Unbiased quantitative assessment of Her-2 expression of circulating tumor cells in patients with metastatic and non-metastatic breast cancer

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Background: Circulating tumor cells (CTCs) can provide the basis for a liquid biopsy and may guide the use of targeted therapies. We report on unbiased quantification of Her-2 protein expression of CTCs.

Patients and methods: Her-2 assessment of CTCs was carried out using the CellSearch® system in 103 metastatic (M1) and 88 non-metastatic (M0) breast-cancer patients. Expression of Her-2 on CTCs was determined by a manual review and an automated algorithm using Her-2-fluorescein isothiocyanate (FITC) fluorescence of leukocytes to determine the Her-2-expression threshold in each sample.

Results: Her-2 expression of CTCs varied greatly within and among patients compared with Her-2 expression of leukocytes. In M1 patients, a threshold of 75% of Her-2 positive CTCs in patients with ≥5 CTCs was set. Applying this threshold, 9% of M1 patients with Her-2-negative primary tumors had Her-2-positive CTC status and 29% of M1 patients with Her-2-positive primary tumors had Her-2-negative CTC status. No Her-2 discrepancy was observed between CTCs and primary tumors in M0 patients.

Conclusions: Our findings demonstrate that Her-2 expression is heterogeneous among CTCs within each patient. We show the feasibility of unbiased quantitative and reproducible assessment of treatment targets on CTCs, opening a path towards personalized treatment.

Key words: automated analysis, breast cancer, circulating tumor cells, Her-2, quantitative assessment

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introduction

Treatment of metastatic breast cancer took a leap forward with the introduction of trastuzumab, which targets the human epidermal growth-factor-receptor type 2 (Her-2) gene [1, 2]. Her-2 is amplified and overexpressed in ~15% of invasive breast cancers, and is usually assessed using immunohistochemistry and/or in situ hybridization on tissues obtained from the primary tumor [3]. At the metastatic stages of this disease, the Her-2 status is considered to be globally stable, but numerous reports have shown some discrepancies in the Her-2 status of patients between the primary tumor and the following metastatic relapses [4, 5]. Currently, metastasis biopsies are often carried out to reassess the therapeutic targets expressed by cancer cells, but are not always feasible and are associated with discomfort and risk to the patient.

Detection and characterization of tumor cells, circulating in the blood at the time of treatment, may resolve this issue [6]. Several studies have established that the presence of circulating tumor cells (CTCs) in metastatic (M1) breast-cancer patients is associated with a poor outcome, either at baseline or during therapy [7–11]. Beyond the quantitative analysis of CTCs in blood, the second interest of CTC detection is that molecular targets can be assessed on CTCs [12–15]. However, the classification of CTCs and quantification of treatment targets, such as Her-2, for CTCs can be subjective as they are morphologically heterogeneous [16, 17]. At present, expression of Her-2 on CTCs using the clinically validated CellSearch® system for CTC enumeration relies on visual inspection of auto-scaled Her-2-fluorescein isothiocyanate (FITC) images from CTCs [18, 19]. However, this method is poorly reproducible and the auto-scaled images may mislead a human reviewer. To overcome this, we automated the classification of CTCs and the quantification of their Her-2-expression levels.

materials and methods

patients

Patients with M1 breast cancer were part of the multicenter prospective IC 2006-04 study (NCT00898014). From June 2007 to September 2009, 267 patients were included [11], and 174 patients had ≥1 CTC detected by a manual review (mCTC) in 7.5 ml of blood at baseline; of these, 103 patients were further screened for Her-2 expression on detected CTCs and were part of this analysis. Patients with non-metastatic (M0) breast cancer were part of two prospective phase II sister studies assessing the neoadjuvant treatment efficacy on inflammatory (T4dNxM0) breast cancer either Her-2 negative (BEVERLY 01, NCT00820547 [20]; n = 38 patients assessable for both CTC detection and Her-2 expression on CTCs) or positive (BEVERLY 02, NCT0071405 [21]; 50 patients assessable at baseline for both CTC detection and Her-2 expression on CTCs). The IC 2006-04, BEVERLY 01 & 02 studies were approved by national ethics board and all patients provided written informed consent.

