as a loading and was followed by a weekly 2 mg/kg maintenance dose of trastuzumab [24]. All patients included in the present analysis were required to have bi-dimensionally measurable (with both diameters >1.0 cm and at least one lesion with both diameters >1.5 cm) disease (excluding previously irradiated or bone lesions as the only site of measurable disease) with clearly defined margins and radiologically (CT and/or MRI and/or ultrasound) documented tumour progression before initiation of trastuzumab-based treatment. Evaluation of response and restaging was performed every 6–8 weeks (depending on the therapeutic regimen) or earlier in case of clinical disease progression by independent review of patients' records and radiology reports in accordance with the Southwest Oncology Group response criteria and endpoint definitions. Further inclusion criteria consisted of the availability of intact paraffin embedded tissue from which the original assessment of Her-2/*neu* overexpression had been performed and the availability of patient sera obtained immediately before and during weekly trastuzumab-based treatment. Adequate amounts of sera stored at −80 °C (as previously described [25]) before analysis were available from 33 patients. A signed informed consent has been obtained from all patients included in the present analysis in accordance with our institutional ethical committee guidelines.

2.2. Immunohistochemistry

All testing was performed on identical tissue material used for initial patient selection for trastuzumab therapy (Herceptest©, DAKO, Glostrup, DK). Expression of EGFR was assessed as previously described [26]. In brief, 4 μm formalin-fixed paraffin embedded tumoural tissue sections were deparaffinized/hydrated by repeated washes in xylene, 100% methanol and in 95% ethanol. This was followed by rehydration. Sections were then pre-treated by digestion with 0.1% protease XIV (Sigma Aldrich, Vienna, Austria) for antigen unmasking. To block endogenous peroxidase activity, sections were incubated in 2% hydrogen peroxide for 10 min. Tissue specimens were then blocked with AB-Block (Vector Laboratories, Burlingame, CA) and incubated with the primary anti-EGFR antibody (clone 31G7, Zymed Laboratories, San Francisco, CA; diluted 1:10 in PBS) for 30 min at room temperature. Following this, the secondary anti-mouse IgG antibody (Vector Laboratories) was applied for further antigen visualization using the StrepHRP system (DAKO) and 3,3′-diaminobenzidine (DAB) according to the manufacturers protocol. Slides were then counterstained with hematoxilin and cover-slipped. Protein expression levels were assessed using the EGFR scoring guidelines (DAKO cytomation pharmDX).

Her-2/*neu* protein expression was evaluated by usingthe HercepTest kit (DAKO A/S, Glostrup, Denmark) for immunoenzymatic staining in accordance with the protocol described in the manufacturer's guide. Explained in brief, tissue sections were deparaffinized in xylene, rehydrated through ethanol to distilled water and immersedin Epitope Retrieval Solution (DAKO) at 95 °C following incubation in a waterbath at 95 °C for 40 min. Sections were then cooled down to room temperature (RT). Following this, tissue specimens were incubated at RT with the primary rabbit polyclonal antibody to the Her-2/*neu* oncoprotein (supplied by the kit manufacturer) on a DAKO Autostainerfor 30 min followed by application of peroxidase-blocking reagent. Antibody visualization was achieved via the DAKO Visualization Reagent

using horseradish peroxidase-conjugated goat anti-rabbitimmunoglobulins for 30 min (DAKO Autostainer). Sections were finally incubated with diaminobenzidine (DAB) as chromogen and counterstained with hematoxylin. Three cell pellets of formalin-fixed, paraffin-embedded human breast cell lines with staining intensity scores of 0, 1+, and 3+ (supplied by the kit manufacturer) were included in each staining run as positive controls. Negative controls were performed by substitution of the HER-2/*neu* primary antibody by normal rabbit serum (DAKO Negative Control Reagent). Only membrane staining intensity and pattern were evaluated using the 0 to 3+ scale as illustrated in the HercepTest kit scoring guidelines. All assessments were performed independently by two experienced pathologists who were blinded to the clinical course of patients.

