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ORIGINAL ARTICLE

Reproducibility of human epidermal growth factor receptor 2 analysis in primary breast cancer – A national survey performed at pathology departments in Sweden

LISA RYDÉN[1], MONICA HAGLUND[2], PÄR-OLA BENDAHL[3], THOMAS HATSCHEK[4], ALEKSANDRA KOLARIC[5], ANIKÓ KOVÁCS[6], ANN OLSSON[7], HANS OLSSON[8], CARINA STRAND[3], MÅRTEN FERNÖ[3] & SWEDISH HER2 ANALYSIS GROUP

[1]*Department of Surgery, University Hospital, Lund, Sweden,* [2]*Department of Pathology, Malmö University Hospital, Malmö, Sweden,* [3]*Department of Oncology, University Hospital, Lund, Sweden,* [4]*Department of Oncology, Karolinska University Hospital in Solna, Stockholm, Sweden,* [5]*Department of Pathology, University Hospital, Örebro, Sweden,* [6]*Department of Pathology, Sahlgrenska University Hospital, Gothenburg, Sweden,* [7]*Department of Pathology, Karolinska University Hospital in Solna, Stockholm, Sweden and* [8]*Department of Pathology, University Hospital, Linköping, Sweden*

Abstract

Background. HER2 is a treatment predictive factor for the effect of trastuzumab and associated with poor prognosis in breast cancer. The analysis of HER2 must be performed with good quality, with regard to both the immunohistochemical (IHC) and *in situ* hybridization (ISH) analysis. *Material and methods.* A tissue microarray (TMA) including 11 breast cancer samples was sent twice (once in 2005 and again in 2006) to 24 pathology departments in Sweden. A questionnaire was also sent to the departments in 2006. *Results.* With IHC, all departments reported the same results (0/1+ *vs.* 2+ *vs.* 3+) for three (2005) and six samples (2006). The mean kappa-value increased from 0.67 to 0.77, indicating a good reproducibility at both occasions. With fluorescence-ISH (FISH), the 11 departments using this technique reported the same results (amplified *vs.* normal) for nine (2005) and ten samples (2006). The mean kappa-value showed very good reproducibility both 2005 and 2006 (0.92 and 0.96, respectively). Based on the answers from the participating departments, the questionnaire revealed that 31% of primary breast cancer diagnosed in 2006 (n = 5 043) were 2+/3+. FISH analysis of 2+ confirmed 12% of the samples to be amplified. The corresponding figure for 3+ was 90%. In total, 14.3% of the samples were HER2 positive (2+ and amplified, or 3+). *Discussion.* The results obtained in this study indicate that the reproducibility for HER2 analysis is good (IHC) and very good (FISH) between the pathology departments in Sweden using TMA-based tumor samples. In 2006, 14.3% of invasive breast cancers were HER2 positive.

The human epidermal growth factor receptor 2 (HER2) is a tyrosine kinase receptor associated with an aggressive tumor type and poor prognosis [1–5]. Approximately 15–25% of patients with breast cancer have tumors with overexpression and/or gene amplification of HER2 [6–9]. HER2 is also an important treatment predictive factor, which can be targeted by monoclonal antibodies, for instance trastuzumab. Treatment with trastuzumab in combination with chemotherapy has shown efficacy both for metastatic [10,11], locally advanced [12], and primary breast cancer [13,14]. Today, international guidelines recommend that testing of HER2 status should be performed in all newly diagnosed primary breast cancers [15–17]. For patients with no overexpression/normal gene copy number, the effect of trastuzumab can be neglected. It is therefore of outmost importance that the analysis of HER2 status with immunohistochemistry (IHC) and *in situ* hybridization (ISH) is performed with standardized methods with good reproducibility. The level of HER2 protein expression with IHC is assessed semi-quantitatively by the intensity and percentage of cells with membrane staining and scored on a scale from 0–3+, whereas results obtained by ISH are given as amplified or normal. High concordance has been achieved between IHC and ISH [18–20]. Standardized procedures should

Correspondence: Mårten Fernö, Department of Oncology, Clinical Sciences, University Hospital, SE 221 85 Lund, Sweden. Tel: +46 46177565. E-mail: Marten.Ferno@med.lu.se

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be used for all steps of the HER2-testing, including pre-analytical (type and duration of fixation, etc.), analytical (pre-treatment performance, choice of antibody, etc.), and evaluation of the results. National guidelines for the analysis and interpretation steps have been in place for several years [17,21].