Her-2 assessment of the primary tumor

Patients had the Her-2 status of their primary tumor determined using immunohistochemistry, according to the current recommendations [5]. Her-2 intensity was scored as being null (0+), weak (1+), intermediate (2+) or strong (3+). Tissues that were 0+ and 1+ were considered as Her-2-negative, and 3+ as Her-2-positive. In doubtful cases (2+), FISH was carried out to determine the Her-2-amplification status (Her-2/CEP17 ratio ≥2.2) [3]. In a few patients included in the IC 2006-04 study, the Her-2 status remained unknown due to lack of tumor material.

manual CTC enumeration and Her-2 assessment

The CellSearch® system (Veridex LLC, Raritan, NJ) was used to enumerate CTCs [11, 22]. Briefly, Epcam-enriched cells are stained with phycoerythrin (PE)-conjugated antibodies C11 and A53-B/A2 directed against cytokeratins (CKs) 8, 18 and 19, respectively, an allophecoycyanin-conjugated (APC) antibody H130 to CD45 and nuclear dye 4,6-diamidino-2-phenylindole (DAPI). FITC-conjugated antibody HER81 (Veridex LLC [11]) was added to the staining cocktail to identify Her-2. A four-color semi-automated fluorescence microscope then captured and stored 8-bit digital images of the four different fluorescent dyes. mCTCs were manually counted by trained technicians, according to the manufacturer’s instructions. A review of the thumbnails of the Her-2 staining of CTCs was used to assign the mCTC as either Her-2-negative or Her-2-positive. This was done by visually comparing images obtained with spiked cell lines of known Her-2 status, as already described by others [17, 18].

Six breast-cancer cell lines were used: SKBR3, BT474, MDA-MB361, ZR75-1, BT20 and MCF7. Early passage cells were used from these cell lines that were originally obtained from the American Type Culture collection (Manassas, VA). Their Her-2 expression (c811 clone) was scored by senior cytologist Dr J. Klijianenko as 3+, 3+, 2+, 1+, 0+ and 0+, respectively.

automated CTC enumeration and Her-2 assessment

The electronic files of the 191 samples were reanalyzed using an automated algorithm developed in Matlab 2009a (Mathworks, Natick, MA), using the DIPimage toolbox (TU-Delft, The Netherlands, http://www.diplib.org). This algorithm was previously described in prostate-cancer patients [23]. Briefly, a dynamic threshold was determined using the CK–PE channel-image histogram of the imaging area selected by the algorithm. Applying this threshold to the CK–PE images gave the outline, size and location of objects. In the next step, the standard deviation of the CK–PE channel and the peak values of both the DNA–DAPI and CD45–APC channels were measured on every object using locations and outlines revealed by the thresholding procedure. Finally, classification of every object was carried out and the objects were counted as automated CTCs (aCTCs), where (i) the CK–PE standard deviation was >50 counts; (ii) it was sized between 75 and 2000 pixels (34–898 µm²); (iii) the DNA–DAPI peak value was >170 counts and (iv) CD45–APC peak was <60 counts. The criterion for the maximum size of aCTC size was raised from 500 to 2000 pixels to accommodate for the larger size of CTCs from breast-cancer patients compared with CTCs from prostate-cancer patients [24]. The image-analysis algorithm does not use the FITC channel for aCTC recognition, and thus permitted determination of Her-2–FITC expression on the outline covering the area where the aCTCs were present. The mean value, summed with two times the standard deviation of the value of the Her-2–FITC channel within the outline of each aCTC, was measured to quantify Her-2 expression.

assessment of a threshold for automated Her-2 assessment

To arrive at a threshold for Her-2–FITC staining within each sample, the distribution of Her-2–FITC staining of leukocytes present in the enriched sample was used as an internal control. To identify leukocytes in the sample, thresholding of the CD45–APC channel was carried out to select the outline of the leukocytes. A leukocyte classifier was created that
Her-2 signals derived with the algorithm for each of the cell lines are shown in panel A of Figure 2. As expected, the Her-2 (3+) SKBR3 and BT474 cells showed the highest Her-2 levels, followed by the Her-2(2+) MDA-MB361 cells; Her-2 expression was lower in Her-2(0 or 1+) ZR75-1, MCF7 and BT20 cells.