2.3. *Her-2/neu ECD and sEGFR ELISA*

Serum values of the Her-2/*neu* extracellular domain (ECD), assessed using a sequential solid phase sandwich human Her-2/*neu* ELISA (Oncogene Science, Cambridge, MA), were available from a previous study from our laboratory [25]. For the present study, the concentration levels of the EGFR extracellular binding domain were determined by a sandwich quantitative enzyme-linked immunosorbent assay (EGFR Microtiter ELISA; Oncogene Science, Cambridge, MA) according to the manufacturer's instructions. Microplates of the EGFR ELISA were washed manually, whereas microplates of the Her-2/*neu* ELISA were washed using an automated washer (Dias Microplate Washer; Dynex Technologies, Denkendorf, Germany). Colorimetric quantification was performed using an automated spectrophotometer reader (FLUOstar Galaxy; BMG Labtech GmbH, Offenburg, Germany). Following the creation of a standard curve, EGFR and Her-2/*neu* serum concentrations were calculated using the Fluoscan Galaxy software (versions 4.20–0; BMG Labtech GmbH). Intra- and inter-assay precision of the both assays were below 5% coefficient of variation.

2.4. *Statistical analysis*

Fisher's exact test was used to compare frequencies of patients' characteristics. Comparisons between patient cohorts with/without response, clinical benefit and progression of disease and corresponding baseline measurements of the Her-2/*neu* ECD and sEGFR (defined by upper normal limits) were calculated by Fisher's exact test and log-rank test. Comparisons of differences between the sEGFR values and grade of EGFR expression were analysed by Mann–Whitney-*U* test. Pharmacodynamics of sEGFR values throughout the treatment with trastuzumab were analysed by Wilcoxon-signed ranks test and a local linear regression analysis. In order to investigate the predictive value of monitoring changes in sEGFR and Her-2/*neu* ECD values over the time of treatment with trastuzumab-based therapy with respect to response, clinical benefit, progression-free and overall survival, multiple logistic regression analysis and multiple Cox regression models were performed using EGFR and Her-2/*neu* serum levels as independent variables. Odds ratios (OR) was calculated with the proportional hazard method in respect of Cox regression and logistic regression. For all analyses, a *P*-value <5% was considered statistically significant. SPSS statistical software system (SPSS Inc., Chicago, IL, version 10.0) was used for all calculations.

3. Results

3.1. *Study population*

A total of 33 patients with Her-2/*neu* overexpressing metastatic breast cancer (MBC), who received trastuzumab-based treatment at our institution between March 2000 and October 2002 were included in the study. Our analysis covered a median observation period of 41.5 (range 35.2–52.8) months, during which 10 (30%) objective responses (including four complete responses) were observed. Eleven patients (33%) experienced disease stabilization ($\geqslant$3 months, with stable disease lasting $\geqslant$6 months in 6 patients). Twelve patients (36%) experienced primarily progressive disease under trastuzumab-based therapy. As of July 2004, 31 (94%) patients had experienced disease progression and 23 (70%) deaths had occurred, all of which were attributed to disease progression. No patient was lost to follow-up. Median progression-free and overall survival calculated from survival function were 4.6 (95% CI 0.0 to 9.3) and 17.4 (95% CI 9.3 to 25.4) months, respectively. Patients' characteristics are shown in Table 1.

3.2. *EGFR and Her-2/neu protein expression*

Immunohistochemical detection of EGFR receptor revealed weak protein expression (1+) in 5/33 cases (15.1%), moderate staining (2+) in 5/33 samples (15.1%) and strong (3+) expression in 1/33 (3%) tumour samples. Twenty-two out of 33 specimens (66.6%) did not express EGFR. Since Her-2/neu overexpression was the selection criterion for trastuzumab therapy, all tumours expressed either moderate (18%) or high amounts (82%) of Her-2/*neu* (see Table 1).

3.3. *Baseline sEGFR and ECD serum concentrations and response to trastuzumab*

When baseline serum EGFR and serum Her-2/*neu* (ECD) levels were measured before the initiation of a trastuzumab-based therapy, median sEGFR levels were 52.8 ng/ml (35.0–111.5) while median ECD levels were 53.7 ng/ml (5.2–6076.2). Twenty-six of 33 patients (79%) had serum ECD levels higher than 15 ng/ml, and were thus considered to have increased ECD [27]. In contrast, only 8 of 33 patients (24%) had sEGFR values above 65 ng/ml and, based on previous reports on the mean serum levels of EGFR in healthy controls [19], were therefore considered to have elevated sEGFR.