In addition, to ensure compliance with validated protocols for testing, it is recommended that laboratories participate in regular internal and external quality control procedures, e.g. the United Kingdom National External Quality Assessment Scheme for immunohistochemistry (UK-NEQAS-ICC) [22] or NordiQC [23]. The quality control work may also include comparisons between local and central laboratories. In one study, the concordance was 81.6% for HercepTest (n = 1 063) and 75.0% for non-HercepTest (n = 636) when tumors locally classified as 3+ were re-analyzed centrally with HercepTest [24]. For fluorescence-ISH (FISH), *HER2* amplification was confirmed in 88.1% after central testing (n = 813; [24]). The authors of this study claimed that laboratories with a small number of assays per months (<99) were one reason for discordances. If this is generally the case, it will have implications for the organization of HER2 analyses in many countries with decentralized pathology units. However, even when only including five highly experienced international pathology testing centers in a slide-exchange program, it was shown that equivocal (2+) immunohistochemistry and borderline FISH cases were difficult to interpret [25]. In another comparison between local and central testing, the concordance was high for FISH, but lower for IHC [9].

The aim of our study was to perform a repeated reproducibility study for HER2 testing, using TMA and standardized methods according to local standards, including almost all (24/26) pathology laboratories in Sweden. Furthermore, a questionnaire was sent to the departments concerning production and results of routine testing on a one-year basis.

Material and methods

Reproducibility study

Study design. A tissue microarray (TMA) including 11 breast cancer samples (0.6 mm diameter) was sent twice (once in 2005 and again in 2006) to 24 of the in total 26 departments in Sweden performing HER2 analyses. The TMA was prepared at the Department of Pathology in Malmö from primary tumors, surgically removed 2004–2005. The samples were blinded to the participants, who were not aware of that the TMA from 2006 included the same samples as the TMA from 2005 and that a comparison between the results in 2005 and 2006 would be performed. The samples were put in a different order on the arrays in 2005 and 2006, and furthermore in 2006 an additional sample was put on the array. The cases were chosen in order to represent different levels of HER2 content. These results were obtained from the previously performed routine analyses, using IHC and FISH according to recommended guidelines from the National Swedish HER2 Analysis Group for HER2 testing [26]. For IHC all 24 laboratories used their own antibodies and staining procedure, according to kit instructions, which include positive and negative controls. HercepTest™ (DAKO) and Pathway® were used by the majority of the 24 laboratories. The results were scored 0 − 3 + according to the protocol. FISH was only performed by those 11 laboratories routinely performing this analysis. PatVision™ (Vysis/Abbot) was most commonly applied, but INFORM® HER-2/neu (Ventana) and *HER2* FISH pharmDx™ (DAKO) were also used. For methods including separate probes for HER2 and the centromer on chromosome 17, the ratio between *HER2* and *CEP17* was calculated. Samples with ratios higher than two were considered to be amplified. For methods with no centromer probe, more than six gene copies of HER2 were required for the sample to be considered amplified.

Kappa statistics. The degree of concordance between laboratories was quantified as the chance-corrected measure of agreement, known as kappa [27]. The latter measure is zero if the concordance is equal to that expected by chance. The overall mean kappa for all 24 laboratories and also the mean kappa value per laboratory were calculated. No absolute definition for the interpretation of different kappa values exists, but one commonly applied is according to the following: <0.20 Poor; 0.21−0.40 Fair; 0.41−0.60 Moderate; 0.61−0.80 Good; 0.81−1.00 Very good. In an Italian study of interobserver reproducibility of IHC HER2 assessment, only kappa-values ≥0.80 were proposed to be satisfactory [28].

