substantial effect on statins of SkC is therefore unlikely or is masked by natural fluctuations in SkC.

The similar changes in SkC concentrations in the control and treatment groups should be analyzed more precisely. Potential explanations include inappropriate drug intake as well as technical failures in test performance. The hypothesis of noncompliance with drug intake in the statin groups can be dismissed in light of the significant decrease in serum lipid concentrations (~30%). Technical failure in the execution of the skin test is also not a probable explanation because the mean (SD) SkC concentrations in our study are comparable to previously published data (6–8). Another potential explanation might be the effect of seasonal variation, but a significant effect seems unlikely, especially if we consider that SkC fluctuations of 11%, even within 1 day, have been reported and that comparable natural fluctuations of 12% were observed in the present study over the total study period of 9 months (6). Nevertheless, based on our present study design, we cannot completely exclude the hypothesis of an additional seasonal influence on SkC.

In summary, SkC concentrations exhibit significant biological fluctuations, and statin therapy leads to comparable decreases in SkC concentrations.

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References

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Comparison of Pre- and Postsurgical Concentrations of Blood HER-2 mRNA and HER-2 Extracellular Domain Reflects HER-2 Status in Early Breast Cancer, Benedetta Salvadori,1 Pamela Pizziati,1 Vito Distanza,1 Donato Casella,2 Simonetta Bianchi,2 Milena Paglierani,2 Vania Vezzosi,2 Rainer Neumann,4 Luigi Cataliotti,3 Mario Pazzagli,1 and Claudio Orlando1* (1 Clinical Biochemistry Unit, Department of Clinical Physiopathology, 2 Department of Human Pathology and Oncology, and 3 Department of Surgery, University of Florence, Florence, Italy; 4 Medical Department, Bayer Vital GmbH, Leverkusen, Germany; address correspondence to this author at: Clinical Biochemistry, Department of Clinical Physiopathology, University of Florence, Viale Pieraccini 6, 50139 Florence, Italy; fax 39-055-4271413, e-mail c.orlando@dfc.unifi.it)

The HER-2 gene (HER-2/neu or c-erbB-2) encodes an 185-kDa transmembrane glycoprotein that is a member of the type I family of growth factor receptors. HER-2 is constitutively activated by overexpression and contributes to cell growth, angiogenesis, survival, and metastasis (1). The assessment of HER-2 status in breast carcinomas provides valuable prognostic and predictive information. Immunohistochemistry, fluorescence in situ hybridization, chromosomal in situ hybridization, and quantitative reverse transcription-PCR (RT-PCR) may be used for this purpose. Other approaches have been proposed for the assessment of HER-2 status in peripheral blood, including evaluating either circulating HER-2 extracellular domain (ECD) or nucleated cell-associated HER-2 mRNA. Some studies have indicated that circulating ECD/HER-2 is frequently increased in metastatic disease (2–4). In addition, high concentrations of ECD/HER-2 are associated with cancer aggressiveness (5) and predict response to trastuzumab (6–8) and antiestrogen (4) therapies in advanced breast cancer. Recently, Martin et al. (9), using an array to assess circulating mRNA, pointed out that HER-2 mRNA was generally low in the blood of healthy individuals but was increased in 31% of patients with untreated invasive breast cancer. Almost all of these studies evaluated blood markers in patients with advanced or metastatic breast cancer using a single presurgical sample.

Our study included 40 consecutive patients (median age, 58 years; range, 29–80 years) undergoing surgery for early breast cancer. Patients did not receive any systemic therapy before surgery and provided informed consent for the study. According to tumor size, 28 patients were classified as T1, whereas the remaining 12 were T2 or T3. Twenty-two were node negative, and 35 were positive for estrogen receptors. From each patient, we collected 12 mL of venous blood in EDTA tubes and divided the blood into two 5-mL aliquots. The first was centrifuged, and the plasma was recovered for ECD/HER-2 measurement. The second was used for isolation of nucleated cells by density gradient. Nucleated cell RNA was extracted by TRIzol (Invitrogen), and HER-2 mRNA was measured by real-time RT-PCR. A reference interval was defined in our laboratory using a group of healthy women matched for age with our patients (n = 40). For the breast cancer
patients, we collected a random tissue sample from the center of the tumor after dividing it at the maximum diameter, and simultaneously, we took an unaffected sample from healthy tissue adjacent to the quadrant bearing the tumor or from the opposite quadrant in case of mastectomy. Total RNA was extracted from surgical specimens with the RNeasy Mini Kit (Qiagen). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was measured in all samples (tissues and nucleated cells) by RT-PCR to check for the presence of normally amplifiable RNA.