We did not detect a significant difference in sEGFR levels of patients who expressed EGFR (median 69.7, range 36.4–111.5 ng/ml) when compared with patients who did not show tumoural EGFR protein expression (median 51.2, range 35.0–94.9 ng/ml, $P = 0.109$ Mann–Whitney *U* test). However, as previously described, we did find that in the same patients 3+ Her-2/*neu* overexpression was associated with significantly higher serum ECD levels when compared to 2+ overexpres-

Table 1 – Patient's and treatment characteristics

Patient's characteristics	$n = 33$
Median age (range) years	53.5 (27.6–74.2)
Her-2/*neu* expression	
Grade 2+	6 (18%)
Grade 3+	27 (82%)
Estrogen receptor status	
Positive	11 (33%)
Negative	22 (67%)
Progesterone receptor status	
Positive	7 (21%)
Negative	26 (79%)
Histologic type	
Ductal	26 (79%)
Lobular	5 (15%)
Other	2 (6%)
Grading	
1	2 (6%)
2	5 (15%)
3	26 (79%)
Previous adjuvant chemotherapy	
Yes	21 (64%)
Sites of active disease	
Breast[a]	6 (18%)
Axilla	3 (9%)
Liver	22 (67%)
Lung	13 (39%)
Skin/soft tissue	4 (12%)
CNS	1 (3%)
Distant lymph nodes	12 (36%)
Bone	16 (48%)
Other	7 (21%)
Number of organs affected by metastatic disease	
2	7 (21%)
3	8 (24%)
4	8 (24%)
More	20 (61%)
Metastatic disease to visceral organs	27 (82%)
Median (range) recurrence-free interval (months)	22.3 (0.0–108.6)
Number of previous chemotherapeutic regimens for metastatic disease	
0	22 (67%)
1	9 (27%)
2 or more	2 (6%)
ECOG performance status	
0	9 (27%)
1	17 (52%)
2	7 (21%)
Treatment	
Single agent trastuzumab	7 (21%)
& Vinorelbine	14 (42%)
& Docetaxel and epirubicin	1 (3%)
& Docetaxel	2 (6%)
& Paclitaxel	4 (12%)
& Paclitaxel and gemcitabine	1 (3%)
& Capecitabine	4 (12%)

a Primary or local recurrence.

sion (median 71.4, range 5.2–6076.2 vs. median 11.5, range 9.3–245.4, $P = 0.048$), which is consistent with our earlier observation in a larger cohort [25].

When evaluating the influence of sEGFR serum levels on clinical response parameters, we did not find patients with elevated (i.e., >65 ng/ml) sEGFR levels to have altered response rates or clinical benefit rates when compared to patients with sEGFR levels of ⩽65 ng/ml (3 of 8, 38% vs. 7 of 25, 28% $P = 0.673$ Fisher's exact test and 4 of 8, 50% vs.17 of 25, 68% $P = 0.420$, respectively). In these patients, elevated ECD levels also did not result in altered response rates (9/26, 35% vs. 1/7, 14%, Fisher's exact test $P = 0.397$) and clinical benefit rates (17/26, 65% vs. 4/7, 57%, $P = 0.686$) when compared to patients with ECD levels below 15 ng/ml. Furthermore, neither increased sEGFR nor increased ECD were found to be associated with a significantly poorer median progression-free survival (PFS) (3.9 months, (95% CI 3.0–4.9) vs. 4.9 months (95% CI 0.0–11.1), log-rank $P = 0.876$, and 4.9 months (95% CI 0.0–14.4) vs. 4.2 months (95% CI 0.2–8.2), log-rank $P = 0.995$). Likewise, we did not find that elevated sEGFR serum concentrations were associated with an altered median overall survival (OS) when compared to sEGFR levels of <65 ng/ml (19.8 months (95% CI 5.2–34.4) vs. 17.3 months (95% CI 9.8–24.9); log-rank $P = 0.956$). We have previously shown that the same was also true for ECD [25].

3.4. sEGFR serum concentrations during trastuzumab-based treatment

Since EGFR activation is thought to lead to increased shedding of sEGFR, we investigated whether trastuzumab-mediated inhibition of the Her-2/*neu*/EGFR system might lead to a decrease in systemic sEGFR levels. Interestingly, in the overall population, we did not observe a decrease but rather an increase of sEGFR during the first week of trastzuzmab treatment from 52.8 to 65.3 ng/ml ($P = 0.011$, Wilcoxon's signed rank test, Table 2). A slight but significant further increase to 74.0 ng/ml was also seen throughout the ensuing weeks (week 2–10: $P = 0.049$; week 11–20: $P = 0.003$; Table 2). A local linear regression analysis of the overall sEGFR serum concentrations showed no significant alterations of systemic sEGFR after week 20 (data not shown). The same model was applied to the group of patients who experienced a clinical benefit under trastuzumab-based treatment (Fig. 1A) and to those patients who did not (Fig. 1B) and no significant differences in serum concentrations during the time course were observed (data not shown).