Questionnaire

This part was performed to get information on the proportion of primary breast cancer during 2006 tested for HER2 status by IHC and/or FISH, and also the results of the analysis (0/1+/2+/3+ and amplified/not amplified). For several reasons, not all of the laboratories had the opportunity to answer all of these questions. In the questionnaire, the laboratories also

indicated whether they followed the national guidelines or not.

Results

Reproducibility study (2005 and 2006)

For IHC, all departments reported the same results (0/1 + *vs.* 2 + *vs.* 3 +) for three (2005) and six (2006) of the 11 samples (Table I). For four (2005) and two (2006) of the remaining samples only one department reported diverging results. The overall mean kappa-value increased from 0.67 in 2005 to 0.77 in 2006, indicating a good reproducibility at both occasions. The ranges for the mean kappa-value for the individual departments were 0.34 – 0.77 (2005) and 0.54 – 0.82 (2006). When comparing the results from 2005 and 2006, it can be seen that it was the same three samples showing the most heterogeneous results (cases 4, 7, and 11; Table I).

For FISH, the 11 departments using this technique reported the same results (amplified *vs.* not amplified) for nine (2005) and ten samples (2006; Table II). The overall mean kappa-value showed very good reproducibility both 2005 and 2006 [0.92 (range: 0.80 – 0.96) and 0.96 (range: 0.82 – 0.98), respectively].

For clinical decision making, the final consideration of HER2 status is based on a combination of IHC and ISH. Considering the 264 (24 × 11) IHC analyses in the present study it is possible to see how many have resulted in a diverging final judgement of HER2 status. In 2005, the number of diverging analyses was ten (considering the majority to be correct). Department 5 scored case 4 as 1 + and department 4 scored case 10 as 0, despite the fact that they were amplified ("false negative" IHC analyses; Tables I and II). Similarily, seven departments scored case 11 as 0 or 1 + with IHC, despite being amplified in nine of ten analyses. For case 9, department 4 scored it as 3 +, but it was normal with FISH at all laboratories ("false positive"). The number of diverging analyses was reduced to one in 2006 (department 5, case 11).

Questionnaire (2006)

The answers from the participating departments showed that 90% of all primary breast cancers diagnosed in 2006 in Sweden were analyzed for HER2 status. Twenty-five departments reported the total number of primary breast cancer cases diagnosed in 2006 that were analyzed for HER2. A total of 69% of the samples from all laboratories taken together were 0/1 + (3392/4940), 20% 2 + (1009/4940) and 11% 3 + (539/4940). This distribution varied between the departments; for 1 + between 58% and 84%, for 2 + between 8% and 27%, and for 3 + between 7% and 15%.

FISH analysis of 2 + confirmed 12% of the samples to be amplified. This figure varied between the departments, from 0/38 and 1/73 in the lowest range to 11/46, 4/9, and 3/6 in the highest range. According to the recommendations from the National Swedish HER2 Analysis Group, 3 + should also be verified with ISH. Of the samples, from the 17 departments following this recommendation, 90% (326/364) of 3 + cases were amplified. The fraction of amplified 3 + cases varied between 6/8 and 7/9 in the lower range to 15/15, 16/16 and 47/49 in the higher range.

When considering both IHC and FISH analyses 14.3% of the samples were HER2 positive (either 3 +, or 2 + and amplified).

Discussion

The results obtained in this study, using TMA-based tumor samples, indicate that the reproducibility for HER2 analysis is good for IHC and very good for ISH between 24 pathology departments in Sweden. However, for a few (n = 3 in 2005 and n = 1 in 2006) of the included departments, the concordance for IHC was lower (kappa value < 0.60). The total number of laboratories performing HER2 analyses was 26. All laboratories followed the National guidelines. According to the study design the laboratories were allowed to use their own preparation and staining methods, but still had to follow the National guidelines [26]. Samples from the same primary tumors were sent out twice, enabling us to compare the results over time. The concordance was somewhat increased for both IHC and ISH, when comparing the results from 2005 and 2006. In 2005 five laboratories reported three (n = 3) and four (n = 2) IHC results diverging from the majority, respectively. In 2006 two laboratories reported three diverging results. Reproducibility using ISH was very good overall, which is in agreement with the experience in other groups [9]. Difficulties may exist, especially in the low amplification range [25]. "False positive" ISH results may be explained by gene amplification in the *in situ* component in tumors with a non-amplified invasive cancer.