Each RNA sample was reverse-transcribed with use of TaqMan RT-PCR reagents (Applied Biosystems). Primers and probe for HER-2 were purchased as Pre-Developed Assay Reagents (Applied Biosystems). PCR amplification was performed with the ABI Prism 7700 Sequence Detector. Results were expressed as pg SKBR3 RNA/µg of total RNA. Imprecision, evaluated on the basis of the calibration curve analysis performed in triplicate [threshold (Ct) values], was always <2%. The ECD of the HER-2 protein was measured by ELISA using the Oncogene Science microtiter plate HER-2/neu assay with 20 µL of plasma (10). HER-2 results were expressed as µg/L. We adopted the manufacturer's cutoff of 15 µg/L. Pre- and postsurgical samples were assayed in triplicate in the same assay run. The imprecision of the assay in the range 2–50 µg/L was always <5%. Immunohistochemical (IHC) staining was carried out on 5-µm tissue sections with use of the HerceptTestTM Kit (Dako Cytomation A/S), and HER-2 immunoreactivity was evaluated according to the Dako protocol. Tumors classified as 0 or 1 were considered “negative,” and those scored as 2+ or 3+ were classified as “positive” for protein staining.

A preliminary study was performed in 10 patients to study the postsurgical kinetics of disappearance of ECD/HER-2 and HER-2 mRNA. For this purpose, ECD/HER-2 and HER-2 mRNA concentrations were measured in blood samples obtained on the day of surgery and 1, 3, and 5 days after surgery. We found a rapid and significant decrease in both markers (P = 0.01) in the sample collected the first day postsurgery, followed by a slight decrease on the following days (Fig. 1). On the basis of these results and for better patient compliance, we used direct comparison of baseline and 24-h postsurgery samples.

In 39 of 40 patients, the baseline presurgical concentrations of ECD/HER-2 were within the fixed reference interval (median, 8.0 µg/L; range, 6.0–11.4 µg/L) but were significantly decreased (P = 0.001) in postsurgical samples (median, 7.2 µg/L; range, 4.9–9.2 µg/L), whereas in the remaining patient, the presurgical concentrations (22.0 µg/L) remained unchanged in the postsurgical sample (22.8 µg/L).

In presurgical blood nucleated-cell fractions, HER-2 mRNA (median, 181.1 pg SKBR3 RNA/µg of total RNA; range, 3.8–1141.1 pg SKBR3 RNA/µg of total RNA) was significantly higher (P = 0.001) than in the age-matched group of 40 healthy women (median, 48.7 pg SKBR3 RNA/µg of total RNA; range, 22.5–249.0 pg SKBR3 RNA/µg of total RNA). In the postsurgical samples from the same patients, we observed a significant decrease (P = 0.005) of HER-2 mRNA [68.8 (3.1–559.6) pg SKBR3 RNA/µg of total RNA], reaching a range of values superimposable on those for the control group.

In cancer tissues, HER-2 mRNA expression was significantly higher [P = 0.001; median (range), 1566.1 (13.8–5700 pg SKBR3 RNA/µg of total RNA)] than in the paired, unaffected control tissues [116.7 (0.5–1230) pg SKBR3 RNA/µg of total RNA]. We considered that mRNA in breast cancer was up-regulated when its concentrations were 3.3-fold (~1 Ct unit) more than in the paired adjacent, unaffected control tissue. HER-2 mRNA was up-regulated in 22 patients (RT-PCR positive), whereas in the remaining patients there was no difference between cancer and noncancer tissue (RT-PCR negative). In RT-PCR-positive patients, the postsurgical concentrations of both ECD/HER-2 and HER-2 mRNA were significantly lower (P = 0.004 and 0.04, respectively) than the corresponding presurgical values. On the other hand, in the RT-PCR-negative group, we found no difference between pre- and postsurgical concentrations of both ECD/HER-2 and HER-2 mRNA (Table 1).