3.5. sEGFR and ECD serum concentration kinetics and response to trastuzumab-based treatment

We have previously demonstrated that in patients experiencing both response and clinical benefit to trastuzumab, a selective and significant decrease of Her-2 ECD serum levels could be observed within the first two weeks of treatment [25]. We therefore used a multivariate logistic regression model to investigate whether an alteration in sEGFR serum concentrations could similarly be used to discriminate those patients who profited from trastuzumab in the

Table 2 – Median concentrations of sEGFR during the first 20 weeks

	N	Median	Minimum	Maximum	P-value*
sEGFR at baseline (ng/ml)	39	52.8	35.0	111.5	–*
sEGFR of week 1 (ng/ml)	30	65.3	36.8	102.1	0.011
Mean sEGFR from week 2 to 10 (ng/ml)	17	66.3	40.1	106.4	0.049
Mean sEGFR from week 11 to 20 (ng/ml)	19	74.0	56.0	134.7	0.003

* The *P*-value was calculated with Wilcoxon's signed rank test in comparison to EGFR at baseline.

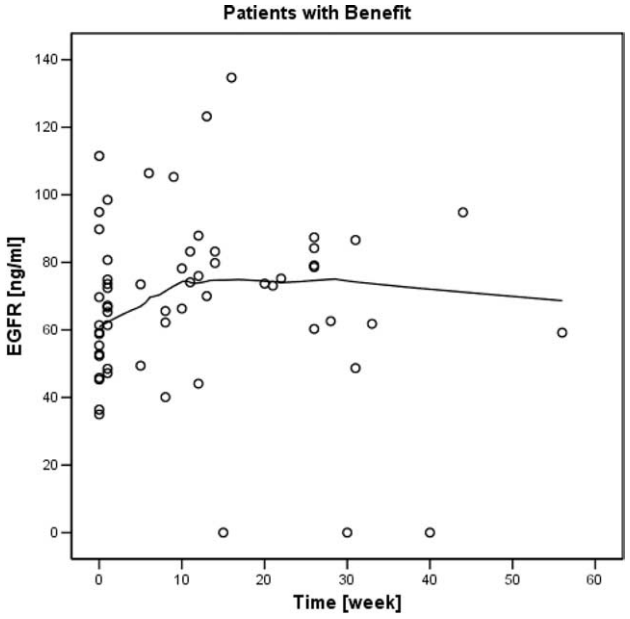

Fig. 1A – Serum EGFR levels in patients with clinical benefit from trastuzumab-based therapy.

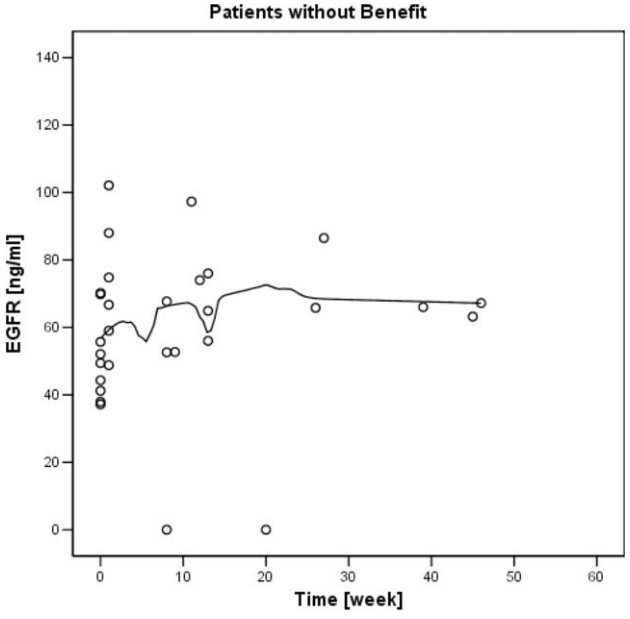

Fig. 1B – Serum EGFR levels in patients without clinical benefit from trastuzumab-based therapy.

metastatic setting. We were however unable to find significant alterations in the serum levels of sEGFR in women who showed a response or clinical benefit (data not shown). Furthermore, sEGFR was not significantly correlated with PFS or OS.