It has been claimed that laboratories with a small number of analyses (< 99 assays per month) are responsible for discordant results [24]. In the small series of samples in the reported study here, however, we found no obvious association between the mean kappa-values for the individual departments and the number of HER2 analyses performed per year. It may not be the number per department which is important, but rather the number of evaluations for each

Table I. Immunohistochemical analyses of HER2 (11 breast cancer samples) at 24 pathology departments in a) 2005 and b) 2006.

a)

Department	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8	Case 9	Case 10	Case 11
1	0	0	0	2+	0	3+	1+	3+	0	3+	2+
2	0	0	0	2+	0	3+	1+	3+	0	3+	2+
3	0	0	0	2+	0	3+	0	3+	0	3+	2+
4	0	1+	1+	2+	1+	3+	2+	3+	3+	0	2+
5	0	0	0	1+	0	2+	0	2+	0	3+	0
6	0	0	0	2+	0	3+	1+	2+	0	3+	1+
7	0	0	0	2+	0	3+	1+	3+	0	3+	2+
8	0	0	0	2+	0	3+	1+	3+	0	3+	2+
9	0	0	0	2+	0	3+	1+	2+	0	3+	1+
10	0	0	0	2+	0	3+	0	3+	0	3+	2+
11	0	0	0	3+	0	3+	1+	3+	0	3+	2+
12	0	0	0	2+	0	3+	0	3+	0	3+	1+
13	0	1+	1+	3+	1+	3+	2+	3+	0	3+	3+
14	0	1+	0	2+	1+	3+	1+	3+	0	3+	2+
15	0	1+	0	3+	1+	3+	2+	3+	0	3+	2+
16	0	1+	0	3+	2+	3+	2+	3+	0	3+	3+
17	0	1+	1+	3+	1+	3+	1+	3+	0	3+	3+
18	0	0	0	3+	0	3+	1+	3+	0	3+	1+
19	0	0	0	2+	0	3+	0	3+	0	3+	1+
20	0	0	0	3+	0	3+	0	3+	0	3+	2+
21	1+	1+	1+	3+	1+	3+	2+	3+	0	3+	2+
22	0	1+	0	3+	1+	3+	2+	3+	0	3+	3+
23	0	1+	1+	3+	1+	3+	1+	3+	0	3+	2+
24	0	0	0	2+	0	3+	0	3+	0	3+	1+

b)

Department	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8	Case 9	Case 10	Case 11
1	0	0	0	3+	0	3+	2+	3+	0	3+	3+
2	1+	1+	2+	3+	2+	3+	2+	3+	0	3+	3+
3	0	1+	0	3+	1+	3+	2+	3+	0	3+	2+
4	1+	1+	0	3+	1+	3+	2+	3+	0	3+	2+
5	0	0	0	2+	0	3+	0	3+	0	3+	1+
6	0	1+	0	3+	1+	3+	2+	3+	0	3+	3+
7	0	1+	1+	3+	1+	3+	2+	3+	0	3+	2+
8	0	0	0	3+	1+	3+	2+	3+	0	3+	3+
9	0	0	0	2+	0	3+	0	3+	0	3+	2+
10	0	0	0	N/A	0	3+	1+	3+	0	3+	2+
11	0	0	0	3+	0	3+	1+	3+	0	3+	2+
12	0	0	0	3+	0	3+	1+	3+	0	3+	3+
13	0	1+	1+	3+	1+	3+	2+	3+	0	3+	2+
14	0	0	0	2+	0	3+	1+	3+	0	3+	2+
15	1+	0	1+	3+	1+	3+	2+	3+	0	3+	2+
16	0	0	0	3+	0	3+	1+	3+	0	3+	2+
17	0	1+	1+	3+	1+	3+	2+	3+	0	3+	3+
18	0	0	0	3+	1+	3+	1+	3+	0	3+	2+
19	1+	1+	1+	3+	1+	3+	2+	3+	0	3+	3+
20	0	1+	0	3+	1+	3+	2+	3+	0	3+	3+
21	0	0	0	2+	0	3+	1+	3+	0	3+	2+
22	0	1+	0	3+	0	3+	1+	3+	0	3+	3+
23	1+	1+	1+	3+	1+	3+	2+	3+	0	3+	2+
24	0	0	0	3+	0	3+	1+	3+	0	3+	2+