Results from the Dako test indicated that HER-2 protein was overproduced in eight patients (IHC positive: five with scores of 3+, and three with scores of 2+). In IHC-positive patients, the postsurgical concentrations of ECD/HER-2 and HER-2 mRNA were significantly lower (P = 0.007 and 0.03, respectively) than the corresponding presurgical values. In the IHC-negative patients, pre- and postsurgical con-
Table 1. Relationships between ECD/HER-2 and HER-2 mRNA in blood and HER-2 status in cancer tissue.

<table>
<thead>
<tr>
<th>HER-2 expression in tissue</th>
<th>Median (range) blood HER-2 mRNA, pg SKBR3 RNA/μg of total RNA</th>
<th>Median (range) ECD/HER-2, μg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Presurgical</td>
<td>Postoperative</td>
</tr>
<tr>
<td>RT-PCR +c (n = 22)</td>
<td>183.1 (3.8–946)</td>
<td>65.9 (3.1–274)</td>
</tr>
<tr>
<td>RT-PCR –b (n = 18)</td>
<td>79.1 (4.2–1141)</td>
<td>82.1 (4.5–282)</td>
</tr>
<tr>
<td>IHC+ (n = 8)</td>
<td>171.6 (27.7–950)</td>
<td>68.5 (8.4–133)</td>
</tr>
<tr>
<td>IHC- (n = 32)</td>
<td>163.3 (3.8–1141)</td>
<td>73.4 (3.1–559)</td>
</tr>
</tbody>
</table>

*Wilcoxon matched-pairs signed-rank test (presurgical vs postsurgical measurements).

b HER-2 mRNA expression in tumor vs healthy tissues: +, patients with higher HER-2 mRNA in tumor; −, patients with higher HER-2 mRNA in healthy tissue.

c NS, not significant.

d HER-2 IHC-positive (+) and -negative (−) patients.

Concentrations of ECD/HER-2 were significantly different (P = 0.001), but circulating HER-2 mRNA concentrations were not (Table 1).

According to our results, in postsurgical blood samples, cell-associated HER-2 mRNA is decreased to a median of 25% of the baseline samples. The contribution of primary tumors to the circulating concentrations of ECD/HER-2 seems less relevant and accounts for ~10%. This disparity seems attributable to the different biological significances of the two markers, but it seems to confirm that the presence of circulating cancer cells is quantitatively relevant in early breast cancers. It also important to note that several features of the primary tumor, such as tumor burden and vascularization, can affect the peripheral concentrations of these circulating markers. Another important aspect to consider is the effective expression of HER-2 in the primary tumor, both as protein and mRNA. In the case of mRNA, the measurement of HER-2 mRNA in blood nucleated-cell fractions is simultaneously affected by the number of circulating cancer cells and by the concentration of the specific mRNA target in the primary tumor. On the other hand, for the HER-2 protein, although IHC and ELISA represent separate approaches to detect the same protein in two distinct functional and biological milieu, it is possible to expect a quantitative relationship between the two forms.

In conclusion, these results seem to suggest a possible role of this approach in the definition of HER-2 status in breast cancer through the measurement of peripheral markers. Even if the use of circulating markers of HER-2 status cannot be considered, at this time, as an alternative to the approved techniques for the evaluation of HER-2 activation in primary tumors, the blood approach has the potential advantage of allowing rapid determination of serial changes in circulating HER-2 in response to physiologic or therapeutic changes and could add valuable information on initial staging of patients. In particular, even if the baseline concentrations of ECD/HER-2 in early-stage breast cancers can be considered as in the reference interval in most cases, the comparison of its pre- and postsurgical measurements seems to add new insights in defining HER-2 status. A similar trend was more evident for circulating HER-2 mRNA.

Further confirmatory studies are needed to verify if this approach can define a subgroup of early breast cancer patients who are affected by a relatively advanced stage of disease and may benefit of a selected therapy.

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References


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