4. Discussion

To date, several studies have demonstrated that the growth potential and invasive behaviour of breast cancer cells is significantly altered by the expression of Her-family members [28,29]. Within this, the crosstalk between EGFR and Her-2/*neu* appears to be crucial for active receptor signalling and transformational properties of heterodimeric Her-receptor complexes. For example, the activity of c-Src, a non-receptor tyrosine kinase required for phosphorylation of the major mitogenic tyrosine residue tyr845 of EGFR, can be significantly triggered via co-association with Her-2/*neu* [30]. Furthermore, EGFR signalling largely depends upon the integrity of the Her-2/*neu* tyrosine kinase residues [31,32], whereas inhibition of EGFR kinase activity may be attenuated by Her-2/*neu* overexpression [33]. As a consequence, the anti-tumoural effects of Her-2/*neu*-targeting trastuzumab might strongly be influenced by the expression of a functional EGF/EGFR system [16].

Several lines of evidence indicate that the biological activity of the Her-family receptor network and its blockade by trastuzumab is reflected by serum kinetics of the cleaved ECD of Her-2/*neu* [25,34–36]. Based on the assumption that the serum levels of the EGFR might similarly correlate with Her-2/*neu*-linked EGFR activity, signalling strength and thereby susceptibility to the anti-tumoural effects of trastuzumab, we investigated the clinical significance of changes in serum concentrations of both, Her-2/*neu* ECD and sEGFR during the course of treatment of MBC patients with trastuzumab. In accordance with previous works, serial measurements of the Her-2/*neu* ECD clearly predicted the therapeutic efficiacy of trastuzumab in terms of response, clinical benefit and progression-free survival in patients with decreasing Her-2/*neu* ECD concentrations as a result of administration of trastuzumab. However, the parallel determination of the kinetics of sEGFR levels did not allow for the prediction of response or clinical benefit or for the probability of prolonged PFS or OS of patients receiving trastuzumab and did not provide additional information on the therapeutic potential of the drug. One simple explanation for these findings might be that trastuzumab exclusively influences Her-2/*neu* but not EGFR receptor signalling and corresponding serum concentrations.

Although some studies have provided information for the impact of EGFR activation on the cytocidal effects of trastuzumab, other possible heterodimeric receptor combinations with Her-3 and Her-4 might finally determine the net result of receptor-mediated signal transduction and its blockade by trastuzumab. Support to this hypothesis is given by a recent study demonstrating that the follow up of sEGFR serum concentrations in EGFR overexpressing non-small cell lung cancer was indeed able to reflect the pharmacological activity of a tyrosine kinase inhibiting drug (Iressa) specifically targeting EGFR, but not Her-2/*neu* [20]. In addition, all patients included in the present study overexpressed Her-2, but expressed EGFR (>0) in only 11/33 cases (33.3%) with rates of overexpression (⩾2+) in only 6/33 patients investigated (18.1%). Serial determinations of sEGFR might therefore only be of predictive value in patients simultaneously overexpressing EGFR and Her-2/*neu* – a precondition that again reduces an already pre-selected patient population.

The insignificant changes of sEGFR concentrations between therapy-responders and non-responders could also be explained by the fact that the cleavage of the extracellular domain of Her-2/*neu* receptors only confers receptor activation and signal transduction in the case of Her-2/*neu* [11,37], but not EGFR expressing breast tumours. As a consequence, the measurements of sEGFR kinetics in breast cancer would neither reflect active EGFR signalling nor the inhibition of EGFR activation possibly achieved through trastuzumab. The fact that sEGFR is measurable in the serum of breast cancer patients does, however, suggest that shedding of sEGFR is a common mechanism in Her-2/*neu* overexpressing malignant breast tumours. Inhibition of Her-2/*neu* and EGFR signalling by a combination of trastuzmab and the anti-EGFR antibody cetuximab (Erbitux®), or by the newly developed dual Her-2/*neu*/EGFR tyrosin kinase inhibitor lapatinib might therefore turn out to be successful anti-neoplastic strategies whose efficacy could potentially be monitored by serial ECD and sEGFR measurements.

In conclusion, the results of the present work strongly underline the potential of monitoring serum Her-2/*neu* levels to predict the clinical outcome of patients receiving trastuzumab for Her-2/*neu* overexpressing MBC. The additional evaluation of soluble EGFR levels, however, does not provide further useful information on the efficacy of trastuzumab-based therapy.

Conflict of interest statement

None declared.

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