N/A = not analyzed.

individual pathologist. In 2006 the median number of HER2 analyses of breast cancer specimen per month for the laboratories in Sweden was around 20–25.

Another way to compare HER2 results between different departments is to collect data from the routine analyses of all primary breast cancer cases during a certain time period (e.g. one year), in order to compare the distribution of IHC scoring and ISH testing. Such a questionnaire was sent out to the laboratories in Sweden for collecting data from

Table II. Fluorescence *in situ* hybridization (FISH) analyses of HER2 (11 breast cancer samples) at 11 pathology departments in a) 2005 and b) 2006.

a)

Department	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8	Case 9	Case 10	Case 11
1	Not ampl	Not ampl	Not ampl	Ampl	Not ampl	Ampl	Not ampl	Ampl	Not ampl	Ampl	N/A
2	Not ampl	Not ampl	Not ampl	Ampl	Not ampl	Ampl	Not ampl	Ampl	Not ampl	Ampl	Not ampl
3	Not ampl	Not ampl	Not ampl	Ampl	Ampl	Ampl	Not ampl	Ampl	Not ampl	Ampl	Ampl
4	Not ampl	Not ampl	Not ampl	Ampl	Ampl	Ampl	Not ampl	Ampl	Not ampl	Ampl	Ampl
5	Not ampl	Not ampl	Not ampl	Ampl	Ampl	Ampl	Not ampl	Ampl	Not ampl	Ampl	Ampl
6	Not ampl	Not ampl	Not ampl	Ampl	Not ampl	Ampl	Not ampl	Ampl	Not ampl	Ampl	Ampl
7	Not ampl	Not ampl	Not ampl	Ampl	Not ampl	Ampl	Not ampl	Ampl	Not ampl	Ampl	Ampl
8	Not ampl	Not ampl	Not ampl	Ampl	Not ampl	Ampl	Not ampl	Ampl	Not ampl	Ampl	Ampl
9	Not ampl	Not ampl	Not ampl	Ampl	Not ampl	Ampl	Not ampl	Ampl	Not ampl	Ampl	Ampl
10	Not ampl	Not ampl	Not ampl	Ampl	Not ampl	Ampl	Not ampl	Ampl	Not ampl	Ampl	Ampl
11	Not ampl	Not ampl	Not ampl	Ampl	Not ampl	Ampl	Not ampl	Ampl	Not ampl	Ampl	Ampl

b)

Department	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8	Case 9	Case 10	Case 11
1	Not ampl	Not ampl	Not ampl	Ampl	Not ampl	Ampl	Not ampl	Ampl	Not ampl	Ampl	Ampl
2	Not ampl	Not ampl	Not ampl	Ampl	Not ampl	Ampl	Not ampl	Ampl	Not ampl	Ampl	Ampl
3	Not ampl	Not ampl	Not ampl	Ampl	Not ampl	Ampl	Not ampl	Ampl	Not ampl	Ampl	Ampl
4	Not ampl	Not ampl	Not ampl	Ampl	Not ampl	Ampl	Not ampl	Ampl	Not ampl	Ampl	Ampl
5	Not ampl	Not ampl	Not ampl	Ampl	Not ampl	Ampl	Not ampl	Ampl	Not ampl	Ampl	Ampl
6	Not ampl	Not ampl	Not ampl	Ampl	Ampl	Ampl	Not ampl	Ampl	Not ampl	Ampl	Ampl
7	Not ampl	Not ampl	Not ampl	Ampl	Not ampl	Ampl	Not ampl	Ampl	Not ampl	Ampl	Ampl
8	Not ampl	Not ampl	Not ampl	Ampl	Not ampl	Ampl	Not ampl	Ampl	Not ampl	Ampl	Ampl
9	Not ampl	Not ampl	Not ampl	Ampl	Not ampl	Ampl	Not ampl	Ampl	Not ampl	Ampl	Ampl
10	Not ampl	Not ampl	Not ampl	Ampl	Not ampl	Ampl	Not ampl	Ampl	Not ampl	Ampl	Ampl
11	Not ampl	Not ampl	Not ampl	Ampl	Not ampl	Ampl	Not ampl	Ampl	Not ampl	Ampl	Ampl