**Her-2 background staining of leukocytes as internal control**

To arrive at a threshold for Her-2 expression in aCTCs, the Her-2 expression of leukocytes within each sample was investigated. Her-2 intensity, in the areas where leukocytes were present, was determined for all patients. By determining the background distribution in a sample with a large number of leukocytes, it was determined that the threshold should be set at the 91st percentile of the leukocyte distribution, 9% of leukocytes exhibiting a higher nonspecific Her-2 intensity. Median and 91st percentile of the Her-2-FITC intensity from the leukocyte populations in the samples of each of the 90 M1 breast-cancer patients are displayed in panel B of Figure 2. The median fluorescence intensity of these internal controls shows a 4.0 fold variation and highlights the importance of an internal control for assessment of Her-2 expression.

**Her-2 expression of CTCs from M1 breast-cancer patients**

At least one aCTC was identified in 90 of the 103 IC 2006-04 patients, and Her-2 expression of aCTCs in these patients is shown in panel B of Figure 2. Her-2 expression of each aCTC is given on the left y-axis and the percentage of Her-2-positive aCTC (i.e. aCTCs expressing Her-2 above the background staining of leukocytes) is on the right y-axis. The patients were sorted according to percentage of Her-2-positive aCTCs. The correlation between the number of Her-2-positive CTCs retrieved in each sample by automated and manual assessments was good, with an $R^2$ between automated and manual Her-2 assessment of 0.979 (slope = 1.78, intercept = −30.64). Figure 3 shows examples of the Cytokeratin, DAPI, CD45 and Her-2 images of five aCTCs from different samples with the relative fluorescence Her-2 intensity and the background Her-2 intensity of the leukocytes in the sample the CTCs were detected.

**primary tissue Her-2 expression versus CTC Her-2 expression in M1 patients**

The primary tumor was Her-2 positive in 28 patients (27%), Her-2 negative in 69 patients (67%) and the Her-2 status was unknown in six patients (6%). The Her-2 status of tissues from metastatic patients, in whom aCTCs were detected, is indicated at the bottom of Figure 2B. This figure strongly suggests that a threshold of 75% of the aCTCs expressing Her-2 above the background staining of leukocytes may accurately identify the Her-2 status of the primary tumor of the patients.

We further investigated whether the number of aCTCs detected and/or the primary tumor Her-2-staining had an impact on this Her-2 status assessment. Figure 4A and B shows percentage of patients (y-axis), divided into three groups...
according to the Her-2 staining of the primary tumor versus the percentage (x-axis) of Her-2-positive aCTCs. M1 patients with a low aCTC count (i.e. 1–4 aCTCs, A) were plotted apart from those with high aCTC counts (≥5 aCTCs, B). For patients with known Her-2-negative (0, or 1+, or 2+/FISH-) tumors, Her-2-positive rates of aCTCs declined steadily with increased percentage (threshold) of Her-2-positive aCTCs. Second, comparison of panel A with panel B shows that this observation was most clear in the subgroup of patients who had ≥5 aCTCs. At the bottom of the figure, the primary tumor-tissue status of each patient is plotted (bright pink = Her-2-positive (3+ or 2+/FISH+), other colors = Her-2-negative (0+, 1+ or 2+/FISH-), x = unknown).

**validation in M0 breast-cancer patients**
We analyzed the images of the 88 M0 patients included in Beverly 01 (Her-2-negative primary tumors) and 02 (Her-2-positive primary tumors) studies. In this inflammatory non-metastatic (T4dM0) breast-cancer population, 55% of patients had ≥1 and 15% had ≥5 aCTCs/7.5 ml detected before the start of neoadjuvant chemotherapy. Correlation between mCTCs and aCTCs was good with an $R^2$ of 0.979 (slope = 1.41, intercept = −1.57). Figure 5A and B shows that several patients in both studies seemed to have a discrepancy of CTCs and primary tumor Her-2 status. However, Figure 4C and D shows that high discrepancy rates were observed only in patients with 1–4 CTCs; No discrepancy between the Her-2 status of the primary tumor and their corresponding CTCs was seen in M0 patients with ≥5 aCTCs, giving support to the validity of our Her-2 assessment method.

**discussion**
Her-2 amplification/overexpression in cancer cells is a key predictive factor of response to anti-Her-2 drugs such as trastuzumab, lapatinib and the more recently developed pertuzumab and T-DM1. Several studies showed that clinically relevant discrepancy rates between breast-cancer primary tumors, the later metastases [4, 5, 25] and CTCs, detected at
either M0 [20, 26] or M1 stages [14, 18, 27, 28, 29]. Reliable tools for detecting Her-2 on CTCs that might effectively drive therapeutic decisions in breast-cancer patients are eagerly awaited by clinicians. However, CTCs are rare in patients, and when present, Her-2 expression is heterogeneous and its determination is encumbered by inter- and intralaboratory

**Figure 3.** Examples of CTCs and the Her-2 expression of five CTCs from five different patients. The last three columns show intensity values for aCTC Her-2, the 91% expression level of leukocytes within the same sample and the final Her-2 status of the cell. The scale bar applies to all images.

**Figure 4.** Comparison of Her-2 expression in tissues and aCTCs. (A) and (B) show comparisons of M1 patients with 1–4 and ≥5 aCTCs, respectively (IC 2006-04 study). (C) and (D) show comparisons of M0 patients with 1–4 and ≥5 aCTCs, respectively (BEVERLY 01 and 02 studies pooled). The dotted line indicates the threshold of patients with >75% Her-2-positive aCTCs.
variations. The difficulties a reviewer faces when judging if a CTC is Her-2-positive by immunofluorescence include estimating Her-2 contrast, and determining whether or not it is above the staining intensity of, for example, a Her-2-expressing cell line. Furthermore, the reviewer has to judge whether or not there is a variation in the intensity based on the differences in Her-2 staining or sensitivity of the microscope, the latter being influenced by the time the mercury arc lamp has been used. Previous reports on Her-2 staining of CTCs from breast-cancer patients have mostly relied on visual estimates of the 'global level' of Her-2-FITC fluorescence from the 'pool' of CTCs detected in each patient [19]. This approach implicitly prevents any further study on the clinical implications of Her-2 heterogeneity within the pool of CTCs regarding their responses to anti-Her-2 therapies. A H-score-based approach has also been proposed to provide a weighted score based on the proportion of CTCs with a given level of fluorescence [27]. The H-score is routinely used to analyze the immunohistochemistry of tumor tissues, but requires that a large number of cancer cells (typically >10^2 to 10^3 cells) are assessed. This scoring system should, therefore, be used with care for CTC analysis, as visual estimates of proportions may be poor for most samples due to the low number of detected CTCs. A third method has been proposed, based on the signal/background-ratio calculation using image-analysis software [18]. This method uses the low-resolution ‘capture screen’ function of the computer to manually export each CTC picture to the image-analyzer software, and is not suitable for samples that contain a large number of CTCs. Moreover, this method has no internal control and is heavily impacted by the automatic scaling of the image intensity done by the CellSearch® system. Interestingly, a FISH-based determination of the Her-2 status in CTCs has been recently published with another detection technique, showing that Her-2 gene amplification can be also detected in CTCs with heterogeneous cytokeratin expression, suggesting that some CTCs might not be detected by system that relies exclusively on visual detection of cytokeratin-positive CTCs [28].

Here, we present a new method to quantify the Her-2 status of CTCs, using an automated algorithm that was tested in a large cohort of patients, with a good correlation between aCTC and mCTC counts (Figure 1). The variability of aCTC count and Her-2 staining evaluation using the algorithm was 0%, whereas a previous report by our group and others showed that interreader variability ranged from 4% to 31% for mCTC counts (median 14%) [17]. In our quantitative measurements of Her-2 on aCTCs within each patient, we observed great heterogeneity in the Her-2 intensity level. Interestingly, the spread of Her-2-intensity was greater in patients than in cell lines, suggesting the presence of several subclones with different Her-2 expression levels in the patients’ blood. It also indicates that, in order to define tumor Her-2 status by means of aCTC analysis, thresholds need to be set for both

Figure 5. (A) shows a scatter plot of aCTCs and median leukocyte Her-2–FITC signal intensities, together with the percentage of aCTCs that were >91% of the baseline leukocyte Her-2 expression levels for 23 M0 patients who had at least one aCTC (pink line) and negative Her-2 tissue status. At the bottom of the figure, the primary tumor-tissue status of each patient is plotted (bright pink = Her-2-positive (3+ or 2+/FISH+), other colors = Her-2-negative (0+, 1+ or 2+/FISH), x = unknown). (B) shows the same scatter plot for 27 M0 patients who had at least one aCTC and positive Her-2 tissue status.
Her-2-intensity level and the proportion of Her-2-positive CTCs.