N/A = not analyzed; Ampl = amplified; Not ampl = not amplified

2006. Twenty-five departments participated in filling out the questionnaire. The questionnaire results showed that 90% of all primary breast cancer cases were analyzed for HER2 in 2006. The explanation to the 10% of cases not being routinely analyzed are regional indications not including node negative and patients older than 70 years.

Based on approximately 5 000 primary breast cancer cases diagnosed in 2006, the mean percentages of 0/1+, 2+, and 3+ IHC scoring were 69%, 20%, and 11%, respectively. These figures varied between the laboratories, as did the figures for the fraction of amplified 2+cases, from 0–50%. Several factors may have contributed to the differences in distributions between the departments, including variations in the pre-analytical steps (the duration and conditions before fixation, the nature and duration of fixation, the temperature of the embedded material, and the length of storage of unstained slides before assessment [29]), differences in the analyses (e.g. pre-treatment condition, antibody, and its dilution), and inter-observer variations. Difficulties in interpreting 2+IHC have previously been reported in an international proficiency-testing ring study [25]. Since the analytical step and the interpretation of results are included in our reproducibility study, the greater variation in HER2 distribution reported in the questionnaire on a one-year-base than in the reproducibility study suggest that differences in the pre-analytical steps are important. Results from 12 Italian laboratories participating in two quality control programs (one focused on the pre-analytical phase and one on the analytical phase) indicated that different laboratories may either have problems with the analytical phase, the pre-analytical phase, or both phases [30]. Only one laboratory in that study showed satisfactory results overall. It should be noted that a kappa-value of ≥ 0.80 was required for results to be considered satisfactory, and furthermore that the samples were separated into four groups (0, 1+, 2+, 3+), whereas we considered only three (0/1+, 2+, 3+). It is harder to obtain high kappa-values when increasing the number of subgroups. One comment in this publication was that each laboratory should be aware of their performance in the different phases of HER2 assessment [30]. Another explanation, albeit a less plausible one, for the diverging results in our study is that there are hereditary differences between breast cancers appearing in different regions of Sweden,

resulting in differences in the percentage of HER2-positive breast cancer cases. Epidemiological studies are needed to further elucidate this possibility. Finally, it should be taken into consideration that the differences were obtained at random and that the filling out of the questionnaires was not a fully standardized process, since data was not uniformly collected at the departments, including manually and computer-based techniques.

The reported percentage of HER2-positive cases in the questionnaire (14.3%) is lower than presented in the literature [6–9]. In a recent Danish nationwide study this figure was 23% [31]. The difference may be explained by the inclusion of a consecutive series of primary breast cancer cases without selection and that mammography screening has been implemented in Sweden as a routine clinical procedure for many years. Similar figures of HER2-positive primary breast cancers in Sweden were also obtained at the corresponding evaluations in 2005 and 2007 (14.0% and 12.5%, respectively).

In conclusion, the results show good reproducibility for IHC and very good reproducibility for ISH for the laboratories involved in HER2 analysis in Sweden, and that this reproducibility is stable over time. The improvements indicate that quality work is worth the efforts. Based on a questionnaire concerning the result of routine HER analysis, it was revealed that 14.3% of primary breast cancers in Sweden were HER2 positive in 2006, but also that differences exist in the distribution between the participating pathology departments.

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