First, a quantitative Her-2 threshold was set for CTCs in each sample, based on Her-2 staining of the leukocytes in each sample, which acted as an internal control. Figure 3 illustrates the importance of an internal control: if a fixed-intensity value is set, then the CTCs from row 4 could be counted as Her-2-negative, whereas the Her-2-intensity of the CTCs is actually well above the leukocyte threshold. In contrast, the CTCs in row 5 could have been judged as Her-2-positive, whereas their Her-2 intensity is below that of the leukocytes in the sample. This threshold allowed the determination of HER(2+) and HER-2(3+) cell lines as Her-2-positive, but not HER-2(0+) and HER-2(1+) cell lines.

Second, we set the percentage of Her-2-positive aCTCs at >75% to arrive at a minimal discrepancy between the Her-2 status of the primary tumor tissue and aCTCs in the training cohort consisting of M1 breast-cancer patients, before the start of first-line chemotherapy. Interestingly, the global discrepancy rate decreased when taking into account patients with ≥5 aCTCs detected. To our opinion, this result suggests that Her-2 positivity percentage has to be assessed in a significant number of CTCs (here, no other threshold than ≥5 CTCs was tested).

We finally conclude, using our method, that CTCs and primary tumors have discordant Her-2 statuses in 29% of the M1 patients with ≥5 CTC count and Her-2-positive primary tumor and 9% of the patients with ≥5 CTC count and Her-2-negative primary tumor.

Our CTC Her-2 scoring method was then validated in M0 patients. Biologically, it is expected that the discrepancy between the primary breast tumor and CTCs is lower with CTCs retrieved at M0 stage (i.e. CTCs likely released by the primary tumor) than with CTCs retrieved at M1 stage (CTCs derived from metastatic masses made of highly selected tumor cells). Our results showed high Her-2 discrepancy rates when all M0 patients were analyzed, in the range of those reported in a previous publication by German groups in the neoadjuvant GEPARQUATTRO trial [19]. However, when looking at the M0 patients with ≥5 CTC count, we observed no discrepancy between CTCs and primary tumor Her-2 status, in line with our previous hypothesis. This also suggests that discrepancy reports in M0 patients may be flawed by the low number of CTCs assessable in this setting.

Finally, this study, based on immunocytofluorescence analysis of a total of 191 M1 and M0 patients, provides a quantitative, reproducible, and fast method to determine Her-2 status of CTCs. Our method eliminates intra- and inter-reviewer, as well as inter-laboratory variations, and enables standardization of Her-2 assessment in CTCs, and is a promising tool for further interventional studies [30] to determine in M1 patients whether Her-2 status changes in CTCs are clinically relevant.

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disclosure

JYP and FCB are consultants for Roche and Veridex. LWMMT is a consultant for Veridex. All remaining authors have declared no conflicts of interest.

references

Bias in reporting of end points of efficacy and toxicity in randomized, clinical trials for women with breast cancer

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Background: Phase III randomized, clinical trials (RCTs) assess clinically important differences in end points that reflect benefit to patients. Here, we evaluate the quality of reporting of the primary end point (PE) and of toxicity in RCTs for breast cancer.

Methods: PUBMED was searched from 1995 to 2011 to identify RCTs for breast cancer. Bias in the reporting of the PE and of toxicity was assessed using pre-designed algorithms. Associations of bias with the Journal Impact Factor (JIF), changes in the PE compared with information in ClinicalTrials.gov and funding source were evaluated.

Results: Of 164 included trials, 33% showed bias in reporting of the PE and 67% in the reporting of toxicity. The PE was more likely to be reported in the concluding statement of the abstract when significant differences favoring the experimental arm were shown; 59% of 92 trials with a negative PE used secondary end points to suggest benefit of

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